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Novel Approaches To Improve DNA Vaccine Induced Responses Against Difficult Infectious Disease Targets

Megan Wise
University of Pennsylvania, megan.c.wise@gmail.com

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Abstract
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NOVEL APPROACHES TO IMPROVE DNA VACCINE INDUCED RESPONSES AGAINST DIFFICULT INFECTIOUS DISEASE TARGETS

Megan Christine Wise

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Supervisor of Dissertation

_____

David B. Weiner, Ph,D.
Professor & WW Smith Chair in Cancer Research
Director Wistar Vaccine Center
Executive Vice President, The Wistar Institute
Professor Emeritus, University of Pennsylvania

Graduate Group Chairperson

_____

Daniel S. Kessler, Ph.D.
Associate Professor of Cell and Developmental Biology

Dissertation Committee
Scott Hensley, Ph.D. Associate Professor Wistar Institute (Chair)

David M Allman, Ph.D. Associate Professor of Pathology and Laboratory Medicine, Chair of Immunology Graduate Group

Terri M Laufer, M.D. Associate Professor of Medicine

Phillip Scott, Ph.D. Professor Microbiology and Immunology, Vice Dean for Research and Academic Resources University of Pennsylvania School of Veterinary Medicine

NOVEL APPROACHES TO IMPROVE DNA VACCINE INDUCED RESPONSES AGAINST DIFFICULT INFECTIOUS DISEASE TARGETS

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ABSTRACT

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David B Weiner

Vaccines have significantly improved human health through decreasing morbidity and mortality associated with infectious diseases. Through the use of vaccines, we have seen the eradication of Small Pox and the control of numerous other diseases which once crippled the society. However, older vaccine technologies have not been successful in tackling many remaining infectious diseases including Human Immunodeficiency Virus (HIV), Malaria, Tuberculosis (TB), emerging diseases or therapeutically impacting cancer. Due to the complexity of these targets, novel vaccine platforms are needed. DNA vaccines were first reported in the early 1990s and demonstrated significant success in small animals. However, due to their lack of robust immunogenicity in large animals and human subjects, excitement was quickly tempered. After years of optimizations and improvements, DNA vaccines can now generate responses as high or higher than other vaccine platforms in these species. Here we explore some of the strength of DNA vaccine technology to improve vaccine-induced responses further. First, due to the ease of production and ability to formulate multiple plasmids into a single immunization, we explore the relationship between vaccine breadth and the coverage of induced responses. Formulations of multiple plasmids encoding the HIV-1 surface protein, Envelope, were able to induce superior responses compared to a single plasmid formulation. These

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CHAPTER 1 INTRODUCTION

1.1 Vaccines

Microorganisms are constantly bombarding humans. Upon infection, the host can usually mount an immune response against these microbes, which in many instances could be protective. Vaccines harness the effectiveness of this immune response to protect people from primary infection. The first vaccine was developed by Edward Jenner in 1798 when he collected Cowpox (*Variolea vaccinae*) from cows to immunize subjects for prevention of the deadly Smallpox (1). Pasteur almost 70 years later, expanded this concept to inactivated vaccines (1). Since then, vaccines have profoundly changed the infectious disease landscape represent one of the most effective public health tools. Smallpox, which claimed over 300-500 million lives in the 20th century, has now been eradicated by vaccination(2). Currently, the World Health Organization (WHO) has licensed 25 vaccines against infectious diseases (**Table 1.1**) (3). For the ten year anniversary of the Vaccines for Children (VFC) program, the Centers for Disease Control (CDC) released a report stating that vaccines prevented over 700,000 childhood deaths, 322 million cases of childhood illness and saved over $295 billion in direct hospital cost (4, 5). Vaccines are extremely cost effective and are very useful in resource-limited settings as they require limited training to deliver, only entail 1-4 injections, and can lead to rapid protection. However, even though there are numerous clinically approved vaccines, there remains a need to develop vaccines against many modern infectious diseases including Human Immunodeficiency Virus (HIV), Malaria and Tuberculosis as well as emerging infectious diseases like Ebola, Dengue, and Zika (6).

In general, previously clinically approved vaccines are developed for the prophylactic setting to prevent disease. Few vaccines induce sterilizing immunity, completely preventing infection, but most instead prevent dissemination and morbidity/ mortality (1, 6, 7). Vaccines work by priming and creating an antigen-specific memory response. Upon infection, the recall responses rapidly and effectively expand to prevent the appearance of infection and disease (3, 7, 8). Most clinically approved vaccines have humoral correlate of protection, however, there are some exceptions such as BCG which induced cellular responses leading to cytokines production, macrophage activation and control of Tuberculosis in the young (3, 9). Even in vaccines which have a humoral correlate of protection, cellular responses should not be dismissed. The need for antigen-specific CD4 T cells to induce highly avid and specific antibodies as well as the ability of CD8 T cells to control and kill infected cells are essential for vaccine protection (3). Depending on the type of vaccination, the immune response will be differentially skewed.

While there are two main types of vaccines, live or inactivated/ fractioned preparations, there are many different forms of vaccines including whole inactivated, live attenuated, polysaccharide conjugated, recombinant subunit, recombinant vectors, virus-like particles, peptides, and nucleic acids. A description of the WHO-approved vaccines and their corresponding vaccine platforms are listed in **Table 1.1**.

1.1.1. Live attenuated vaccines

Live attenuated vaccines are the closest platform to active infection. They are created by mutating a part of the pathogen to render it unable to replicate to the capacity of its unmutated parent (1, 10). Live attenuated vaccines produce both cellular and

humoral responses and can induce lifelong immunity with one or two shots(6). However, there are some drawbacks for live attenuated vaccines. First, there is always the potential that the pathogen could mutate back to its active, virulent form (11). Additionally, people who are immunocompromised cannot receive these vaccines due to the inability of the immune system to control the vaccine (12). Furthermore, attenuated vaccine strains can spread from person to person. Another potential drawback is the creation of these vaccines which can be difficult, especially for larger pathogens like bacteria (13). Traditionally, live attenuated vaccines for viruses like Measles and Mumps were created by passaging the virus numerous times in cells which do not support normal replication (10, 11). To grow in these hostel conditions, the virus must mutate, thus weakening its growth in the human host. However, bacteria which have more genes than viruses are much more complicated and may require the mutation or removal of multiple genes.

1.1.2. Whole Inactivated Vaccines

Whole inactivated vaccines are the killed form of the infectious microbe (1, 3, 6). Methods of inactivation include heat, radiation or chemical such as the use of formalin (6). As long as the microbe is carefully purified and inactivated fully, killed vaccines are very safe. Unlike Live attenuated vaccines, whole inactivated vaccines have no risk of reversion to a virulent form. Additionally, due to the lack of replication, there are less adverse reactions. However, these vaccines tend to have lower immunogenicity than live attenuated vaccines (6). This lack of immunity leads to the need of additional booster vaccinations to maintain immunity. Additionally, inactivation of the microbe can lead to

misfolding of antigen proteins, thus creating an immune response which is ineffective against the pathogen (14).

1.1.3. Polysaccharide conjugate vaccines

Polysaccharides are an ideal target for many bacterial microbes since they are expressed on the surface and differ from self-polysaccharides. However, the immune response induced against polysaccharides are extremely limited. B cells can undergo two differentiation pathways, T cell dependent or independent (1, 3). T cell independent B cells become activated without the aid of CD4 helper T cells. These responses can occur when the B cell receptor binds to polysaccharides which are not presented on MHCs. Thus, there is limited to no T cell specific responses to this antigen, eliminating T cell help to the B cell (15). B cells which undergo T cell independent activation produce a rapid response but this response wanes quickly and induces limited memory (15). Additionally, these B cells have undergone fewer rounds of maturation and thus produce lower affinity antibodies (16, 17). On the other hand, during T cell dependent B cell responses, the B cell obtains adequate CD4 help, undergoes rounds of maturation and develop high-affinity antibody responses as well as memory. Conjugate vaccines can overcome the inability of the immune system to induce a potent and long-lasting humoral response against polysaccharides. In a conjugate vaccine, a strong antigen is chemically attached to a polysaccharide (1, 3, 16, 17). The T cells then respond to the strong antigen and provide the necessary CD4 help to polysaccharide specific B cells. Dr. John B. Robbins created the first conjugate vaccine against *Haemophilus influenza* type B

establishing a pathway for numerous other conjugate vaccines including the Pneumococcus vaccine (18, 19).

1.1.4. Subunit vaccines

Subunit vaccines are similar to whole inactivated vaccines but instead of containing the entire microbe, only contain a specific protein or group of proteins. Of all of the proteins expressed by the microbe, the ones predicted to be the most immunogenic or important for protection are included in the vaccine (7, 20). However, determining which proteins to include is one of the most difficult aspects of a recombinant subunit vaccine. Since the vaccine includes only a specific set of antigens, there tend to be less off target immune responses which decrease the risk of adverse events (20). These vaccines are made from purified antigen from the whole inactivated microbe or by using recombinant DNA technology (21-24). Most subunit vaccines are formulated with an adjuvant to increase the vaccine-induced immune response. Adjuvants will be discussed in detail in a later section.

1.1.4.1. Toxoid vaccines

Toxoid vaccines can be viewed as a subclass of subunit vaccines. Upon infection, many bacteria release toxins which are the main cause of symptoms. This includes infections of Tetanus, Diphtheria, and Pertussis bacteria (25, 26). During infection of these pathogens, neutralization of the toxin would decrease morbidity and mortality. To induce an immune response to neutralize the toxin, vaccinologists inactivate the toxin to create a toxoid (6). These toxoids retain their antigenic characteristics while having no functional properties. Thus, a toxoid vaccine primes the immune system against the toxin,

inducing neutralizing antibodies that will block the activity of the toxoid before side effects are observed.

1.1.4.2. Virus-Like Particle (VLP) vaccines

Virus-like particle vaccines are an additional class of subunit vaccines. VLPs self-assemble into small particles which look like a virus but are not infectious. Due to the characteristics of certain viral proteins, VLPs can naturally form upon expression and purification. These VLPs can display more native-like epitopes compared to a single protein molecule. Furthermore, the self-assembly can lead to increased stability and capacity to target specific cells within the body. Additionally, VLPs can be used to carry different cargo into cells. For example, if a plasmid of DNA is in solution when VLP assembly occurs, this plasmid can be encapsulated within the VLPs. Currently, there are three WHO approved VLPs vaccine all targeting HPV (Cervertex® and Gardasil®/ Gardasil9®). These vaccines include the major capsid protein, L1, which self-assemble into a VLP. There are additional VLP vaccines in preclinical and clinical development including the Hepatitis E vaccine which is approved in China but not recommended by WHO.

1.1.5. Viral vectors

Currently, there is no WHO approved licensed viral vector vaccine with most viral vector research focuses on gene therapy. However, there are numerous different phase 1, 2, and 3 clinical trials which harness the use of viral vectors for vaccination (27). The main viral vectors used in vaccine research are Adenovirus, Vaccinia, Pox, and Adeno-associated virus (AAV) (27, 28). Viral vectors have numerous similarities to live

attenuated vaccines including replicating infection and expression of antigen in cells. However, unlike live attenuated vaccines, the vectors only express a small amount of the pathogen and thus are much safer and potentially easier to produce than live attenuated vaccines (28). Viral vectors are limited in the length of the insert they can accommodate, restricting the number or types of genes which can be encoded. Additionally, if the viral vector retains it native surface proteins, the vector's tropism may not be ideal for the type of immune response needed for protection (28). The lack of tropism can be overcome or modified by including the surface protein of the pathogen of interest (29, 30). Further, immune responses induced by viral vectors can be limited by the host immune response to the vector itself. This prevents repeat administration of the vector to increase responses (27, 31-33). Many researchers overcome this hurdle by creating prime-boost strategies or by using vectors which do not circulate in humans (33-36).

1.1.6. Peptide vaccines

There are no peptide vaccines approved for clinical use. Nevertheless, there are over 400 clinical trials using peptide vaccines mostly targeting cancer antigens (37). Additional targets include infectious diseases, allergy, diabetes and Alzheimer's disease (37). Peptide vaccines gained popularity due to the ease of manufacturing and the ability to specifically target the immune response. Peptide vaccines come in two different flavors, short epitope-specific peptides and synthetic long peptides (SLPs). SLPs are usually 15-35 amino acids in length and encode both a CD4 and CD8 epitope (37). Due to their length, SLP vaccines do not require a subject HLA typing or epitope prediction which are necessary for short epitope peptides (38). These SLP peptides can be

formulated with adjuvants or lipids similar to recombinant subunit vaccines and are taken up by antigen-presenting cells (APCs). After intracellular processing in the APCs, these peptides can be presented on both HLA class I and II, leading to induction of both CD8 and CD4 help. However, there is a potential for off-target effects as the peptides are expressed out of the context of the rest of the antigen and may be processed and presented in a different manner (39). Short peptides are usually 8-11 amino acids in length and are restricted to subjects HLA type (37). Thus they require epitope mapping or prediction using sophisticated algorithms. However, these algorithms are limited as even for the most well studied MHC allele; the prediction is usually only 60% correct (40). Additionally, these prediction models do not adjust for the abundance of the T cells and could predict a rare T cell epitope (41). Also, if the peptide epitope only targets class I, these CD8 T cells will have suboptimal and short-lasting responses due to the lack of help. There are additional prediction models for HLA class II peptides which could be included in the vaccine to stimulate an adequate amount of help (42, 43).

1.1.7. Nucleic acids

Like viral vectors, there are no WHO approved nucleic acid based vaccines. However, there are many different clinical trials which are exploring the use of nucleic acid vaccines. Both RNA and DNA-based strategies are in development and due to advances in technology have recently seen a resurgence.

1.1.7.1. DNA vaccines

DNA vaccines were first reported in the early 1990s (44). Initially, the vaccine platform saw great success in small animal models (45-48). However, once progressed

into non-human primates and people, the vaccines induced limited immunity (49-52). After years of optimizations, DNA vaccines are now major contenders for novel vaccine development.

The premise of DNA vaccination is to use plasmids which carry an insert encoding the antigen of interest. Like other vaccine platforms, typically DNA vaccines are administered to the muscle or in the dermis. The muscle or dermal cells are then transfected with the plasmid which will be exosomally transcribed and translated into the antigenic protein (53). The protein will then be processed and presented on MHC class I or secreted leading to cross-presentation on MHC class II (54). Any resident APCs can be transfected or APCs which translocate into the site of vaccination, will then drain to the local lymph node and stimulate cellular and humoral responses (53, 54). Due to the high ability to present on MHC class I, DNA vaccines are potent inducers of cellular immune responses.

Unlike viral vectors, DNA vaccines are not hindered by vector serology and thus can be given multiple times with repeated boosting of responses (31). DNA vaccines are also extremely safe as they contain minimal elements on the plasmid backbone and only encode for a specific antigen of interest (53). Since they are non-live, non-replicating, and non-spreading, there is no fear of reversion, and very limited side effects have been observed (51, 55-58). There are additional benefits to the DNA vaccine platform including vaccine stability, the ease of manufacturing plasmid as vaccines as well as a short time frame for insert development (53). Moreover, unlike recombinant subunit vaccines, since antigen encoded by DNA vaccines are produced in host cells, the protein

may contain native glycosylations and processing making the resulting immune responses of more relevance.

The main drawback for DNA vaccines is the lack of immunogenicity. After years of optimizations, DNA vaccines can now induce cellular responses as good, if not high than, viral vectors (31). These optimization strategies include modifications made to the insert itself, the backbone and the mode of delivery (53). The different plasmid optimization strategies are described in **Table 1.2**.

Even with all of these modifications, vaccine-induced immunity increased but was still significantly lower than viral vectors (59). One of the largest improvements in DNA vaccine platform was the use of novel delivery strategies to increase the transfection efficiency. Delivery methods include using gene gun (a biolistic particle delivery system), encapsulation in nanoparticles, VLPs or the use of electroporation (EP) (58-63). Electroporation is the use of electrical pulses to create small transient pores in the cell membrane. Additionally, it creates an electrical gradient which drives the negatively charged DNA into the positively charged cell (64). The transient pores then reseal with the plasmid successfully in the cell. Electroporation can increase the transfection efficiency of DNA vaccination 100-1000 fold and revolutionized the DNA vaccine field (31, 58, 64). All of these optimization and delivery modifications led to DNA vaccines with more consistency inducing strong and potent vaccine-induced responses.

1.1.7.2. RNA vaccines

Similar to DNA vaccines, RNA-based vaccines were first reported in the early 1990s (44). After success in small animals for the prevention of infectious disease and cancer, initial excitement was quickly dampened by the lack of stability and difficulty in manufacturing RNA-based products (65-67). However, almost 20 years later, after numerous improvements in synthesis technology and production, interest in RNA vaccines has been revitalized. Nevertheless, these improvements while exciting are behind those of DNA vaccines.

There are multiple different ways in which RNA vaccines are delivered into a host. The first and most developed is the use of dendritic cells isolated from a patient, transfected *ex vivo* with mRNA encoding the antigen of interested, and then fused back into the patient (67-69). Clinical trials using these *ex vivo* transfected DCs have been mainly for oncogenic targets including colorectal, pancreatic, neuroblastoma, and melanoma (68). Regarding RNA vaccines, this method is the most developed and frequently used approach in the clinic. It is extremely time-consuming, costly and requires patient-specific cell preparations (67). RNA vaccines can also be delivered as naked, formulated with proteins or encapsulated by lipids or viral/ bacterial vectors (69, 70). RNA is extremely susceptible to degradation by nucleases. To overcome this, formulation and encapsulating strategies have aided in the stability of the RNA by preventing degradation (71). Additionally, complexing can increase the targeting of the RNA to certain cell types allowing for the tailoring of the vaccine-induced responses (70).

There have been additional improvements for the RNA transcript as well. Traditionally, the RNA is manufactured using a DNA plasmid encoding the template for the mRNA transcript in combination with bacteriophage (like T7) polymerase (71). All mRNA must include a 5' cap and a poly(A) tail which can be added during transcription or enzymatically afterward (70). Without these two elements, the host cell will not translate the mRNA. Additionally, there has been abundant research into superior sequences in the 5' and 3' untranslated regions. These regions can increase the stability of the RNA and translation but can also contain destabilizing elements such as microRNA binding sites and AU-rich regions (72-74). A single chromatographic step can be used to purify the RNA leading to increased expression (75).

The two most prominent and well-studied RNA vaccines include the self-amplifying RNA and RNActive® produced by CureVac. The self-amplifying RNA is based on the alphavirus genome using its RNA replication machinery. The structural proteins which would produce an infectious virus have been replaced by the antigens of interest (76). Similar to naked RNA, the RNA template is transcribed *in vitro* off of a plasmid DNA template by T7 RNA polymerase (77). To increase the stability of the RNA *in vivo* and increase transfection efficiency, the self-amplifying RNA is usually encapsulated by lipids or a viral replication particle composed of zwitterionic lipids, cationic lipids, cholesterol and PEGylated lipid (77). The other major platform, RNActive® vaccines, is a product of CureVac. These RNA vaccines use *in silico* modifications of the mRNA sequence in both the 5' and 3' UTRs to increase stability and translation. These changes use only natural nucleic acids and have been shown to

increase the duration of expression (78). For example, unmodified RNA has a peak expression of 6-8 hours after injection which quickly diminishes. With RNActive®, the peak expression is extended to 24 hours with prolonged expression to up to 9 days (78). Additionally, RNActive® is complexed with protamines or small arginine-rich proteins which are found in the nucleus and have similar roles to histones during spermatogenesis (79). Mechanistic studies have demonstrated that naked mRNA can induce a Th2 like responses (80). However, when mRNA is complexed with protamine/RNA complex, it acts as a danger signal and stimulated a Th1 response (81). This stimulation occurs via the TLR7/8 recognition of the complex leading to innate immune activation and expression of key inflammatory cytokines like type 1 interferons (78). Additional studies into uptake pathways have demonstrated a difference between the localization in the cell of the complexed vs. naked RNA, further supporting the induction of different immune sensors (70, 82). Nonetheless, these complexes are extremely tight and thus limit the amount of antigen which can be translated. To overcome this lack of translation, RNActive® uses a specific ratio of complexed to naked RNA (78). The RNActive® platform has seen success in the clinic against both prostate cancer and non-small cell lung cancer (78, 83, 84).

1.1.7.3. Similarities and differences between RNA and DNA vaccines

There are many similarities between the two nucleic acid vaccine platforms. First, both vaccines initially sparked interest due to the ability to have indefinite boosting without the inhibition of seroconversion (31, 78). Nucleic acid vaccines harness the power of host cell expression, allowing for modifications which might not be achievable

during *ex vivo* expression. Due to this intracellular expression, both platforms efficiently induce strong CD8 T cell responses due to MHC class I processing but can also lead to cross-presentation on MHC class II and induction of humoral responses. Additionally, nucleic acid vaccines can be easily manufactured and developed, allowing for rapid progression into the clinic during potential outbreak situations. The platforms also allow for the ease of formulation as well as combining multiple RNA or DNA plasmids, increasing the breadth of coverage. Additionally, the manufacturing process is fairly similar between different antigens and thus can be easily scaled or manipulated for novel targets. Furthermore, neither platform is restricted to MHC haplotype as can be observed with peptide vaccines. The immunogenicity for both platforms can also be enhanced or tailored by the use of genetic adjuvants which will be further discussed in the next section.

One of the largest differences between the two platforms is the localization of the nucleic acids. RNA vaccines are translated in the cytoplasm and never enter into the nucleus (67). DNA vaccines, on the other hand, must pass through the nuclear envelope to get transcribed (53). This leads to the possibility of DNA integration into the host genome, an issue not encountered with RNA vaccines. However, over the 30 plus years of DNA vaccine development, no integration events have been observed in both pre-clinical and clinical trials (53). Depending on the sequence of the RNA vaccine template, numerous innate immune sensors including TLR3 and TLR7/8 can be activated leading to the increased production of type 1 IFNs (70). Though this can be positive and lead to a self-adjuvanting vaccine, if not controlled, it could cause increased off-target effects,

decreased adaptive immune responses and produced fever and flu-like illnesses (85). Similar responses have not been observed with DNA vaccine. DNA vaccines also have increased stability compared to RNA vaccines, which was a major limitation during the early development of the platform (85, 86). However, many improvements in technology have increased the stability of RNA vaccines (67, 70). Finally, the expression kinetics of the two platforms are slightly different. Antigen expression of DNA vaccines is theoretically slower than naked RNA vaccines. Antigen expression with DNA vaccines can be observed as early as 1-hour post injection with peak express around 24-48 hours and strong expression observed out to day 7 (78, 87). Thus, RNA vaccines have a much more transient expression than DNA vaccines. Furthermore, RNA vaccines contain minimal coding elements and do not include a promoter, bacterial resistant markers, and origin of replication that are required for DNA manufacturing (67, 70). Overall, these two platforms both have their strengths and weaknesses. However, RNA has a much more limited data set and we have to wait and see its development progress.

1.2. Adjuvants

Though optimizations to the DNA platform did increase vaccine-induced responses, there is still room for improvement and tailoring of those responses. Traditionally, vaccine adjuvants are used in formulation with whole inactivated or subunit vaccines to stimulate an improved immune response to the antigen (88). The first approved and most widely used adjuvant is alum which works by creating a depot that retains the antigen at the site of injection (89, 90). This leads to the “leaking” of the antigen, preventing rapid dissemination and elimination, leading to a continuous stimulation of the immune system.

It also leads to the increased activation of recruited APCs through stimulation of the inflammasome (91, 92). Without the addition of alum, a subunit vaccine would only produce low and short-lived responses (3). Alum is used in the Tetanus, Diphtheria, and Pertussis (DTaP) combination vaccine, *Haemophilus influenzae* type b (Hib), Pneumococcus, HPV and Hepatitis A and B vaccines (93). There are two other clinical approved adjuvants in the United States, AS04 which is a combination of monophosphoryl lipid A (MPL) / alum and an oil and water emulsion AS03 (94). AS04 is currently used in the HPV vaccine, Cervarix®, and works through stimulating Toll-like receptor 4 via MPL as well as having the adjuvant effects of alum (90, 94, 95). MPL is a derivative of Lipid A which is the active portion of the lipopolysaccharide (LPS) found on the surface of gram-negative bacteria (in this case *Salmonella minnesota*) (96). Due to its modifications, MPL induces similar cytokine profile as LPS but is less toxic and thus induces fewer side effects (97). The other approved adjuvant is AS03 which is found only in the US pandemic Influenza vaccine stockpile (98). It is currently not available to the public. It is a combination of α-tocopherol, squalene and polysorbate 80 (98). It works by increases APC uptake of the antigen and the induction of the innate immune system (99).

There are many different reasons to include a vaccine adjuvant. In the case of AS03 and the pandemic influenza vaccine, it was demonstrated that the addition of AS03 increased antibody titers over no adjuvant and would allow for the use of a decreased dose (98). This allowed for dose sparing of the vaccine and thus increased the US supply. Adjuvants can also work by increasing the kinetics of the vaccine response; broadening the breadth; increasing the immunogenicity in newborns or the elderly; decreasing the

number of vaccinations needed and tailoring the immune system to induce a certain type of response (93).

1.2.1. Plasmid-encoded immune adjuvants

DNA vaccines can also harness the power of adjuvants. However, in the case of DNA vaccines, the adjuvant used is slightly different from traditional adjuvants. Plasmid-encoded immune adjuvants harness the power of molecules which, when expressed in a normal setting, would increase or alter the immune responses. These plasmids encode cytokines, chemokines or other immune molecules and are co-formulated with a plasmid(s) expressing the antigen(s) of interest. When the plasmids are injected followed by *in vivo* EP, both plasmids enter the muscle cells and are co-expressed (**Figure 1.1**). Plasmid-encoded immune adjuvants have demonstrated the ability to increase APC activation and antigen presentation; increase the number, breadth or potency of both CD8 and CD4 T cells; and influence the humoral response by increasing class switching, isotype or affinity/ avidity of the antibody response (100, 101). They are a powerful tool to not only increase the immunogenicity of DNA vaccination but also to tailor the response towards the desired pathway. However, not all adjuvants will work as many times atopic expression of certain immune mediators will not affect or will decrease vaccine-induced responses.

1.2.1.1. Success of IL-12 as an immune adjuvant

Interleukin 12 (IL-12) is a cytokine mainly produced by APC which has a role in the induction of naïve T cells into T helper1 (Th1) cells during an immune response and in expanding natural killer (NK) cell function (102). It is a heterodimer cytokine, created

by two subunits, p35, and p40, leading to p70. Binding of IL-12 to its receptors (IL-12Rβ1 and β2) leads to a signaling cascade which upregulates the phosphorylation and dimerization of STAT1, 3, 4, and 5 transcription factors (103). This, in turn, leads to the increased production of key inflammatory cytokines including IFN-γ and TNF-α and polarization of the T cell into long-term Th1 differentiation (104, 105). Additionally, IL-12 plays a role in proliferation and increases the functionality of T cells (106, 107).

Due to its role in Th1 development and increases in CD8 T cell killer function, IL-12 was explored as a plasmid-encoded immune adjuvant. Initial studies examined pIL-12 in combination with an HIV vaccine in mice (108). This study was performed before the use of EP, yet the study demonstrated that when plasmid IL-12 (pIL-12) was used in combination with DNA vaccination, there was an increase in antigen-specific T cell responses and most importantly, an increase in CTL activity (108, 109). Furthermore, these responses persisted into memory and afforded increased protection when mice were challenged (110). In rhesus macaques, the addition of pIL-12 was demonstrated to have dose-sparing effects on an SIV DNA vaccine while increasing both humoral and cellular responses (111). When pIL-12 was combined with EP, an additive enhancement was observed, increasing both cellular and humoral responses in NHPs (59). More specifically proliferation, memory, and polyfunctionality were significantly enhanced when both pIL-12 and EP were combined (59). pIL-12 was then combined with an HIV-1 DNA vaccine (PENNVAX-B: HIV-1 clade B Env, Gag, and Pol) in a multicenter randomized clinical trial. The immunogenicity outcomes of the trials supported many of the pre-clinical findings of pIL-12's adjuvanting effects. The addition of pIL-12 increased

the percentage of subjects which had CD4 or CD8 T cell responses (IFN-γ or IL-2 production after stimulation) after the second or third immunization (**Table 1.3**) (57, 58). The magnitude of the T cell responses did not increase significantly, but the overall rate of response of subjects did. Importantly, there was no increase in adverse events with the addition of pIL-12 (58). This is especially important as the systemic delivery of recombinant IL-12 has shown increased immune activation leading to many side effects and decrease tolerance of the treatment (112).

1.2.1.2. Novel Immune adjuvants

pIL-12 laid the groundwork for the development of other plasmid-encoded immune adjuvants. There has been extensive work to develop additional adjuvants which either alone or in combination with pIL-12 could increase DNA vaccine-induced responses. Some of the more prominent and researched adjuvants include IL-15, Granulocyte macrophage colony-stimulating factor (GM-CSF), and IL-28 (57, 113-117). Within this thesis, I will focus on two kinds of novel adjuvants, mucosal chemokines and the use of CD40 ligand (CD40L). Each adjuvant will be described in detail at the beginning of each chapter.

1.3. Targets of vaccination

Though there are 25 WHO-approved vaccines, there is still a remaining need to develop vaccines against both infectious diseases and cancer targets. For example, HIV, TB, Malaria and neglected tropical diseases (NTD) accounted for 4.3 million deaths worldwide in 2014 (118). There is also a need for the development of vaccines against emerging infectious diseases and zoonotic diseases like Ebola, Middle Eastern respiratory

syndrome (MERS), and Zika (100, 119-121). Additionally, with the increase in global temperature and the expansion of vectors to areas previously uninhabited, the globe is observing a spreading of diseases like Dengue and Chikungunya which previously were isolated to a small area of the world (118).

In addition to infectious targets, there has also been increased interest in using a person's immune system to combat and control cancer growth. These vaccines can be used in either the prophylactic or therapeutic setting. In contrast to many vaccines against infectious diseases which have a humoral correlate of protection, cancer vaccines must induce strong cellular responses (122). These responses are then able to target and kill the cancerous cells, preventing growth and metastasis. However, there are complications for producing a cancer vaccine especially those which are not caused by infectious agents like Human Papillomavirus (HPV). One of the largest hurdles for cancer vaccine development is the ability to induce T cell responses against self-antigens (122). Though there are certain proteins which are overexpressed on oncogenic cells, targets need to be carefully selected to prevent off-target toxicities. Once a target protein is selected, the vaccine needs to break tolerance. Due to the complexity of some of these diseases and cancer targets, vaccine development will likely have to expand beyond the traditional platforms.

1.3.1. Human Immunodeficiency Virus

The first target I will focus on is HIV.

1.3.1.1. Overview of HIV

Human Immunodeficiency Virus (HIV) was first discovered in the early 1980's. In the 30 plus years, HIV has infected over 60 million people worldwide, led to 22 million deaths, and created over 13 million orphans (123-125). It is a retrovirus, which is distinguished from other viruses by two steps in its life cycle. First, Retroviruses use reverse transcription to transcribe its RNA genome into DNA (126). Secondly, retroviruses integrate its cDNA genome into the host cellular DNA (126). There are seven different genera of retroviruses including lentivirus which includes HIV-1, 2 and Similan Immunodeficiency Virus (SIV) (127). There are four key genes encoded by the HIV genome, *gag*, *pol*, *pro*, and *env*. Each of the genes and functions are described in **Table 1.4.**

The receptor for HIV is CD4 and the co-receptors CCR5 or CXCR4 (128). Upon binding, the Env trimer undergoes conformational changes exposing a fusion peptide and leading to the merging of the viral and cellular membrane (129, 130). Viral DNA is then formed by Reverse Transcriptase (RT) and is integrated into the host chromosome by Integrase (131). The new viral RNA produced is then used as both genomic RNA and as a template for viral protein production (132). These proteins are then packaged and assembled into viral particles which then bud from the cellular membrane (132).

HIV-1 and 2 were originally zoonotic diseases transmitted from non-human primates (NHPs) to humans in Africa (133). The pandemic HIV group M (which includes clades A, B, C, D, E, F and G) came from a transmission of SIV in a chimpanzee into a human host (133). The primary route of infection is sexual contact, but HIV can also be

transmitted vertically from mother to child as well as through the blood (134). The most effective transmission rate is through infected blood transfusions (>90%), followed by mother to child (~25%) and then sexual (134). Though sexual transmission accounts for the majority of infections, this route has the lowest efficiency, with transmission occurring between 1 in 200 to 500 unprotected transmission events (134). This rate can be significantly enhanced by the viral load of the donor, co-infections, and circumcision (134). There is a significant bottleneck during sexual transmission, with infection usually being established by 1-4 viruses (134). These viruses seed the infection and rapidly diversify (135). The natural history of HIV infection is characterized by an acute infection with high peak viral loads which are then controlled down by the adaptive immune system to a set point. However, eventually, the immune system loses its limited control, CD4 T cell levels significantly drop, and the person progresses to Acquired Immunodeficiency Syndrome (AIDS) (136). Usually, a person does not die due to HIV but instead due to opportunistic infections in which the diminished immune system can no longer fight or control (136). AIDS is characterized by immune deficiencies in both B and T cell dysfunction which leads to greater opportunistic infections, neoplastic diseases like Kaposi's sarcoma, organ dysfunction and autoimmune dysregulation (136).

1.3.1.2. Protection against HIV

There are numerous different ways in which the immune system can limit HIV viremia. However, to date, there is no natural correlate of protection against HIV. Though these responses are not able to clear the infection, they can control it and prevent sustained high viral loads. There are multiple responses which have been identified and

all likely play some role in control. The best evidence for control of viremia is for cellular immunity. Upon infection, CD8 T cells develop against multiple epitopes in all HIV proteins. The majority of responses are against structural proteins such as Gag and Pol (137). However, the virus is constantly evolving away from epitopes which are recognized by CTLs, limiting their effectiveness. There have been many studies which have demonstrated the ability for the cellular response to control infection in both NHP and humans (138-140). For example, the loss of CD8 T cells during primary SIV infection of NHPs leads to a higher set point viral load and rapid disease progression (141). Additionally, in humans, lower viral loads and better disease course correlated with higher T cell responses and certain MHC class I alleles (142).

Humoral immune responses also develop during HIV infection. The majority of the humoral responses are against the surface glycoprotein Env and a rare number (5-15%) of infected subjects do develop strong neutralizing antibodies (143-145). However, by the time the neutralizing antibodies develop, the virus has already escaped the immune pressure, limiting the effectiveness of the antibody (146). Due to this escape, the antibody is clearly executing pressure on the virus but whether or not these antibodies are contributing to viral control *in vivo* is still unclear (144, 147). In addition to neutralization capacity which will be further discussed below, antibodies have many other roles in preventing the spread of HIV including crosslinking of viruses, trapping at the epithelial surface, phagocytosis, and antibody-dependent cellular cytotoxicity (ADCC) (143, 147). The effects of these antibodies on both protection from infection and control of viremia are beginning to be explored in NHP through passive transfer studies (148).

1.3.1.3. Difficulties in HIV vaccine development

There are many factors causing the development of an HIV vaccine to differ from classical vaccines. For example, classical vaccines mimic natural immunity against re-infection. Currently, there are no cases of natural immunity and elimination of HIV infection in humans. Additionally, it is difficult to determine exactly what a vaccine should induce since we do not know the exact immune response needed to control or prevent the infection. Furthermore, most vaccines protect against disease (morbidity/ mortality) and not against infection (3). Upon infection, HIV will preferentially target activated CD4 T cells (136, 149). Some of these T cells will rest down and become a pool of cells which have the proviral DNA integrated into the cell's DNA (149). Though these cells are not actively creating infectious virions, upon reactivation of the cells, the virus can be produced (149). This causes a major hurdle for HIV infection since very early, a latent reservoir, which is not patrolled by the immune system, will be established (150, 151). Due to integration and the ability for HIV to establish latent reservoirs, the ideal vaccine would create sterilizing immunity with no infection. The ability to eliminate these latent reservoirs is a major focus of the HIV CURE initiative, however, currently a treatment still eludes scientist (150, 151). Furthermore, the most effective vaccines tend to be whole-killed or live attenuated organisms. Some of the first studies to come out of the HIV vaccine field were killed HIV-1 (152). Unfortunately, killed HIV-1 did not retain its immunogenicity and was found to induce non-protective responses (152). Moreover, the use of live attenuated retrovirus is not a safe option as it can easily revert to its fully immunogenic form. Finally, most vaccines protect against infections that are infrequently

encountered (1). In some populations, the HIV could be encountered on a daily basis (153).

There are three potential outcomes of an HIV vaccine. The first and most ideal is sterilizing immunity, leading to complete protection, no detectable HIV, and no transmission. Though this is the desired outcome of vaccination, it is the hardest bar to obtain. The second outcome is a transient infection. In this scenario, infection occurs, but the disease does not progress. There may be detectable levels of viremia early, but at later time points, the immune system has cleared the infection. With this vaccination, transmission may occur only briefly during early infection. The third potential outcome is a vaccine that induces long-term control of infection. The vaccine would play a similar role to that of highly active antiretroviral drugs. It would control viral loads to low or undetectable levels, maintain CD4 T cell levels, prevent disease progression and significantly lower transmission rates. Thus, the vaccine would replace the daily need to take antiretrovirals, preventing the side effects of the drugs and lowering treatment cost.

1.3.1.4. HIV Envelope Diversity and other characteristics

In addition to the above complications in HIV vaccine development, another major hurdle is the diversity of the HIV surface protein Envelope (Env). The major cause of the diversity is the high rates of viral replication, the error-prone reverse transcriptase, and the fact that HIV frequently recombines (135, 154). The amount of diversity observed in the Env in a single asymptomatic individual who has been infected for six years is equivalent to the amount of global diversity in the H3N2 circulating influenza strains (155). This diversity increases when viruses are sequenced from multiple

individuals across the globe. Currently, there are nine different genetic subtypes (clades) (A, B, C, D, F, G, H, J, and K) with two recombinant forms (CRF01_AE and CRF02_AG). The most common clades are A, B, C, D, CRF01_AE, and CRF02_AG. Clades tend to circulate regionally, however, due to travel and the ease of movement, there are many areas in which multiple clades are circulating at one time (156). This restricts the possibility of the development of a clade-specific vaccine to limit some of the diversity which must be covered.

In addition to Env diversity, there are many other factors which lead to difficulty in vaccine development. Firstly, HIV Env is a heterotrimer composed of 3 gp120s and 3 gp41s subunits. Studies have demonstrated that Env is constantly breathing and changing shape on the virus (157, 158). When it binds to CD4 and exposes potentially neutralizing epitopes, it is in a high energy state which is extremely unstable and only last for seconds (158-160). This constant movement shields potential neutralizing epitopes and leads to the immune system responding to areas which will not have any effect on the virus. Additionally, studies have suggested the density of Env on the HIV virion surface is extremely low (161, 162). This lack of antigen could create issues if the antibodies induced by the vaccine do not have high affinity/ avidity for the Env. Since the amount of Env is low, this could eliminate the ability for antibodies to increase their avidity by crosslinking to another Env structure on the virus. An additional ways Env is a difficult target is that is it extremely glycosylated. HIV Env is one of the most heavily glycosylated proteins discovered (163-165). This glycosylation acts as a shield for the Env, preventing antibodies from recognizing and binding to hidden neutralizing epitopes.

Many of the potent antibodies induced to HIV Env have to navigate this glycan shield to reach into its binding epitope (164, 165).

1.3.1.5. Broadly neutralizing antibodies

Even though there are many difficulties in producing a potent humoral response against Env, a small percentage of people (5-15%), over multiple years, do develop potent antibodies (144, 166, 167). These antibodies are terms broadly neutralizing antibodies in that they can neutralize 70-100% of tested viruses (144). The less potent and first of these antibodies were isolated using phage display (168, 169). Thus, they would not necessarily be heavy and light chain combinations which would naturally occur in subjects. However, newer technology has allowed for the sorting of Env positive B cells and high throughput procedures to obtain novel antibody sequences (170, 171). This has led to a flurry of new discoveries of broadly neutralizing antibodies (bNabs). bNabs are classified based off of what epitope they binding to. Currently, there are six major classes (**Table 1.5**) (172-178). The most recent antibodies are highly potent and able to neutralize a wide range of viral isolates at microgram to nanogram levels (172-178). In subjects which develop these bNabs, however, their virus has already escaped from the humoral pressure and thus even though this potent antibody has been created, the person's viral load remains unchanged. This does not mean that these bNabs could not be effective in other people. There have been numerous studies investigating the strength of bNabs in both NHP and the clinic. For example, a single infusion of a bNab into an NHP can significantly increase the number of challenges required to obtain infection (179). Studies in humanized mice and NHPs have demonstrated that infusion of

bNabs into mice with productive HIV/ SHIV infection, significantly decrease viral loads (180-183). Additionally, there have been clinical studies which have used two bNabs, 3BNC117 and VRC01 in subjects with HIV infection. In subjects who had undetectable viral loads while on antiretrovirals (ART), there was no change in the viral reservoir after VRC01 infusion. However, upon a single dose of VRC01, six of eight subjects with detectable plasma viral loads saw a 1.1 to 1.8 log decrease in viral loads (184). Trials have also been performed with treatment interruption of ART to test the efficacy of VRC01 to delay or prevent viral rebound. Though VRC01 did delay the time to viral rebound compared to historical controls, there was no long-term decrease in viral load or prevention of rebounding even though VRC01 levels were high (185). Studies have been performed with shorter durations between VRC01 infusions and had similar outcomes (186). 3BNC117 has been tested for safety and efficacy in both healthy human subjects as well as those HIV infected. The majority (88%) of the HIV-infected subjects in this trial were not on ART and a 0.8-2.5 log reduction in viral load was observed with a single infusion (187). This decrease in viral load was maintained for up to 28 days (187). Rebound studies have also been performed with 3BNC117 with similar delay in rebound as seen with VRC01 treatment(188). In all bNab trials, the infusions were well tolerated with limited to no severe adverse events (184-188). Researchers are further investigating what viral populations rebound, why there is a loss of control, increase in virological resistance as well as effects on the immune system after infusion (184-189). Another avenue of research is in the field of gene therapy, which is exploring the ability to harness

viral vectors to encode these bNabs. Thus, the infected cell would produce the antibody, circumventing the need for the immune system to produce it (190, 191).

Recently there has been increased interest in establishing how these bNabs are developed. Studies have explored the interaction between the viral evolution and the B cell receptor as well as immune characteristics between subjects who do and do not develop these bNabs. Seminal work performed by the Duke CHAVI-ID demonstrated the dance between the virus and the B cell receptor (192-194). The group followed subjects longitudinally and screened for neutralization titers. Once the serum demonstrated bNab characteristics, the subject's samples were retrospectively sequenced for both viral evolution and BCR diversity. Through this study and others, we are beginning to understand that a bNab lineage does not develop on its own, but also may require a helper BCR which forces the virus down a pathway eventually exposing the bNab epitope (193). It has also become evident that many germline BCRs, which eventually lead to bNab development, do not bind to the HIV Env but are instead activated by other antigens. How to potentially stimulate these BCRs is also a field of great interest.

Ideally, a vaccine would induce similar humoral responses as a bNab. However, upon further analysis, bNabs have antibody characteristics which are quite distinct from other vaccine induced antibodies. Within the germinal centers, antibodies undergo rounds of affinity maturation. The level of somatic hypermutation within a variable heavy chain is between 3-12% for an anti-influenza antibody (195). For some of the more potent and broad bNabs, the rate of somatic hypermutation compared to the germline is between 21-36% (196, 197). This high rate of somatic hypermutation likely requires multiple rounds

of affinity maturation. This may require years of antigen presentation and maturation which could explain the length of time required for a subject to develop these antibodies. Furthermore, the region that mainly determines the binding epitope of the antibody, the complement determining region 3 (CDR3), is extremely long (198). Normal CDR3s are around 16 whereas for HIV bNabs it is between 20-34 (198, 199). These long CDR3 are required to reach into the glycan shield to bind to the Env. Many of these CDR3s interact with the glycans and increase the avidity of the antibody (198). How to induce such long CDR3s with a vaccine is an open question in the field.

1.3.1.6. Major clinical trials in HIV vaccine development

There have been over 45,000 human volunteers in over 180 HIV vaccine clinical trials since 1987 (152). The vast majority of these trials have been phase I and II clinical trials. There have been some major phase IIB and III efficacy trials. The first two phase III trials Vax003 and Vax004 were both gp120 recombinant protein vaccines formulated in alum (200-202). The two trials had a combined 7900 subjects and were performed in Thailand (Vax003) and the USA (Vax004). The Thai trial enrolled injection drug users (one of the hardest populations to protect) whereas the US trial focused on men who have sex with men (MSM) and high-risk women. Both trials, unfortunately, had the same outcome of no efficacy (200-202). After these trials and the difficulty in isolating strong neutralizing antibodies, the field began to shift towards a more T cell based vaccine. This led to the HVTN502 Step trial and the HVTN503 Phambili Trial. Both trials use an Adenovirus subtype 5 virus expressing HIV-1 *gag, pol, and nef* genes (203, 204). These two trials were performed in the US and South Africa respectively. The Step trial focused

on MSM and high-risk heterosexual men and women whereas the Phambili Trial focused on heterosexual men and women only. Both studies again did not show any efficacy and also observed transient increases in infection risk in the vaccinated vs. the placebo group (205-207).

Following these two vaccines and the increased risk associated with vaccination, there was a slight shift towards focusing on a vaccine which induced both humoral and cellular responses. The RV144 phase III trial used a modified Canarypox-based vectored vaccine (ALVAC) followed by a gp120 protein boost (AIDSVAX B/E) in alum (208). The trial was performed in Thailand focusing on community population and enrolling over 16,000 subjects. This was also the first prime-boost platform to be tested with the hypothesis that the ALVAC would induce strong cellular responses but limited humoral responses which would be boosted with the protein immunizations. The results of the trial demonstrated moderate 31.2% efficacy – the best efficacy in an HIV vaccine trial to date (208, 209). Unfortunately, there were no observed effects on plasma viral load once a person became infected (209). The vaccine efficacy did wane over time with peak vaccine efficacy post final vaccination around 70% which then contracted down to 31.2% 3.5 years post final vaccination (208, 210). Post-hoc correlates analysis revealed that protection was not mediated by neutralizing antibody titers or T cell responses (211). Instead, IgG antibodies binding to the variable loop 1 and 2 of HIV Env gp120 decreased the risk of infection whereas serum IgA antibodies to Env weakened vaccine efficacy (212). Further analysis demonstrated that these V1/V2 specific IgG antibodies could lead to antibody-dependent cell cytotoxicity (ADCC), virus capture and low levels of

neutralization (213, 214). Additionally, these vaccine-induced antibodies were able to induce a sieve effect in the virus which did break through (215). The role of serum IgA is still under investigation and how this response differs from mucosal IgA. Additional studies have revealed that the primary binding epitope for the serum IgA antibody blocked the binding epitope for the effective IgG response (216, 217). Follow-up studies are in progress to building on the efficacy of the RV144 trial including a phase III trial in South Africa which will test similar vaccine platform and schedule but with modified Envs to better match the local circulating strains. Additionally, there are numerous studies investigating potential methods to increase the level and durability of vaccine efficacy by incorporating additional boost as well as comparing each of the different vaccine-induced responses to further understand how these responses develop and differ.

1.3.1.7. DNA vaccines and HIV

DNA vaccines against HIV were initially attempted as a solo platform immunization. However, due to limited immunogenicity induced by these vaccinations, many of the recent and ongoing clinical trials combine DNA prime with a boost composed of proteins or viral vectors. Similar to other HIV vaccines, initial vaccines were composed of only HIV Env in the hopes of inducing strong humoral responses. During the movement from humoral based vaccines to cellular, the insert antigen progressed from the surface protein Env to the intracellular structural and accessory proteins Gag and Pol. Current vaccine inserts included both Env as well as a combination of Gag and Pol. As DNA delivery technology developed over the years, EP and gene gun were incorporated into the vaccine regimen. There have been 5 phase II studies which

have used DNA vaccination. The first trial, IAVI010 which was a DNA prime (no EP) MVA boost, saw limited induced immune responses (218). Other phase II studies including HVTN 205, RV172, and HVTN204, all DNA prime viral vector boost, demonstrated increased cellular responses compared to IAVI010 (219-221). There has been one phase IIb efficacy study which has incorporated a DNA prime followed by Ad5 boost (HVTN505). In this trial, over 2000 subjects were immunized with a mixture of 6 DNA plasmids encoding Gag, Pol, Nef from clade B (HXB2, NL4-3, NY5/BRU) and HIV Env clade A (92RW020), B (HXB2/BaL), and C (97ZA012) delivered by Biojector 2000® gene gun (222). This was boosted by 4 recombinant Adenovirus serotype 5 encoding clade B Gag-Pol fusion, clade A, B or C Env. This study was prematurely stopped due to lack of efficacy in the vaccine arm. **Table 1.6** includes a description of each of the phase II trials as well as the ongoing phase I clinical trials which incorporate DNA vaccination.

Our lab has been active in the development of HIV DNA vaccines over the years. One recent completed trial of HIV DNA vaccines from our lab, HVTN070, and HVTN080 demonstrated the strongest induction of antigen-specific T cells observed in a DNA only regimen (**Table 1.3**) (57, 58). This study was described in the DNA encoded immune adjuvants section. Additionally, our DNA vaccines are being studied in two ongoing prophylactic phase I trials (RV262 and HVTN098). In HVTN098, the regimen selected is based on the success observed in HVTN080. Subjects in HVTN098 will be vaccinated with a combination of our consensus clade A and C Envelopes (gp140) as well as plasmids encoding consensus M Gag and Pol with or without pIL-12. Also, this

trial will investigate the ability of ID immunizations to increase humoral responses by targeting the skin which is rich with antigen presenting cells.

1.3.2. HPV

The second target I will focus on for my thesis is Human Papillomavirus (HPV)

1.3.2.1. Overview of HPV

The family of Papillomaviruses has evolved for millions of years with their host. Due to this extensive co-host evolution, there is little transmission of species-specific Papillomavirus to other host species (223). Papillomaviruses infect numerous different animal species including birds, reptiles, marsupials and mammals (224). Currently, there are no Papillomaviruses that infect amphibians or lower phylogenetic orders (224). The majority of infections occur at mucosal and cutaneous epithelium except Bovine Papillomaviruses 1 and 2 which infect the mesenchymal tissues (225). There are over 150 different types of Human Papillomaviruses (HPV) which have been sequenced (226). The majority of these do not cause disease and have evolved to cause chronic unapparent infections. However, there is a subset of HPV types which are classified as “high risk” and thus can cause disease specifically cancer (227). Due to their influence on human health, these “high risk” types the focus of most research.

Papillomaviruses are small non-Enveloped icosahedral viruses which have a single circular double-stranded DNA genome. This genome is around eight kilobases in length and usually, is bound to cellular histones. The genome has eight open reading frames with three functional parts: the early genes (E1-E7) are needed for viral replication, the late (L1, L2) are structural and the long control region (LCR) which has

cis elements needed for viral replication and transcription (228). **Table 1.7** describes each of the genes encoded by "high risk" HPV and their general role in the viral lifecycle. To note, there is no E3 protein due to an early sequencing error which suggested a third early open reading frame (228).

The genomes of Papillomaviruses are extremely stable as there are few mutations or recombination events (228). Papillomaviruses are categorized based on their L1 protein which is one of the most conserved proteins in the genome (229, 230). If an L1 protein has greater than 10% diversity from any known Papillomavirus, then it is considered a new type. Diversity between 2-10% is a subtype, and less than 2% is classified as a variant (229). To date, there are 16 different Papillomavirus genuses (228). These genuses are phylogenetically related but could be biologically distinct. There are four genuses that infect humans: alpha, beta, gamma, and delta. **Table 1.8** describes the "high risk" HPV types, as well as two other types included in the HPV prophylactic vaccine, which is described later. Presently, all of the "high risk" HPV types are from the alpha genus (228).

The receptor for Papillomaviruses is currently still unknown. Restriction to the epithelium and particular host suggest a specific receptor. However, the virus can bind to a wide variety of cells *in vitro,* potentially implying that the restriction may be due to cell intrinsic restriction factors (231). Glycosaminoglycans (GAGs), FC receptors and α6 integrins have all been proposed as receptors, but none have been confirmed (231). HPV lesion formation usually occurs after a wound is formed and infection of the basal stem cells of the epidermis. The division of these cells, which is associated with wound

healing, allows for the virus' genome to enter into the nucleus and be episomally maintained (232). These lesions can naturally clear or progress, leading to the precancerous stages called cervical intraepithelial neoplasia (CIN) 1-3. CIN is the definition of abnormal cells in the cervix after detection by histological examination of cervical biopsies (228). The different stages represent the proportion of abnormal cells that compose the sample, with CIN 1 being the lowest and CIN 3 the highest. There are also similar stages and definitions for vaginal (ValN 1-3) and vulvar (VIN 1-3) abnormalities.

Natural immunity to HPV infection does develop. Greater than 90% of infection will clear within two years (223, 233). There are still many remaining questions as to the role of natural immunity in clearance, control of reinfection, and prevention of reactivation. For example, within the 90% of people who will clear infections within two years, only 50-60% of people will develop detectable serum antibodies against HPV (234). It has been suggested that part of the reason natural immunity is limited to HPV infection is due to the virus's ability to surpass recognition by the innate immune system. Initial infection does not create viral loads, cell lysis or death and limits innate pattern recognition receptors (PRR) signals (235). Additionally, the viral proteins E6 and E7 (discussed further below) can counteract many signaling pathways including the STAT pathways and interferon response factor 1 signaling (236-239) as well as downregulate MHC-I expression on the surface (240-242). This leads to a more tolerant phenotype with ineffective activation of antigen presenting cells and cellular responses. Studies in both humans and animals models suggest that clearance of lesions are passive instead of

active, with the clearance caused by the replacement of infected cells by "normal" cells during basal cell division (243-245). These cells can still have viral genomes but are not actively producing viral proteins. However, studies in immunocompromised subjects and animals do suggest a role of the immune response, as reactivation of the virus can occur after immune depletion (223). Other factors that could contribute to viral reactivation include changes in hormone levels or abrasions and wound healing (228).

1.3.2.2. Prophylactic vaccination

There are three WHO approved HPV vaccines to prevent infection, reduce HPV-associated disease and formation of genital warts. All are indicated for females and males ages 9-26 but recommended for ages 11-12. All of these vaccines are based on the L1 gene and the formation of virus-like particles (246, 247). Cervarix® is a bivalent vaccine protecting against HPV16 and 18, Gardasil® is a quadrivalent vaccine which protects against HPV 16, 18, 6 and 11 and Gardasil 9® (approved in 2014) adds to Gardasil® and protects against serotypes 31, 33, 45, 52, 58 (**Table 1.8**) (246-248). The two main types, HPV 16 and 18, account for about 70% of all cervical cancers (223, 228, 249). However, there are many other types of HPV-associated cancers including vaginal, head and neck, and anal. **Table 1.9** describes the prevalence of each of the nine types covered by Gardasil 9® when the cancer is HPV-positive (249-252). HPV 6 and 11 are not "high risk" types but do cause laryngeal papillomas and are a major cause of genital warts.

The prophylactic HPV vaccine uses the L1 gene as the immunogen. The L1 gene will form virus-like particles (VLPs) spontaneously when expressed, which will assemble in a similar manner to virus assembly during natural infection (253, 254). The VPL has a

pentameric structure composed of 72 L1 units (227). Due to the repetitive nature of the VLP, it is highly immunogenic. During natural infection, the L1 exposes major neutralizing epitope and humans will develop limited humoral responses to these epitopes (255). These epitopes are highly variable and thus only induce type-specific antibodies (228, 256). The more conserved epitopes of L1 are hidden inside the VLP, and thus the immune response does not induce cross type neutralizing antibodies (257). If a person has cross type-specific antibodies, it is usually an indication that they have been infected with multiple types and upon cross absorption, the humoral response will be mono-specific (255). Similar, but stronger humoral responses are induced by the prophylactic vaccine and thus are the major reason for the increase in valency between Gardasil® (protecting against four strains) and Gardasil 9® (protecting against nine strains).

These vaccines have demonstrated significant efficacy in protection against the included types. In the phase III efficacy trial for both Cervarix® and Gardasil®, the vaccine prevented 100% of moderate to severe precancerous lesions (CIN2/3) in subjects who were naïve for infection (246, 247). The antibody titers induced by the vaccines were 10-100 time higher than humoral responses induced during natural infection (257). These humoral responses do decrease one log from peak (post final vaccination) to 18 months post final vaccination (153, 258, 259). However, these levels do eventually stabilize and remain higher than after natural infection. The protective antibody levels needed is still unknown, and there is a remaining question as to how long this protection will last or if booster immunizations will be required. Preliminary studies suggest that the prophylactic vaccines induce strong memory responses, but continued surveillance is

required to determine if additional immunizations are necessary (223). The longest follow to date is with the quadrivalent vaccine, which prevented greater than 90% of persistent infections in women who were previously uninfected for up to 5 years (258).

There are three key areas of research to improve the prophylactic vaccine: 1) increasing the immunogenicity, 2) decreasing the cost of production and 3) a universal HPV vaccine. A universal HPV vaccine is ideal and would prevent infection by all HPV types. Within L2, the other protein which assembles into the capsomer, there are neutralizing epitopes which are conserved across types (260, 261). However, most of the L2 is not exposed to the surface but instead situated inside the capsomer (261). There is a small epitope which is exposed, but this epitope is much less immunogenic compared to the L1 epitopes and antibodies induced to this area are much weaker (256, 261). Pre-clinical research is progressing on increasing the immunogenicity of this L2 epitope by linking it to other immunogenic proteins or by adding L2 into the L1 VLPs (228). Additional areas of active research are decreasing the cost of the vaccine and the number of immunizations required. To date, the HPV vaccine is one of the most expensive vaccines. Additionally, the requirement of 3 immunizations limits the rate of complete vaccination and induces sub-protective immune responses. There is ongoing research into using other expression systems like plants, viral vectors or bacteria, and DNA vaccines to increase the vaccine induced response and lower the cost of production (228).

1.3.2.3. Therapeutic vaccination

Unfortunately, these impressive prophylactic vaccines do not treat already established HPV infection. In the phase III trial of Gardasil®, an arm of the study

included women already infected with CIN2/3. The vaccination did not afford any protection or decreased the severity of the disease (153, 258, 259). Due to the low uptake of the HPV prophylactic vaccines and an increase in HPV-associated cancers, there is a need to develop an efficacious therapeutic vaccine. To date, most therapeutic vaccines focus on two key proteins: E6 and E7.

The E6 protein interacts with four types of cellular proteins: transcriptional co-activators, cellular polarity and motility proteins, tumor suppressors and inducers of apoptosis, and finally DNA replication and repair factors (228, 262, 263). All of these interactions are essential in the life cycle of the virus or the immortalization of the cell. Drilling down deeper for the tumor suppressor role, E6 recruits the protein ligase, E6 associated protein, to p53, thus preventing p53's tumor suppressive activity (263, 264). Additionally, E6 leads to the activation of Telomerase, which is a major factor in the immortalization of the cells (265). The main target for E7 is the family of retinoblastoma (Rb) proteins or pocket proteins. E7 will bind to many proteins within the family, but the most established binding partners are p105Rb, p107Rb, and p130Rb (266, 267). Upon binding to E7, the Rb proteins are degraded, eliminating their ability to regulate the cell cycle. p105Rb and p107Rb are important for cell cycle regulation in the basal cells whereas p130Rb is important for cell re-entry in the upper epithelial layers (268-270). E7 can also bind to proteins involved in cell cycle regulation, as well as, the destabilization of the centrosomes leading to mitotic deficiencies (271).

The expression levels of E6/E7 increases as the tissue progresses from CIN 1 to 3 (228). Tissues which display CIN 1 classification still support the viral life cycle of HPV.

The activity of E6 and E7 is not high enough to compromise the integrity of the cell and thus virus production is supported (228). When tissues progress to CIN 2+, the level of E6 and E7 increase, leading to expanded genetic errors, cell cycle dysregulation and increased conversion to oncogenesis (228). It is currently unknown what causes the dysregulation in the expression levels of E6 and E7, but studies have suggested that it could be hormonally related (272). Additionally, stages CIN 2+ have increased the amounts of integrated viral genomes. Though these integration events occur randomly, studies have found that many integrated viral genomes from cancer biopsies have integration events disrupting the E1/E2 ORF (273, 274). This prevents E2 ability to regulate the expression level of E6 and E7, further leading to increased expression of these two proteins (273-275). An integrated HPV genome is not necessary for oncogenic formation. However, studies have found that as much as 70% of HPV16 cervical cancers have integrated genomes (276-279).

Due to E6's and E7's role in malignancy formation and the drastic increase in expression levels in precancerous and cancerous tissues, most therapeutic vaccines have focused on the use of E6/E7 as antigens. To prevent the activity of these two proteins, mutations are usually incorporated to disrupt the pRb and p53 binding sites (40). Additionally, vaccines have also used E2 and E5, two proteins which are expressed early during transformation. Many different vaccine modalities have been used, focusing mainly on the ability to induce cytolytic T cells (CTLs) to control tumor growth. The earliest HPV therapeutic vaccines were focused around protein immunization using recombinant E6 and E7. To increase the limited immunogenicity induced with E6 and E7

alone, these two proteins were fused with other proteins including heat shock protein 65 (Hsp65) or the bacterial protein CyaA of *Bordetella pertussis* (280-282). Many protein conjugate vaccines have demonstrated success in preclinical testing, but few have been moved into clinical trials. The most prominent are Hsp65-E7 linked protein which, in a phase II study, demonstrated 35% complete responses in anogenital intraepithelial neoplasia (282). In an additional phase II study in CIN3 subjects, 13 out of 58 subjects demonstrated complete histological regression (283). However, in both of these studies, the researchers were unsure if the response rate was due to the vaccine or natural regression and clearance (282, 283). Additional recombinant protein vaccine trials have used the HPV L2 protein fused to E7 to increase immunogenicity. When immunized with the TLR-7 agonist, imiquimod, histological regression of VIN2/3 was observed by week 52 in 63% (12 out of 19) of subjects (283). Similar fusion with L2 has been performed with HPV 6 and assessed in over 300 subjects for clearance and prevention of recurrence of genital warts. Though humoral responses were detected in subjects, there was no difference in vaccinated vs. placebo regarding recurrence (284).

Therapeutic HPV vaccines have also harnessed the use of peptide vaccines. Both short epitope specific and synthetic long peptides (SLPs) have been used in clinical trials against HPV disease. In a clinical trial using subjects with VIN3 disease, SLPs against E6 and E7 demonstrated complete and durable responses in 47% of subjects (285). Strong and broad CTL responses were observed in these subjects, which peaked after first vaccination (285, 286). Shorter epitope-specific peptides have also been used. The most common epitopes are $E7_{11-20}$, $E7_{82-90}$, $E7_{86-93}$, $E6_{29-38}$ in combo with adjuvants or lipids

(40). However, there has been very modest success with these short peptides with the highest responses rate observed in high-grade CIN or VIN subjects (18% complete response, 50% partial response) (287). Due to the limited induction of immune responses produced by short peptides, there is abundant research into modifications in their delivery and formulation for vaccination studies.

Dendritic cell-based vaccines have also been used in the HPV therapeutic field. These autologous DCs are usually pulsed with either peptides/ proteins or transduced with nucleic acids or viral vectors encoding the target antigen. The first phase I DC vaccine was performed in 15 late state cervical patients. Overall, the vaccine was well tolerated and 4 out of 15 subjects developed antigen-specific immune responses (288). However, no subject observed clinical responses (288). There have been numerous other DC vaccines with the best response rate observed in a phase I clinical trial of subjects with stage IB/IIA cervical cancer. In this study, DCs were pulsed with HPV 16/18 E7 and keyhole limpet hemocyanin (KLH). All subjects developed antigen-specific CD4 responses with 8 out of 10 developing antigen-specific CTLs (289).

Clinical trials using the viral vectors Vaccinia and Modified Vaccinia Ankara (MVA) have also been performed for therapeutic HPV vaccination. Vaccinia viruses encoding E6/E7 fusion protein for HPV16 and 18 has been used alone and in prime-boost regimens with DNA or protein prime (40). The clinical trial which demonstrated the best response rate was in 18 VIN 2/3 patients, as 13 developed HPV-specific responses and 8 subjects observed partial responses as defined as a regression in lesion diameter by 50% (290). Contrary to many of the other therapeutic trials, most MVA clinical trials have

used an MVA encoding the E2 protein. In a phase I/II clinical trial with 34 CIN2/3 subjects, vaccination with the MVA vector induced complete histological regression in 20 subjects with an additional 11 observing partial reduction by 50% (291). All subjects developed HPV-specific cellular and humoral responses (291). There are many ongoing preclinical studies using other viral vectors including adenovirus, alphaviruses, and lentiviruses (40). Additionally, there have been clinical trials utilizing bacterial vectors encoding HPV E7. The bacterial vector is base off of *listeria monocytogenes* which is a gram positive bacterium that infects macrophages. Due to its bacterial life cycle and ability to escape the phagosomes, the vector can stimulate both MHC class I and II responses (292). To increase the immunogenicity of the vector, E7 was fused with the non-hemolytic fragment of Listeriolysin O protein (LLO). In a phase I trial in late stage metastatic cervical cancer, 4 out of 13 subjects observed a reduction in tumor load (293). This vector is currently being tested in a phase I/II trial in persistent or recurrent cervical cancer, a phase I/II in HPV16 positive oropharyngeal cancer and a phase II randomized, single-blind, placebo-controlled trial in CIN2/3(40, 294).

Finally, DNA vaccines have also been used in the treatment of HPV infection. A plasmid encoding the HPV16 $E7_{83-95}$ epitope formulated with biodegradable polymer microparticles has been used in phase I clinical trials of AIN and CIN2/3. Due to the peptide restriction, the subjects were required to be HLA-A2 positive (295). In the phase I trial in 12 AIN subjects, 10 develop antigen-specific immune responses (295). In the CIN2/3 trial of 15 subjects, 11 developed HPV-specific T cell responses, and 5 had complete histological regression (296). Due to these two successes, a modified DNA

vaccine which included epitopes for both E6/E7 and types 16 and 18 was assessed in a phase II randomized, double-blind, placebo-controlled trial in 123 CIN 2/3 subjects (297). There was higher resolution of lesions in the vaccinated compared to control, but these levels were not significantly higher (297). A plasmid encoding E7 attached to Hsp70 has also been assessed in a phase I/ II trial in CIN2/3 subjects. The Rb binding of E7 was disrupted as well as a signal sequence was added to increase the section of the fused protein. Of the 15 subjects, 8 developed CTL responses, and 3 observed complete responses (298). The same plasmid was used in a prime-boost regimen with the recombinant vaccinia virus described above in CIN 3 subjects. Seven out of twelve subjects developed immune responses with 42% complete response rate (299).

Our group in collaboration with Inovio Pharmaceuticals has also developed an HPV therapeutic DNA vaccine. This vaccine includes the combination of two plasmids expressing RNA and codon optimized E6 and E7 proteins for HPV16 and 18. The E6 and E7 proteins are encoded on the same plasmid but have a furin cleavage site to allow for proper folding and processing. Additionally, both E6 and E7 activity has been ablated by mutating the pRb and p53 binding sites (300). This vaccine is delivered intramuscularly followed by *in vivo* EP. A phase I trial in CIN2/3 subjects after cervical resection demonstrated that 14 out of 18 subjects developed CTL responses and that these cellular responses expressed multiple lytic markers and were able to kill in an *ex vivo* assay (301). Following this success, the vaccine was moved into a phase IIb double-blind, placebo-controlled, efficacy study in women who have CIN 2/3. In the per protocol analysis, 53 out of 107 vaccinated (49.5%) vs. 11 out of 36 placeboes (30.6%) displayed histological

regression (55). This increase in clearance was statistically significant and was the first time a DNA vaccine demonstrated clinical efficacy. Furthermore, vaccination increased virological clearance in cervical biopsy samples, 52.3% vs. 25.7% in controls (55). Additionally, correlates analysis revealed an increase in CD8 T cells in the epithelial and stroma of non-CIN2/3 cervical tissue and increase in peripheral CD8 T cells which displayed an active killing phenotype (CD138, Perforin positive) (55). This vaccine is currently being moved into a phase III clinical trial.

1.4. Thesis objects

Though DNA vaccines have seen large improvements in immunogenicity over the last ten years, there is a continued need for their further development and further enhancement. In chapter 2, we will harness the ability for DNA plasmids to be easily formulated together and investigate if increased breadth of immunization leads to improved cross-clade HIV humoral responses. In chapter 3, we will build on the success demonstrated in chapter 2 and explore the use of primary vs. consensus Envelope immunogens. We will further develop the use of multi-plasmid formulation to enhance humoral responses induced by a DNA vaccination. Chapters 4 and 5, we will investigate novel plasmid encoded immune adjuvants to enhance DNA vaccine-induced responses. The two sets of adjuvants will be mucosal chemokines (chapter 4) and the use of various forms of immune co-stimulation molecule CD40 ligand (chapter 5).

Table 1.1: Description of the WHO-approved vaccines

Vaccine	**Type**
Anthrax	Toxoid (protein)
Cholera	Monovalent vaccine – whole killed *V.cholerae O1* + protein (cholera toxin B subunit) Bivalent – whole killed serogroups O1 and O139
Diphtheria	Toxoid (protein)
Hepatitis A	Inactivated Live attenuated
Hepatitis B (HbsAg)	Subunit
Hepatitis E (currently not recommended by WHO)	Subunit (VLP)
Haemophilus influenzae b	Polysaccharide peptide conjugate
Human Papilloma-Virus	Bivalent, quadrivalent, and 9-valent subunit vaccine (VLP)
Influenza Influenza intranasal	Inactivated subunit Live attenuated
Japanese Encephalitis	Inactivated Live attenuated Live recombinant
Measles	Live attenuated
Meningococcal	Polysaccharide (bivalent – A,C; trivalent – A,C, W135; and quadrivalent – A, C, W135, Y polysaccharide-protein conjugate (monovalent – A or C or quadrivalent (A, C, W135, Y) Protein (serogroup B)
Mumps	Live attenuated
Pertussis, whole cell Pertussis, Acellular	Inactivated Subunit/ toxoid
Pneumococcus	Polysaccharide-protein conjugate (target either 10 or 13 of the most prevalent serotypes)
Polio Sabin Polio Salk	Live attenuated Inactivated
Rabies	Inactivated
Rotavirus	Live attenuated
Rubella	Live attenuated
Tetanus	Toxoid (protein)
Tick-borne encephalitis	Inactivated
Tuberculosis (BCG)	Live bacteria (different subtype)
Typhoid fever	Polysaccharide-peptide conjugate Live attenuated
Varicella Herpes Zoster	Live attenuated
Yellow Fever	Live attenuated

Table 1.2: Description of DNA vaccination optimization strategies

Optimization strategy	***Description***
Plasmid Optimization	**Improve gene transcription and expression**
Strong promoter	Many different promoters, both non-specific and muscle specific, were explored to increase transcription. The most common is the human CMV promoter as it is a burst promoter.
Termination site	Two to three stop codons are usually encoded to ensure no read through
Poly (A) signal site	Added for increase export of the mRNA from the nucleus and proper processing
Enhancer Elements	Increase promoter activity
Gene Optimization	**Enhanced protein production**
RNA optimization	Leads to more efficient translation. Removed any predicted RNA secondary structural or instability elements
Codon Optimization	Uses species-specific codon changes to increase the translational efficiency. There are many algorithms for mouse, nonhuman primates as well as humans.
Kozak Sequence	Increases the translational initiation
Leader Sequence	Improves the stability of the mRNA and leads to increased efficiency of translation and processing. The most common leader sequence is the IgE leader.

Table 1.3: Cellular response rates from HVTN080

Immunization	**% CD4 (number of subjects)**		**% CD8 (number of subjects)**	
	EP	**EP+12**	**EP**	**EP+12**
Second	30.0 (3/10)	67.9 (19/28)	10.0 (1/10)	35.7 (10/28)
third	44.4 (4/9)	80.8 (21/26)	33.3 (3/9)	51.9 (14/27)

Table 1.4: Description of HIV genes and their properties and functions.

Gene	**Properties/ function**
gag	Proteolytically cleaved to form the internal structural proteins Matrix (MA), Capsid (CA), and Nucleocapsid (NC)
pro	Protease (PR)
pol	Proteolytically cleaved into Reverse transcriptase (RT) and Integrase (IN) enzymes
env	Surface glycoprotein which is cleaved into two subunits gp120 and gp41

Table 1.5: Broadly neutralizing antibodies to HIV

Binding site	**Antibody**
V2/ glycan	PG9, PG16, PGT140s, CAP256-VRC26's
V3/V4/ glycans	PGT120s and PGT130s
V3/ CD4i	3BNC176
CD4bs	B12, VRC01, NIH45-46, 12A12, 3BNC117, VRC-CH30's
Face of contiguous areas of gp41 and gp120	35O22, PGT150's
MPER	2F5, 4E10, 10E8

Table 1.6: HIV DNA vaccine trials

type	Study protocol	Candidate Vaccine	EP or gene gun	Phase	#	result
DNA – Pox	IAVI010	DNA-HIVA/ MVA-HIVA (A)	N/A	IIa	115	Rare IFN-γ ELISpot responses (218)
DNA-Pox	HVTN205	GeoVax JS7 DNA/ MVA HIV62 (B)	N/A	IIa	225	93.2% subject developed antibodies in DDMM arm CD4 T cell – 66.4% CD8 – 21.8% (219)
DNA-Ad5	RV172	DNA (VRC-HIV DNA016-00-VP)/ rAd5 (VRC-HIVADV014-00-VP (A, B, C))	Biojector 2000®	I/IIa	324	IFN- γ ELISpot in 63% of volunteers (220)
DNA-Ad5	HVTN204	DNA (VRC-HIV DNA016-00-VP)/ rAd5 (VRC-HIVADV014-00-VP (A, B, C))	Biojector 2000®	IIa	480	IFN- γ ELISpot in >60% of volunteers (221)
DNA-Ad5	HVTN505	DNA (VRC-HIV DNA016-00-VP)/ rAd5 (VRC-HIVADV014-00-VP (A, B, C))	Biojector 2000®	IIb	2504	Premature stop due to lack of efficacy(222)
DNA-Pox	HVTN104 NCT02165267	DNA Nat-B, Con-S Env, Mosaic Env MVA- CMDR	Biojector 2000®	I	105	Ongoing
DNA-VSV	HVTN087 NCT01578889	DNA: HIV-MAG + pIL-12 VSV HIV gag	Ichor TriGrid®	I	100	Ongoing

Table 6 (con): HIV DNA vaccine trials

type	Study protocol	Candidate Vaccine	EP or gene gun	Phase	#	result
DNA-Pox	RV262 NCT01260727	PENNVAX-G MVA CMDR	Cellectra 5P® Or Biojector 2000®	I	92	Ongoing
DNA	HVTN098 NCT02431767	PENNVAX-GP + IL-12	Celletra 5P® and 3P®	I	94	Ongoing
DNA	CRO2049 NCT02075983	GTU multiHIV B clade	IM+ EP – Ichor TriGrid® No EP – IM+ ID and IM+ Transcutaneous	I	30	ongoing
DNA-protein	CUTHIVAC002 NCT02589795	DNA-C CN54ENV; CN54gp140	Ichor TriGrid®	I	24	ongoing
Pox-Ad DNA-Ad - Pox	IAVI N0004/HIVCORE004 NCT02099994	DNA: SG2.HIV consv MVA: HIVconsv rAd35: GRIN	Ichor TriGrid®	I/II	72	ongoing
DNA-Pox-Protein	UKHVCSpoke003 NCT01922284	DNA: CN54ENV and ZM96GPN Protein: CN54ENV gp140 MVA-C		I	40	ongoing

VRC-HIV DNA016-00-VP : mixture of 6 DNA plasmids – Gag, Pol, Nef from clade B (HXB2, NL4-3, NY5/BRU) and HIV Env – clade A (92RW020), B (HXB2/BaL) and c (97ZA012)
rAd5 (VRC-HIVADV014-00-VP (A, B, C))- 4 recombinant serotypes 5 – clade B Gag-Pol fusion, clade A Env, Clade B Env and Clade C Env

PENNVAX GP – Gag, Pol, Env – clade A and C
PENNVAXG- Gag, Env clade A, C, D

MVA CMDR – CM235 ENV/ CM240 Gag/Pol

GTU multiHIVB clade – Multiantigen- synthetic fusion protein build by full-length polypeptides of Rev, Nef, Tat, p17 and p24 with 20 epitopes for Protease, Reverse Transcriptase and gp160 (HAN2 HIV-B)

MVA-C: Gag-Pol-Nef and gp120 from clade C 97CN54

GeoVax – DNA: pGA2/JS7 DNA – Gag, Protease, Reverse Transcriptase, Tat, Rev, and Vpu – clade B HIV-HXB2/BH10 and Env (ADA sequence)

MVA – MVA62B – HIV Gag, Protease, RT, Env from the same sequence as DNA prime

Table 1.7: HPV genes and role in viral lifecycle

Promoter	Gene	Role
Early	E1	Recognize origin of replication with E2, has helical-like activities
	E2	Recognize origin of replication with E1, role in viral transcription, replication and genome partitioning Dependent on interactions with cellular proteins
	E4	Acts later in lifecycle, induces cytoskeleton rearrangement, G2 arrest, viral assembly and escape from epithelial surface
	E5	Increases cell proliferation, activates protein kinases, inhibits MHC trafficking to cell surface, inhibits apoptosis
	E6	Target regulators of cell cycle – p53, induces telomerase, prevents cell differentiation
	E7	Target regulator of cell cycle – retinoblastoma Rb proteins
Late	L1	Major capsid encodes the neutralizing epitope
	L2	Minor capsid– required for encapsulation of the HPV DNA

Table 1.8: "High Risk" + HPV6/11 types and risk level

Genus	species	Type species	Other papillomaviruses	risk
Alpha	5	HPV 26	HPV 51	High-risk mucosal lesions also in benign lesions
	6	HPV 53	HPV 30 HPV 56 HPV 66	High-risk mucosal lesions also in benign lesions
	7	HPV 18	HPV39 HPV 45 HPV 59 HPV 68 HPV 70	High risk mucosal lesions
	9	HPV 16	HPV 31 HPV 33 HPV 35 HPV 52 HPV 58 HPV 67	High risk malignant mucosal lesions
	10	HPV 6	HPV 11 HPV 13 HPV 44 HPV 74	Benign mucosal lesions, lower risk
	11	HPV 34	HPV 73	High risk mucosal lesions

Blue: Included in Cervarix®, Gardasil® and Gardasil 9®
Green: Included in Gardasil® and Gardasil 9®
Purple: Included in Gardasil 9®

Table 1.9: Percent prevalence of each type in HPV-positive cancers from distinct tissues.

	Invasive cervical cancer (%) (249)	Anal carcinomas (%) (251)	Invasive vaginal cancer (%) (252)	Vulvar cancer (%) (250)	Oropharyngeal (302)
Attributable to HPV (%)	100	90	40	40	12
HPV 6	<1	2	1	<1	
HPV 11	<1	<1	<1	<1	
HPV 16	61	76	59	73	60.2
HPV 18	10	3	5	5	
HPV 31	4	1	5	1	5.7
HPV 33	4	2	5	7	
HPV 45	6	1	4	3	
HPV 52	3	<1	3	2	
HPV 58	2	2	4	1	

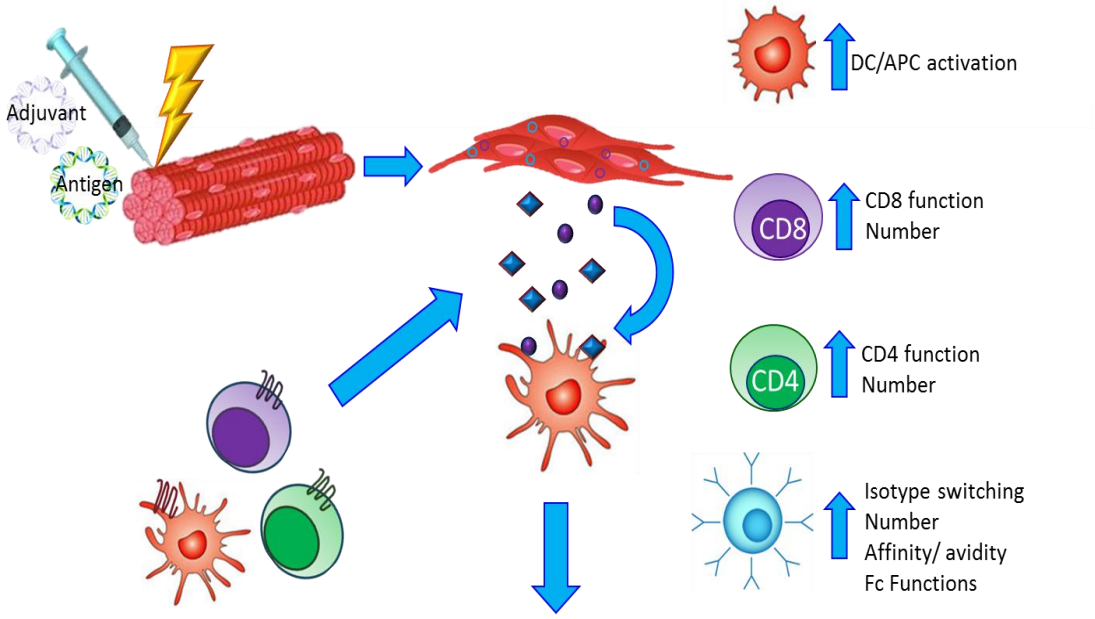

Figure 1.1: Diagram of how plasmid encoded immune adjuvants work. A plasmid encoding the antigen of interested is co-formulated with a plasmid encoding immune modulators. These two plasmids are injected into the same site of the muscle followed by *in vivo* electroporation (EP). The muscle cell will then produce both the antigen and immune adjuvant leading to changes in the vaccine induced response.

CHAPTER 2 ENHANCED SYNTHETIC MULTI-CLADE DNA PRIME INDUCES IMPROVED CROSS-CLADE REACTIVE FUNCTIONAL ANTIBODIES WHEN COMBINED WITH AN ADJUVANTED PROTEIN BOOST IN NON-HUMAN PRIMATES

2.1 Introduction

Even with effective anti-retroviral drugs, HIV remains an enormous global health burden. Vaccine development has been problematic in part due to the high degree of diversity and poor immunogenicity of the HIV Env protein. Studies suggest that a relevant HIV vaccine will likely need to induce broad cellular and humoral responses from a simple vaccine regimen due to the resource-limited setting in which the HIV pandemic is most rampant. DNA vaccination lends itself well to increasing the amount of diversity included in a vaccine due to the ease of manufacturing multiple plasmids and formulating them as a single immunization. By increasing the number of Envs within a formulation, we were able to show increased breadth of responses as well as improved functionality induced in a non-human primate model. This increased breadth could be built upon, leading to better coverage against circulating strains with broader vaccine-induced protection.

The induction of broad multifunctional antibody responses with an HIV vaccine in non-human primates (NHP) has been challenging. Previous studies of neutralizing antibodies have focused on rare tier 1 HIV-1 viruses that are relatively easy to neutralize, due to the difficulty to induce neutralizing responses against the more common tier 2 HIV-1 isolates (303, 304). Current vaccines using a nucleic acid prime followed by a

protein or viral vector boost usually induce either no or minimal neutralizing antibodies after the priming immunizations and require one or more boosts to observe induction of substantial titers (305-311).

It has also been reported that vaccine-induced non-neutralizing Fc-mediated antibody functions could be important in providing protection from HIV-1 infection (312). Among these functions, ADCC has been reported to play a relevant role in control. In fact, several studies have correlated protection from SIV and SHIV infection with the presence of ADCC activity (313-315). Moreover, in the ALVAC/AIDSVax® RV144 clinical trial, which provided a modest 31.2% protection from infection, the ADCC responses in vaccinees with low-level anti-Env plasma IgA responses correlated with a lower risk of infection (211, 217). Moreover, in the course of natural infection, ADCC responses have been associated with a delay in disease onset, virus control, and the status of long-term non-progressors (316-318). In a previous study with DNA prime-MVA boost, DNA prime-MVA and gp140 boost, MVA prime-MVA and gp140 boost, and four doses of gp140 alone, all failed to induce any ADCC activity in NHP sera (319). However, recently it has been shown that DNA or MVA priming followed by a gp140 boost based on a transmitter founder virus (HIV C.1086) can induce ADCC activity following the protein boost (320). However, it remains unclear if similar activity can be achieved by DNA only prime or in combination with a boost containing unmatched Envs.

Though DNA has previously been relegated to a priming role due to its inability to induce strong humoral responses, many advances in the field, including the use of DNA and RNA optimization strategies as well as the use of electroporation (EP), and better

formulations, have increased the effectiveness of this platform in stimulating primary immune responses (31, 58, 61, 101, 301, 321, 322). This once modest platform is now capable of inducing T-cell responses as good as or better than viral vectors (31). Additionally, these improvements have enabled DNA to induce humoral responses in NHP and humans. For example, in a recent phase I clinical trial, all subjects (18/18) responded to at least two vaccine antigens by ELISA after receiving a DNA vaccine for HPV (301). Despite these advances in the immunogenicity achieved with DNA alone, improvements are still needed, especially for the induction of functional protective antibodies and increased breadth of responses. One of the strengths of DNA vaccination is the ability to include multiple Env constructs in a single injection with ease of manufacturing and formulation. We have recently reported on the ability to combine multiple consensus Env immunogens to increase both humoral and cellular responses above those induced by the individual constructs in small animals (323). The antibody responses were further boosted with protein immunization and were able to neutralize a panel of tier 1 viruses with neutralizing titers of greater magnitude than either platform alone (323).

This chapter further examined how an improved DNA prime capable of inducing strong antibody responses impacts the magnitude and quality of the HIV Env-specific antibody response when combined with an adjuvanted recombinant protein boost in rhesus macaques (RhM). Building upon small animal studies, I determine if increased polyvalency of Env constructs would increase the breadth of immune responses in NHP. Compared to the single Env construct, including multiple consensus Env immunogens

significantly increased cross-clade binding titers. I observed broad binding antibodies and tier 1 neutralization titers averaging 10^2 with the improved synthetic multi-Env (ME) DNA prime alone. With just a single protein boost, neutralization titers increased 10- to 100-fold, and ADCC activity was observed against SF162 coated targets. Together these data demonstrate the utility of improved synthetic ME DNA priming used in combination with protein boosts.

2.2 Materials and Methods

Plasmids: All plasmids were RNA and codon optimized and included an IgE leader sequence for efficient transcription and translation. The inserts were all consensus immunogens created as previously described (57, 58, 323-326) and cloned into the pVax backbone under the control of the human cytomegalovirus immediate early promoter/enhancer. The cytoplasmic tail of all Env antigens was truncated to prevent Env recycling.

Vaccination: Fourteen Indian rhesus macaques were housed at The Children's Hospital of Philadelphia (Philadelphia, PA) according to the standards of the American Association for Accreditation of Laboratory Animal Care and all animal protocols were IACUC approved. Five animals (B group) were vaccinated at week 0, 6, 12 and 18 with an HIV DNA consisting of consensus plasmids expressing multiclade Gag and Pol with clade B Env. Five animals (ME group) were vaccinated at weeks 0, 6, 12 and 18 with an HIV DNA vaccine consisting of consensus plasmids expressing multiclade Gag and Pol with clade A, B, C, D, and A/E Env. All DNA was formulated in water at 1.0mg for Gag and Pol and 1.5mg for each Env constructs. Both groups along with an additional four

naïve animals were boosted with 100ug recombinant SF162 gp140 formulated in MF59 adjuvant at week 32. DNA was delivered to a single site in the quadriceps followed by *in vivo* EP with the constant current CELLECTRA® device (Inovio Pharmaceuticals, Plymouth Meeting, PA) with 3 pulses at 0.5A constant current, a 52ms pulse length and 0.2s rest between pulses. Recombinant protein was delivered in a single IM injection.

Blood Collection: Animals were bled two weeks following each immunization. Blood (20mL at each time point) was collected in EDTA tubes, and PBMCs were isolated using standard Ficoll-Hypaque procedure with Accuspin tubes (Sigma-Aldrich, St. Louis MO).

Intracellular staining of PBMCs: Intracellular staining of PBMCs was performed as previously described (61). Briefly, after isolation, PBMCs (1-2 x 10^6) were stimulated with individual pools of either Gag, Pol, Env A, B, C, or D for 6h in a 96 well U-bottom plate. Each peptide pool contained approximately 1μg of each peptide. Media only (R10) and PMA (0.1μg/ml) and ionomycin (0.5μg/ml) were used as negative and positive controls respectively. All stimulations were performed in the presence of secretion inhibitors brefeldin A (1μg/ml BD Biosciences, San Jose, CA) and monensin (1μg/ml; BD Biosciences). After stimulation, cells were washed with phosphate-buffered saline (PBS) and stained with violet amine-reactive dye Live/Dead stain (Life Technologies, Carlsbad, CA) for 5min followed by surface staining for 30min at room temperature. Cells were washed with PBS and fixed/permeabilized with BD Cytofix/Cytoperm (BD Biosciences) for 15min at room temperature. Following washing with BD Perm/Wash buffer, cells were stained with intracellular antibodies for 1hr at room temperature, washed and fixed with 2% paraformaldehyde. Cells were analyzed using a modified BD

LSR II (BD Biosciences) and analysis performed with FlowJo 9.2 (Tree Star, Ashland, OR).

Endpoint binding ELISA: The ELISA assay was performed as previously described using 1µg/mL HIV consensus clade A, B or C gp120 (Immune Technology, New York, NY) in PBS-T (PBS with 0.5% Tween 20) (327). Endpoint titers were determined as previously reported (328). Briefly, the upper prediction limit of HIV-specific IgG antibodies was calculated using the Student t-distribution where the mathematical formula that defines the upper prediction limit was expressed as the standard deviation multiplied by a factor based on the number of naïve controls and a 95% confidence interval. The endpoint titer was reported as the reciprocal of the lowest dilution that remained above the upper prediction limit.

Multi-Env Binding ELISA: A similar protocol as above was used to determine binding specificity against multiple different Envs. Briefly, plates were coated with 1µg/mL of each of the specific Envs: ZM197, A244, 92RW020, HXBC2, TRJO455, SF162, and gp41 (HXBC2) (Immune Technology, New York, NY) in PBS. After blocking, serum from pre-bleed, two weeks post final DNA immunization, or 2 weeks post final protein immunization were diluted 1:50 in 1% FBS in PBS-T and allowed to incubate for 1hr at room temperature. Mouse anti-NHP IgG HRP (Southern Biotech, Birmingham, AL) at a 1:5000 dilution in 1% FBS in PBS-T was used. Plates were then developed using SIGMAFAST™ OPD (o-Phenylenediamine dihydrochloride) tablets according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). The reaction was stopped after 15min with the addition of 2N H_2SO_4. Plates were then read at an optical density of

450nm on a GloMax® 96 Microplate Luminometer (Promega Madison, WI). All samples were plated in duplicate.

V1/V2 and V3 binding specificity ELISA Cyclic V1/V2 or V3 binding specificities were determined as previously described (329). Briefly, plates were coated with 1µg/mL cyclic V1/V2 and V3 constructs and incubated at 37ºC for 1.5hrs. After washing with 1X PBS-T, serum from pre-bleed, week 20 (post final DNA immunization) and week 34 (post protein boost) were diluted 1:35 and incubated for 1.5hrs at 37ºC. Secondary anti-human Alkaline Phosphatase conjugated Ab (Southern Biotech Birmingham, AL) was diluted 1:2000 and incubated for 1.5hrs at 37ºC. After washing, plates were developed with the pNPP substrate (1mg/ml in 10% Diethanolamine) and incubated for 30min at room temperature. Plates were then read at OD405nm.

Individual Peptide Mapping: Overlapping 15-mer peptides were obtained from the NIH AIDS Reagent Program for consensus clade C HIV-gp160 (catalog number 9499). Peptides were resuspended in PBS to obtain a concentration of 1mg/peptide in 1ml. Plates were coated with 1µg/ml of peptide and ELISA was performed as described above.

Avidity Index ELISA: The avidity of the humoral responses were assessed against consensus clade A, B, and C gp120 two weeks after each DNA vaccination for ME group, two and eleven weeks after protein boost for clade B only DNA, ME DNA and protein only groups. Plates were coated with 1ug/ml of either consensus clade A, B, or C gp120 (Immune Technology, New York, NY) in PBS. After blocking, serum was diluted 1:50 or for the dilution curves 1:50 and then four-fold from there in 1% FBS in PBS-T.

Each sample was run in quadruplicate where half of the wells were treated, and half were untreated. After an 1 hour incubation, plates were washed five times with PBS-T. Half of the wells for each sample were incubated with a denaturing reagent, 8M urea, for 5 minutes while the others were incubated with PBS. Plates were washed and incubated with mouse anti-NHP IgG HRP (Southern Biotech, Birmingham, AL) at a 1:5000 dilution in 1% FBS in PBS-T. Plates were then developed as described above and OD450 values were obtained. The avidity index was determined by dividing the OD450 values of the treated by the untreated and multiplying by 100.

Neutralization: Neutralization was determined using the previously described TZM-bl based assay (304). Briefly, pseudotyped viruses were produced by transfecting 293T cells with plasmids expressing HIV *env* and *env* deficient HIV-1 backbone plasmid (pSG3ΔEnv) using FuGene6 (Promega Madison, WI). Media containing viruses were collected 48hrs after transfection, spun and cleared through a 0.45μm filter. The virus was then titered to determine the TCID at which infectivity produced 150,000 relative luminescence units (RLU). For neutralization, serum was diluted 1:20 in 10% D-MEM growth medium followed by serial 3-fold dilutions (range 1:20 to 1:43740) and incubated for 1hr at 37^0 C with titered virus. TZM-bl cells were then added (1×10^4/ well in 100μl) with DEAE-Dextran with the final concentration of 10μg/ml. Controls included TZM-bl cells only and cells with virus only. Plates were incubated for 48hrs at 37^0C. Following incubation, 150μl of media were removed, and 100μl of Bright-Glo luciferase reagent (Promega Madison, WI) was added. After a 2min lysis, 150μl of the lysate was transferred into a 96 well black solid plate, and luminescence was measured using a

Victor 3 Luminometer (Perkin Elmer Waltham, MA). The 50% inhibitory dose (ID_{50}) titer was determined as the serum dilution that caused a 50% reduction in the RLU compared to the level in the virus control after subtraction of the cell control background.

ADCC: ADCC activity against SF162 coated target cells was measured using the ADCC-GranToxiLux (GTL) assay as previously described (330). This assay measures the percentage of target cells containing active Granzyme B (%GzB), a principal effector of cell-mediated cytotoxicity (331), after incubation with effector cells and serum samples. Target cells were $CEM.NKR_{CCR5}$ cells (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: CEM.NKR-CCR5 from Dr. Alexandra Trkola) coated with recombinant HIV-1 clade B SF162 gp120 (Immune Technology Corp, New York, NY). ADCC activity against HIV-1 infected cells was measured as a percentage of target cell killing using the Luciferase ADCC assay as described (216). For these assays, the $CEM.NKR_{CCR5}$ target cells were infected with a replication-competent infectious molecular clone designed to encode the SF162.LS (accession number EU123924) *env* gene in *cis* within an isogenic backbone that also expresses the *Renilla* luciferase reporter gene and preserves all viral open reading frames (332, 333). For both ADCC assay methodologies, the effector cells were PBMC isolated from an HIV-1 seronegative human donor heterozygous for 158F/V polymorphic variants of Fcγ receptor 3A. Serum was tested at baseline, week 20 (2 weeks post final DNA immunization), and week 34 (2-week post protein boost). Serum samples were tested using 4-fold serial dilutions ranging from 1:100 to 1:102,400. The results were calculated as maximal % specific Granzyme B (% GzB) and % specific killing for the GTL and Luc ADCC assay respectively after

subtracting the activity observed before immunization (after background subtraction). We also calculated the area under the curve (AUC) of ADCC activity versus serum dilution for the responses observed using each assay after background subtraction. For the Luc ADCC assay, the AUC was calculated above a 10% cut-off.

Statistics: Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc. La Jolla, CA) Analysis among groups was performed using an independent T-test and a Mann-Whitney test depending on the normalcy of data when two groups were being compared and an ANOVA when three groups were being compared. A p-value less than 0.05 was considered statistically significant.

2.3 Results

Multi-Envelope formulation induces potent cross-clade cellular response

In this study, we characterized cellular and humoral response induced by a highly optimized DNA prime and adjuvanted recombinant protein boost in RhMs. To address if multiple consensus Env immunogen are able to broaden vaccine-induced responses, two different priming immunizations were used. One group of five RhMs received four doses of pHIV Gag, Pol and consensus clade B Env gp140 DNA at weeks 0, 6, 12 and 18 followed by SF162 (Clade B) gp140 protein boost formulated in MF59 (adjuvanted protein) at week 32. Another group of five received a multi-Env (ME) DNA prime consisting of four doses of pHIV Gag, Pol and consensus clade A, B, C, D, and AE Env gp140 DNA at weeks 0, 6, 12 and 18 followed by the same protein boost. Four RhMs received the adjuvanted protein only at week 32. All of the consensus DNA immunogens

were created as previously described and included RNA and codon optimization as well as efficient IgE leader sequence (324). All constructs were formulated together in water and delivered in a single intramuscular immunization followed by adaptive EP as previously described (324).

We first assessed how increasing the breadth of the prime impacts cellular immune responses using multiparameter flow cytometry. Peripheral blood mononuclear cells (PBMCs) were stimulated overnight with overlapping Gag, Pol, Env A, Env B, Env C and Env D peptides and the total response calculated as the percentage of cells making IFN-γ, IL-2 or TNF-α. Following four DNA primes, we observed strong T-cell responses induced by both the clade B only and the ME-prime (**Figure 2.1*a, b,* Table 2.1**). The inclusion of additional Env plasmids did not further enhance the magnitude of the response. The total $CD8^+$ response to all antigens was comparable at 0.87 ± 0.38% for a clade B prime and 0.87 ± 0.27% for the ME-prime. In addition to $CD8^+$ responses, the synthetic DNA prime was also capable of inducing robust $CD4^+$ responses averaging 0.47 ± 0.15% for a clade B prime and 0.48 ± 0.13% for the ME-prime. There was a shift in the frequencies of Env responses with the clade B only immunization inducing 0.09% of $CD4^+$ and 0.10% of $CD8^+$ T-cell responses compared to 0.40% and 0.74% respectively for the ME DNA vaccination. Thus, within the ME DNA responses, Env accounts for 84% and 85% $CD4^+$ and $CD8^+$ responses compared to clade B only immunization where Gag and Pol responses dominate. Following a clade B recombinant protein (SF162) boost, clade B $CD4^+$ T-cell responses were significantly enhanced from the post-DNA prime level for the clade B only prime ($^*p < 0.05$) and trended up in the ME-prime

compared to pre-boost levels ($p = 0.06$) (**Figure 2.1*c***). The post-boost $CD4^+$ T-cell levels were also significantly higher for the clade B primed RhMs compared to the protein only levels ($*p < 0.05$) and trended towards an increase in the ME-prime RhMs ($p = 0.09$). The protein boost was able to slightly affect the $CD8^+$ clade B specific responses in the clade B Env only primed RhMs but not the ME group (**Figure 2.1*d***).

Multi-Envelope DNA vaccine produces superior humoral responses compared to a single DNA Env formulation.

We hypothesized that these $CD4^+$ responses would provide sufficient T-cell help for the induction of functional antibody responses. To assess the induction of antibodies with a single vs. ME-prime, we first determined the magnitude and breadth of antibody binding. The ME DNA prime induced higher and broader binding antibody responses compared with a clade B prime alone (**Figure 2.2**). After just two doses of ME DNA, all five animals produced antibodies which could bind clade A, B and C HIV-1 Env gp120 proteins, whereas no Env-binding antibodies were detected in sera from animals given two doses of the clade B DNA prime. Animals receiving the clade B DNA prime required the protein boost before all five animals produced clade B binding gp120 antibodies as well as cross-reactive clade A and C binding antibodies. Priming with the ME DNA strategy significantly enhanced binding titers against all three Envs after two ($*p<0.05$), three ($**p<0.01$) and four ($***p<0.001$) immunizations compared to the single clade B DNA vaccination. Additionally, ME prime-boost was significantly elevated from both the clade B prime-boost and recombinant protein alone following the protein boost at week 32 ($**p<0.01$ for all gp120s). Although the adjuvanted protein boost was an SF162 clade

B protein, we still observed an enhancement in clade A and C binding titers primed with ME DNA. Though these binding antibody responses do wane in the memory phase, we are still able to detect high binding titers eleven weeks after protein boost, with ME DNA prime-protein boost group having the highest levels.

ME DNA vaccination induces cross-clade binding titers and lower gp41 reactive antibodies compared to protein only immunization.

Having observed enhanced binding titers with the ME-prime, we wanted to understand further the binding epitopes recognized by the induced antibodies. We first tested the ability of antibodies to bind to various primary gp120 and gp41 proteins (**Figure 2.3**). After the DNA priming immunizations, the ME group had high binding antibodies to all gp120 Envs tested, which spanned clades A, B, C, and AE. These responses with ME DNA only were either above or on the same level as the protein only control group (**Figure 2.3*a*-*b***). When analyzing binding antibodies specific to gp41 region of HIV Env, the DNA only groups induced a lower level of binding compared to the protein only controls (**Figure 2.3*c***). Upon receiving the protein boost, gp41 specific antibodies were expanded to levels above the DNA only groups. We do see an increase in binding across multiple different Envs in the clade B DNA prime-protein boost group, these levels are still lower than the ME prime-protein boost and reflect more closely the levels of protein only immunization.

Vaccine-induced antibodies target the V3 loop of gp120

Recently, two key areas in the gp120 protein have been found to important in vaccine protection. Antibodies binding to both V1/V2 and V3 regions contribute to the

moderate efficacy of the RV144 trial (211, 212, 214, 329, 334, 335). Therefore, antibody binding to V1/V2 and V3 epitopes were assessed after DNA prime-protein boost or after protein only immunization (**Figure 2.4**). The V1/V2-gp70 fusion protein carrying the following V1/V2 sequences were tested: strain A2 clade B, consensus A, consensus C and consensus AE. Minimal ELISA binding activity (at a serum dilution of 1:35) was noted with the strain A2 clade B V1/V2-fusion protein using post-boost sera from RhMs that received the prime-boost regimens (data not shown). However, there was substantial reactivity (2 to 40 fold increase in OD values) in the sera of all animals during the immunization regimen against cyclic V3 peptides carrying the sequences of consensus A and C as well as the sequence of clade B strain MN (**Figure 2.4**). Importantly, immunization with ME DNA induced similar levels of binding when compared to the protein only immunization group. These levels are increased significantly when ME DNA was followed by protein boost ($p < 0.001$ compared to pre-boost levels), inducing antibody levels higher than DNA or protein only immunization. To further define the specificity of the antibody binding, we mapped linear epitopes within the V3 loop using overlapping 15-mer peptides derived from the consensus clade C Env (**Figure 2.5*a***). Using a serum dilution of 1:50, we observed the strongest binding to the peptide that spans the V3 crown (peptide 9261) in the sera of animals immunized with the ME DNA prime-protein boost (**Figure 2.5*b***). The sera were also able to bind to a peptide that contains the N-terminal β-sheet and the crown of V3 (peptide 9259).

DNA vaccination drives increase antibody avidity which was boosted upon protein immunization

To further characterize the humoral responses induced by DNA prime-protein boost regimen, we investigate how the antibody avidity to consensus clade A, B, and C gp120 changed over time. The ability to induce highly avid antibody has been shown to be a correlate of protection in NHPs (336) and is especially important in a prophylactic HIV vaccine where Env density is low, potentially preventing the possibility of bivalent binding (337). Due to the limit in antibody titers after DNA vaccination with the clade B only regimen, antibody avidity was determined using the ME DNA group (**Figure 2.6*a***). The avidity index for clades A, B, and C increased, though not significantly, after 2nd and 3rd immunization. After the 4th immunization, the avidity index against clade B gp120 increases to levels significantly higher (*p <0.05) than after the 2nd immunization. For clades A and C gp120, however, the avidity index after 4th DNA immunization was slightly lower than after the 3rd. Upon protein boosting, the avidity index for all clades increased to close to 100, which was significantly higher compared to levels after 2nd, 3rd, and 4th immunization and these levels were maintained into memory. The avidity index after protein boost was compared across clade B DNA only prime, ME-prime and protein only immunization. The ME-prime resulted in significantly higher avidity index after protein boost compared to clade B only immunization (clade A gp120 ***p < 0.001, clade C gp120 ****p<0.0001) and protein only immunization (clade A gp120 **** p<0.0001, clade B gp120 ****p<0.0001, clade C gp120 ****p<0.0001) (**Figure 2.6*b***). Interestingly, for the clade B gp120, even with the low amount of binding antibodies after

DNA only vaccination, the clade B only DNA prime induced significantly higher avidity after clade B protein boost compared to protein only immunization (**$p<0.01$). Memory responses were also investigated and for both the clade B DNA prime-protein boost and the protein only immunization; the avidity index was increased at the memory time point compared to the levels two weeks after vaccination (**Figure 2.6*c***). For all clades, at the memory time point, the avidity index was significantly higher for the ME prime-protein boost group compared to clade B only prime-boost and protein only groups. Since the avidity index for the ME DNA prime-protein boost two weeks post vaccination and memory time points were close to 100, we also investigated at which dilution the avidity index began to decline (**Figure 2.7**). When serum was diluted four-fold lower, the avidity index was slightly decreased and continued to drop down at each sequent dilution. The avidity index curves for both two weeks post protein boost and memory are similar suggesting that these levels are maintained but not necessarily increased.

Monovalent protein boost increases functional antibody titers primed by DNA vaccination

Having characterized binding antibodies, we next sought to determine how DNA, protein and a combined DNA prime-protein boost compared to the induction of neutralizing antibodies. Neutralizing titers were measured two weeks following the final immunization using the TZM-bl assay against clade AE, B and C tier 1 Env-pseudotyped viruses. We observed cross-clade neutralizing antibodies after the ME DNA prime-protein boost compared to either ME DNA, or protein delivered alone (**Figure 2.8*a***). In fact, all five ME DNA prime-protein boost animals had neutralizing titers to MN.3 and

MW965.26 that averaged two orders of magnitude higher than titers induced with ME DNA alone. After protein boost, the neutralization titers against MN.3 (ID_{50} range: 337-4434) were significantly higher ($p<0.05$) compared to DNA only (ID_{50} range: 45-192) and protein only immunization (ID_{50}: baseline). Similar trends were seen with MW965.26 pseudotyped virus, where neutralization titers induced by ME prime-protein boost (ID_{50} range: 256-9379) were increased, though not significantly, compared to DNA only (ID_{50} range: 67-280) ($p<0.13$) and protein only (ID_{50} range: baseline-27) ($p<0.1$). Animals immunized with ME DNA alone or with ME DNA prime-protein boost had low neutralizing responses to the clade AE TH023.6 pseudovirus (ID_{50} range ME DNA: baseline-177; ME + protein: baseline-1897; protein only: baseline). A single dose of gp140 protein alone resulted in essentially no neutralizing antibodies to any of the three pseudoviruses tested. These neutralization titers wane into the memory phase, with ME DNA prime-protein boost having levels below 10^2 for all three pseudotyped viruses.

Lastly, we examined the ability of the different vaccine platforms to induce functional antibody responses capable of inducing antibody-dependent cellular cytotoxicity (ADCC) as an indication of the induced Fc-mediated Ab functions capable of recognizing infected cells. We analyzed samples using HIV-1 clade B SF162 gp120 coated cells, homologous to the recombinant protein boost (330). We observed significantly higher ADCC, measured as both maximal %GzB activity and %GzB activity AUC (**Figure 2.8*b*-*c***), in animals that received the ME DNA prime-protein boost when compared with DNA alone (* $p<0.05$) or with protein alone (** $p<0.01$). We next evaluated the ADCC response using HIV-1 SF162-infected target cells (Luciferase

ADCC assay). The responses detected for the ME DNA prime-protein boost group were higher when compared to the other two groups. However, these differences did not reach statistical significance (**Figure 2.8*d*-*e*).**

2.4 Discussion

There is a critical need for an effective HIV vaccine to induce helper cells to support cytotoxic T-cell responses that can kill virus-infected cells, as well as provide strong B-cell help required for the maturation of antibodies and induction of B-cell memory. The delivery of consensus Env DNAs from multiple clades can enhance the breadth of the T-cell response that could help avoid T-cell escape upon infection. By using the consensus Envs, a DNA vaccine can specifically encode conserved T-cell epitopes which could be included in many different strains. Importantly, the robust responses generated with a synthetic DNA prime were efficiently boosted by the administration of an adjuvanted recombinant protein. While $CD4^+$ T-cell responses were more effectively boosted with protein, modest boosting of $CD8^+$ T-cells were also observed, especially in the animals receiving only the clade B prime, where a larger percentage of the T-cell response was matched to the clade B recombinant boost.

In addition to robust T-cell responses, in this study, we observed for the first time the ability of synthetic DNA to induce high titers of binding antibodies, similar to protein vaccine platforms which have been characterized as being able to induce strong humoral responses (338-340). Though the clade B only group induced lower humoral responses, this was not due to lower protein expression as both $CD4^+$ and $CD8^+$ T cell responses against clade B Env peptides was higher in the B only group compared to the ME DNA

immunization ($CD4^+$: 0.087% vs 0.046%, $CD8^+$: 0.102% vs 0.064% respectively) (**Table 2.1**). These data also suggest that an ME DNA prime can elicit a broad humoral response and both heterologous and homologous responses can be boosted with a single recombinant protein. Also, we observed lower gp41 binding after four doses of DNA immunizations compared to a single dose of recombinant gp140 Env (**Figure 2.3*c***). Though preliminary, this suggests that the DNA vaccination focused the humoral responses to the gp120 region of Env, even when delivered in a full length or truncated gp140 form. This response could be due to the antigen being presented on the surface of transfected cells, leading to less exposure of the gp41 region when compared to recombinant free protein. Additionally, the avidity index of humoral responses was significantly higher when RhMs are primed with DNA followed by protein boost compared to protein only immunization (**Figure 2.6**). Even for the group immunized with clade B only DNA, the avidity to consensus clade B gp120 was significantly higher after protein boost compared to the protein only immunizations. This suggests that DNA vaccination could be inducing adequate help, which is expanded upon boosting with protein. The functional antibody response observed was qualitatively similar to the binding antibody response with the ME DNA prime-protein boost inducing the highest neutralizing and ADCC titers compared to either platform alone (**Figure 2.8**). The ability to boost with multiple protein Envs may further increase antibody breadth, titers, and potency. Studies investigating differences between heterologous/homologous prime-boost immunizations containing multiple Envs are of interest, and this study provides a strong rationale for these.

The ability to induce humoral responses to specific portions of the HIV Env could increase the protective efficacy of the vaccine regimen. In the case of the modestly successful RV144 trial, these antigenic regions include the V1/V2 region as well as the V3 region. We detected minimal to no vaccine-induced antibodies to V1/V2 fusion proteins. These responses may not have been adequately primed by the DNA immunization nor further expanded by the adjuvanted protein immunization. Even though there was limited V1/V2 binding, we did observe substantial induction of antibodies binding to the V3 region of clades A, B, and C (**Figure 2.4**). These responses were highest in the ME DNA prime-protein boost immunized group. Analysis of the RV144 trial demonstrated that antibodies against V3 inversely correlated with risk of infection in a subpopulation of vaccines which had low levels of serum IgA Env-specific antibodies (214). Additional analysis revealed that these antibodies would be able to exert immune pressure against the virus (329, 341). The antibodies were found to predominately bind to a region that included the I^{307} amino acid, which is a key contact point for many V3 binding antibodies (342-344). This amino acid is maintained within our peptides used for linear mapping and was included in the two peptides that indicated the highest binding. Though glycan-independent V3 antibodies are less broad and potent in their neutralization capacity than the glycan-dependent V3 antibodies, the former have been shown to be important in reducing infection in the RV144 trial and in providing sterilizing protection in passive transfer challenges (345-348). Thus, the ability for a vaccine to induce such antibodies may be important for protection.

The improved immunogenicity seen within this study was achieved in the absence of employing immune plasmid adjuvants to increase vaccine-induced responses. The inclusion of molecular adjuvants such as IL-12, IL-28 or CCL28 may further enhance the cellular and humoral responses achieved by a synthetic DNA prime (57, 58, 113, 349-351). Potentially, the inclusion of one of these plasmid adjuvants could help increase memory responses leading to the maintenance of neutralizing antibodies. Additionally, novel delivery devices such as intradermal electroporation have shown promising pre-clinical results and the ability to induce improved humoral responses (60, 61, 321). Lastly, boosting with more than one dose of recombinant protein, protein from multiple clades, or recombinant epitope-scaffold proteins that focus the immune response on particular regions of the Env, as well as the use of alternative adjuvants may further improve the breadth, magnitude and maintenance of the HIV-specific response. These results merit future pre-clinical evaluation to test further the limits of polyvalency from more potent DNA constructs on inducing anti-HIV immune responses. Improved DNA primes may be combined with multiple platforms to improve platforms performance.

Tables

CD3+ CD4+																
	RhM	Gag	Avg Gag	Pol	avg Pol	Env B	avg Env B	Env A	avg Env A	Env C	avg Env C	Env D	avg Env D	total Env	avg total Env	total response	avg total response
B only	1	0.008	0.200	0.035	0.200		0.087								0.087	0.043	0.469
	2	0.154		0.033		0.079								0.079		0.266	
	3	0.210		0.233		0.059								0.059		0.503	
	4	0.094		0.480		0.072								0.072		0.646	
	5	0.533		0.217		0.138								0.138		0.887	
ME only	1	0.091	0.038	0.028	0.038	0.100	0.046	0.200	0.155	0.307	0.092	0.096	0.106	0.703	0.399	0.823	0.476
	2	0.051		0.082		0.015		0.337		0.027		0.160		0.538		0.671	
	3	0.014		0.036		0.057		0.191		0.045		0.207		0.499		0.549	
	4	0.019		0.032		0.044		0.029		0.056		0.057		0.186		0.237	
	5	0.015		0.015		0.015		0.017		0.025		0.013		0.070		0.100	

CD3+ CD8+																	
	RhM	Gag	Avg Gag	Pol	avg Pol	Env B	avg Env B	Env A	avg Env A	Env C	avg Env C	Env D	avg Env D	total Env	avg total Env	total response	avg total response
B only	1	0.105	0.435	0.054	0.349		0.102								0.102	0.159	0.865
	2	0.188		0.198		0.026								0.026		0.412	
	3	0.218		0.081		0.026								0.026		0.325	
	4	0.114		1.031		0.081								0.081		1.226	
	5	1.551		0.379		0.276								0.276		2.206	
ME only	1	0.109	0.065	0.014	0.065	0.065	0.064	1.372	0.450	0.046	0.077	0.239	0.147	1.722	0.738	1.845	0.868
	2	0.086		0.092		0.027		0.316		0.072		0.219		0.634		0.813	
	3	0.066		0.060		0.068		0.278		0.205		0.212		0.763		0.889	
	4	0.045		0.021		0.115		0.065		0.022		0.039		0.241		0.307	
	5	0.017		0.137		0.043		0.219		0.041		0.028		0.331		0.485	

Table 2.1: $CD4^+$ and $CD8^+$ T cell responses induced to each vaccine included antigen. Cellular responses were determined after final DNA vaccination. PBMCs were stimulated with overlapping peptides to Gag, Pol, and Envs from clade A, B, C, and D and stained for polyfunctional flow. Responses were characterized as cells expressing IFN-γ, TNF-α or IL-2.

Figures

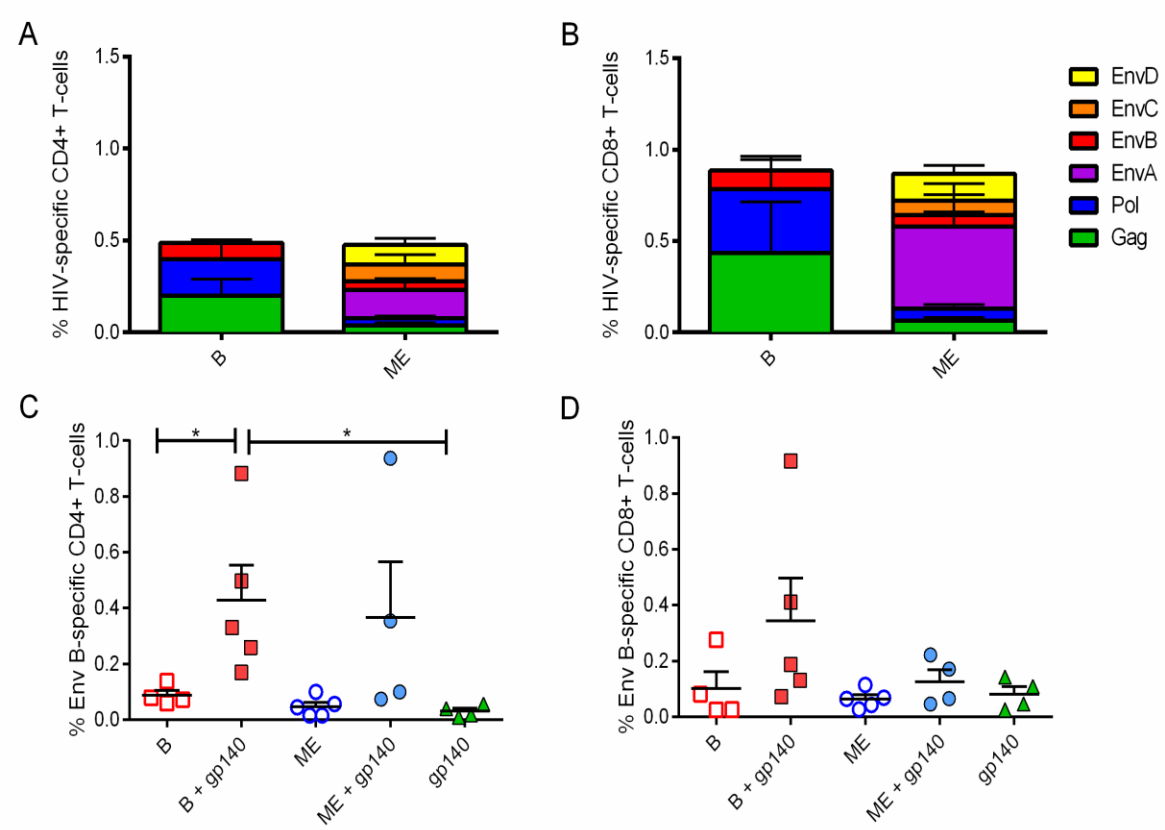

Figure 2.1: DNA prime-protein boost induces robust CD4$^+$ and CD8$^+$ T-cell responses. T-cell responses were measured two weeks following the fourth dose of DNA (B or ME) and after the recombinant gp140 boost using polyfunctional flow cytometry. DNA vaccines consisted of pGag/Pol/Env B (B) or pGag/Pol/Env A, B, C, D, A/E (ME). Responses are the sum of any cells producing IL-2, TNF-α, or IFN-γ. Total CD4$^+$ (***a***) and CD8$^+$ (***b***) T-cell responses to individual HIV antigens following the DNA prime. Total CD4$^+$ (***c***) and CD8$^+$ (***d***) T-cell responses to the HIV Env clade B peptide pools following DNA prime (B or ME), DNA prime plus adjuvanted protein boost (B+gp140 or ME+gp140) or the protein alone (gp140). Bars represent mean with error bars representing standard error. Significance reported as * $p<0.05$ as determined by modified ANOVA.

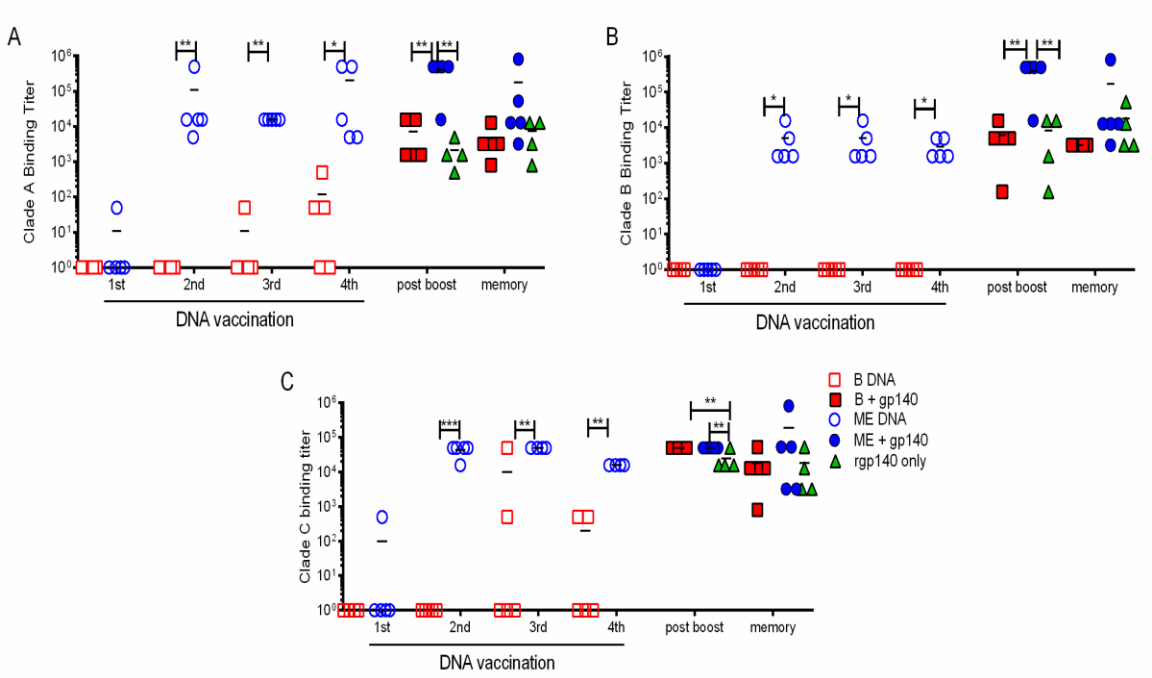

Figure 2.2: ME DNA vaccination induces increased binding titer responses compared to single Env immunization. Responses were measured two weeks after each immunization and eleven weeks after protein boost (memory). Vaccine groups were pGag/Pol/Env B prime-gp140 boost (clade B DNA) (red squares), pGag/Pol/Env A, B, C, D, A/E prime-gp140 boost (ME DNA) (blue circles) or recombinant gp140 boost alone (green triangle). Endpoint binding titers against consensus clade A (***a***), clade B (***b***) and clade C (***c***) gp120 following each immunization and memory time point. Horizontal brackets represent significant differences as determined by modified ANOVA between groups at a given time point (*p<0.05, **p<0.01, ***p<0.001). Bars represent mean.

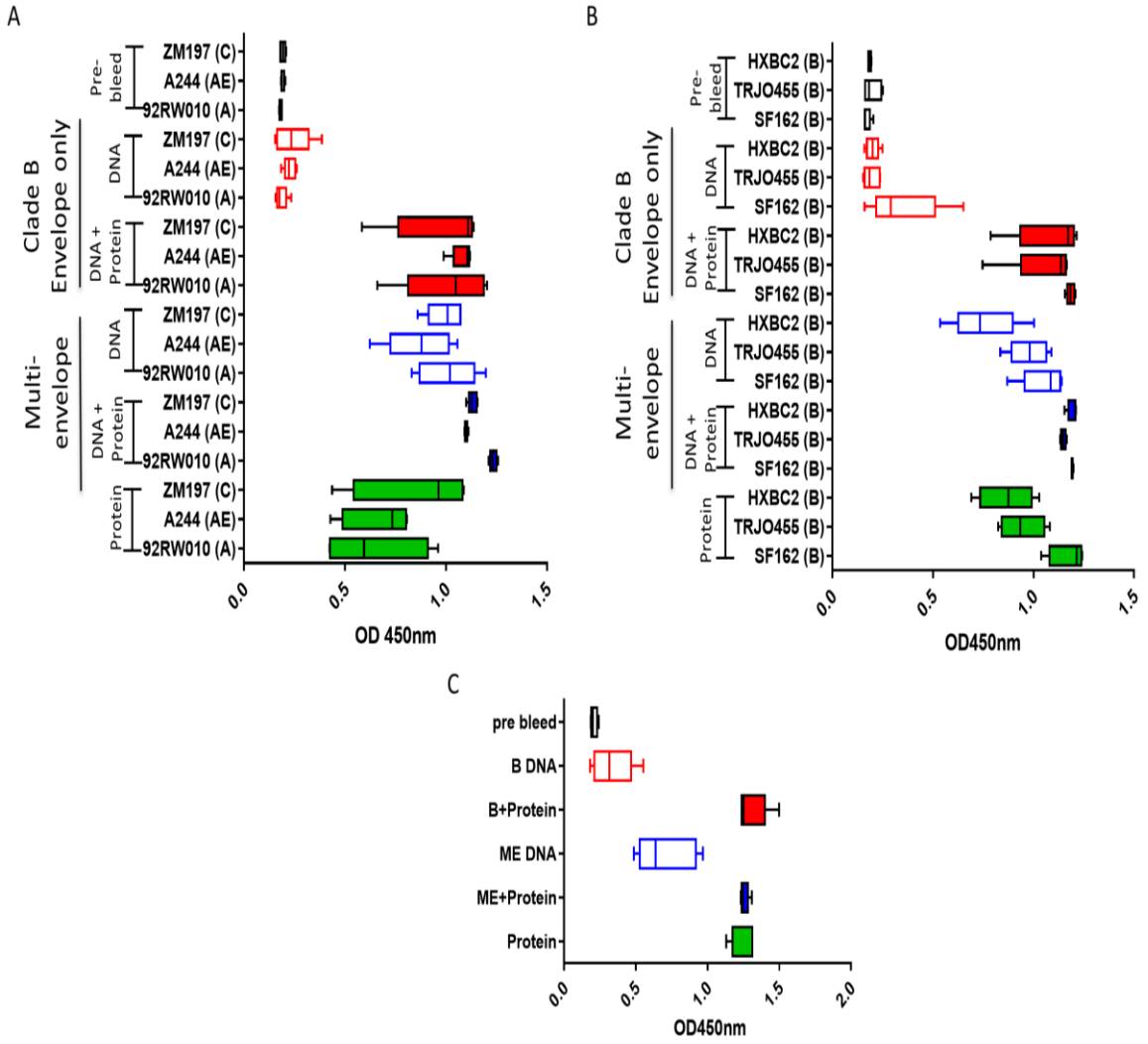

Figure 2.3: ME DNA prime-protein boost increases the breadth of antibody binding compared to single Env prime. Serum binding responses were measured two weeks after DNA only immunization (week 20), DNA + protein boost immunization (week 34) or protein only immunization (week 34) for both single clade B DNA prime and ME DNA prime. Binding antibodies against gp120s spanning clades B (***a***) and clades A, C, and AE (***b***) as well as against whole protein gp41 (HXBC2) (***c***) were determined. Optical density values are reported as box and whisker plots.

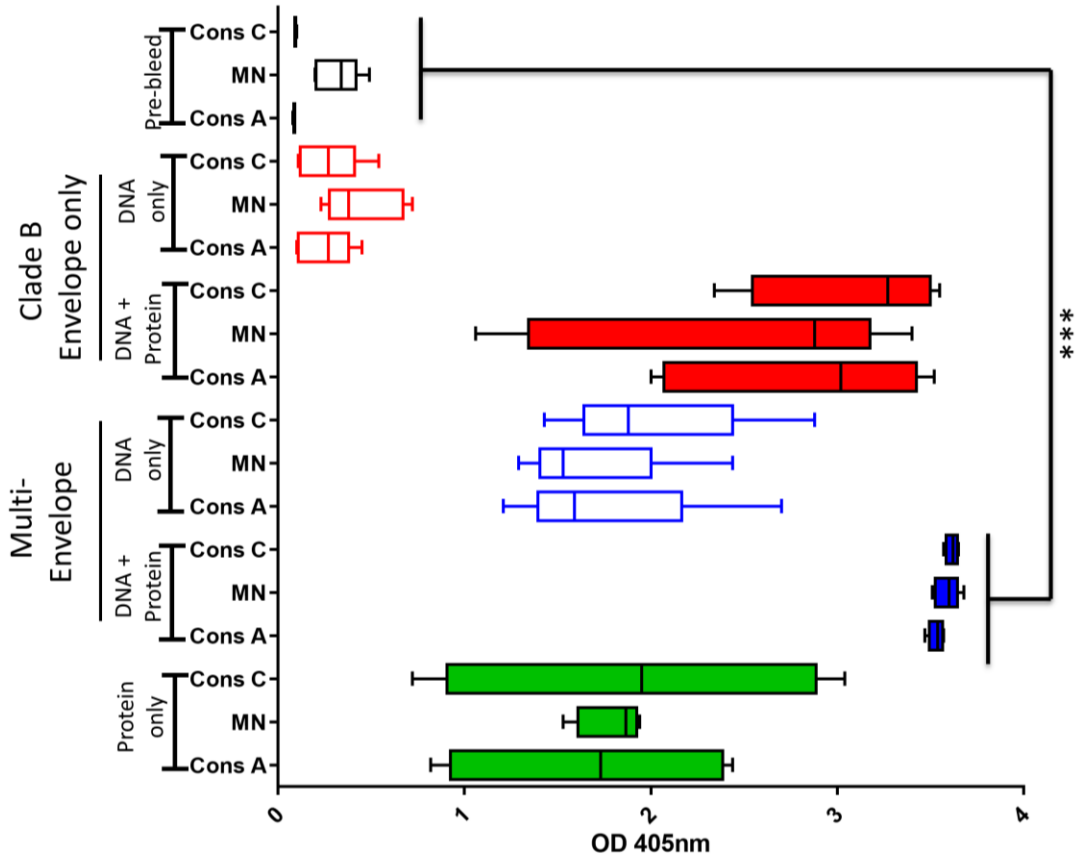

Figure 2.4: V3 binding induced following ME DNA prime and expanded after boost. Serum was diluted 1:35 and analyzed using sera drawn at week 0 before vaccination, two weeks following the fourth DNA prime (week20) and two weeks following the recombinant gp140 protein boost (week34). Binding antibodies were detected against the cyclic V3 peptides representing consensus A and C as well as the sequence from clade B (strain MN). Horizontal brackets represent significant differences as determined by modified ANOVA between groups at a given time point (***$p<0.001$). Optical density values are reported as box and whisker plots.

A

```
9259 CTRPNNNTRKSIRIG
9260     NNNTRKSIRIGPGQT
9261         RKSIRIGPGQTFYAT
9262             RIGPGQTFYATGDII
9263                 GQTFYATGDIIGDIR
9264                     YATGDIIGDIRQAHC
```

B

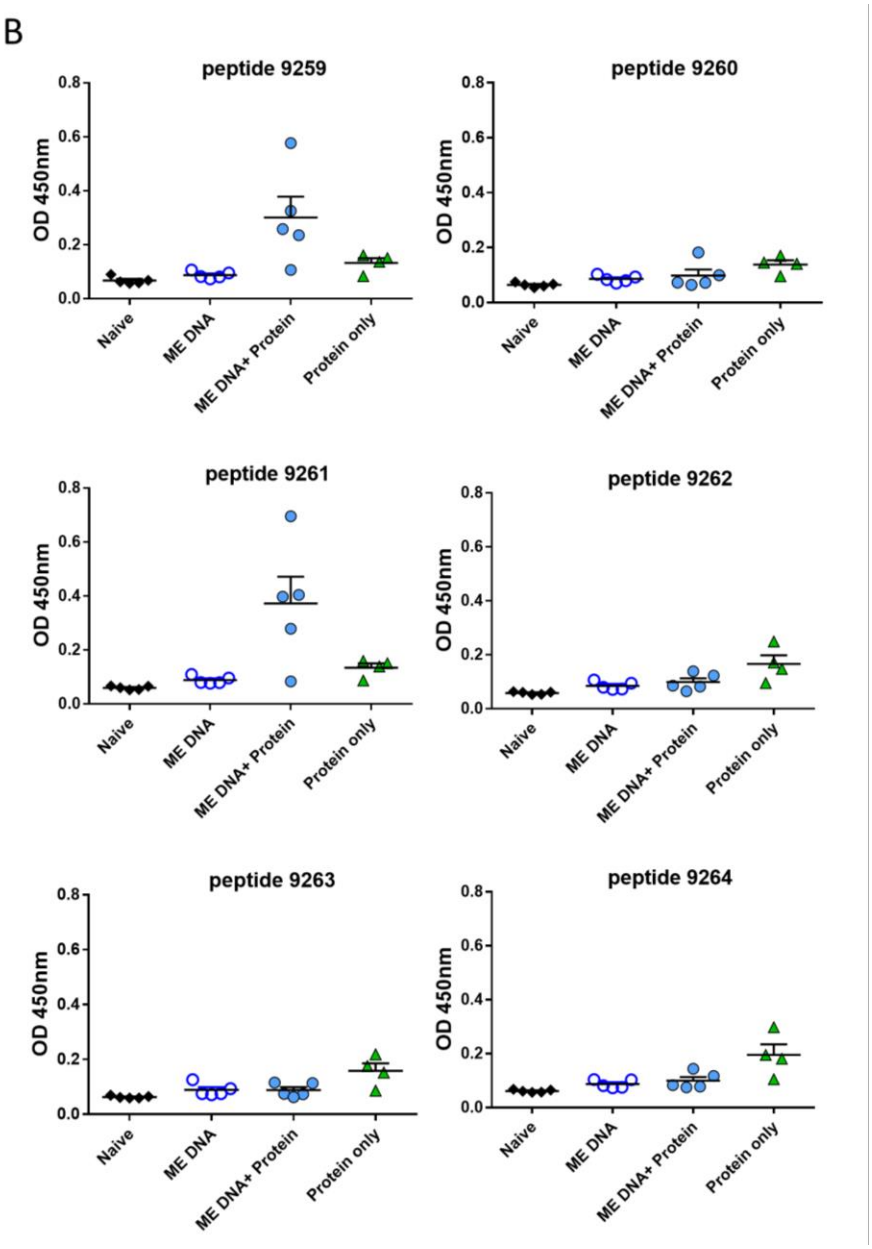

Figure 2.5: Vaccination induces binding antibodies to linear epitopes in the V3 crown and β sheet. Binding antibody epitopes were mapped using a serum dilution of 1:50 two weeks following the ME DNA prime (week 20), ME DNA prime-protein boost (week 34), or protein boost alone (week 34). (***a***) Consensus clade C peptides were used for the mapping of responses. (***b***) The DNA prime-protein boost was mapped to two sequences, 9259 (CTRPNNNTRKSIRIG) and 9261 (RKSIRIGPGQTFYAT). Bars represent mean with error bars representing standard error.

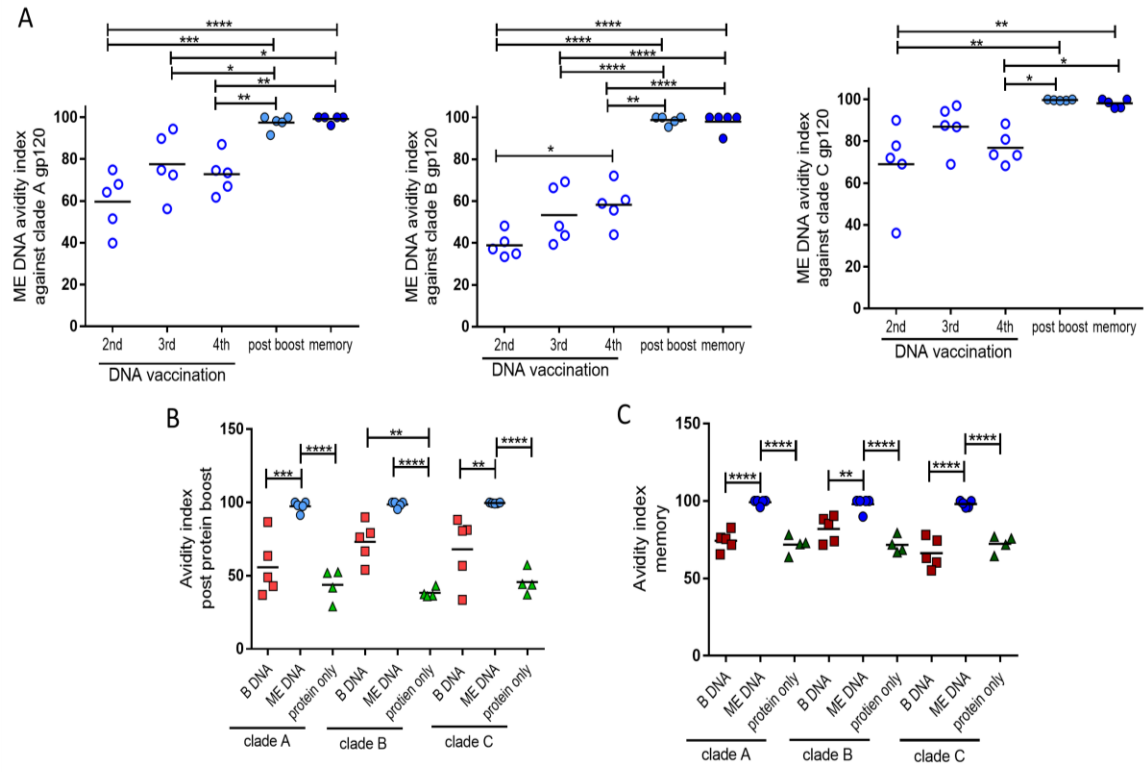

Figure 2.6: ME DNA prime increased the avidity index of humoral responses after protein boost compared to clade B DNA prime and protein only vaccination. The avidity index of humoral responses against consensus clade A, B, and C gp120s were determined two weeks after each immunization and into memory for the ME DNA prime-protein boost group (***a***). The avidity index was also analyzed two weeks after protein boost (***b***) and at a memory time point (***c***) for all three groups against consensus clade A, B and C gp120. Horizontal brackets represent significant differences as determined by modified ANOVA between groups at a given time point (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Bars represent mean.

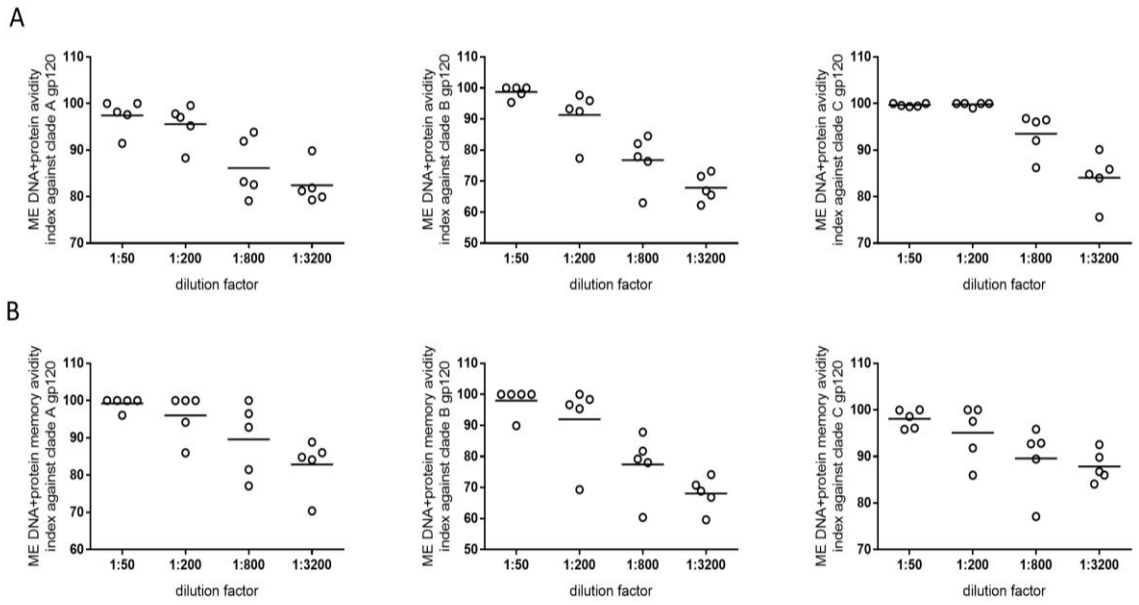

Figure 2.7: Avidity index of ME DNA prime-protein boost responses decreases with increasing dilution. The avidity index of the humoral response induced by the ME DNA prime-protein boost was assessed at decreasing dilutions two (***a***) and eleven (memory) (***b***) weeks post protein boost. Responses were determined against consensus clade A, B, and C gp120 proteins.

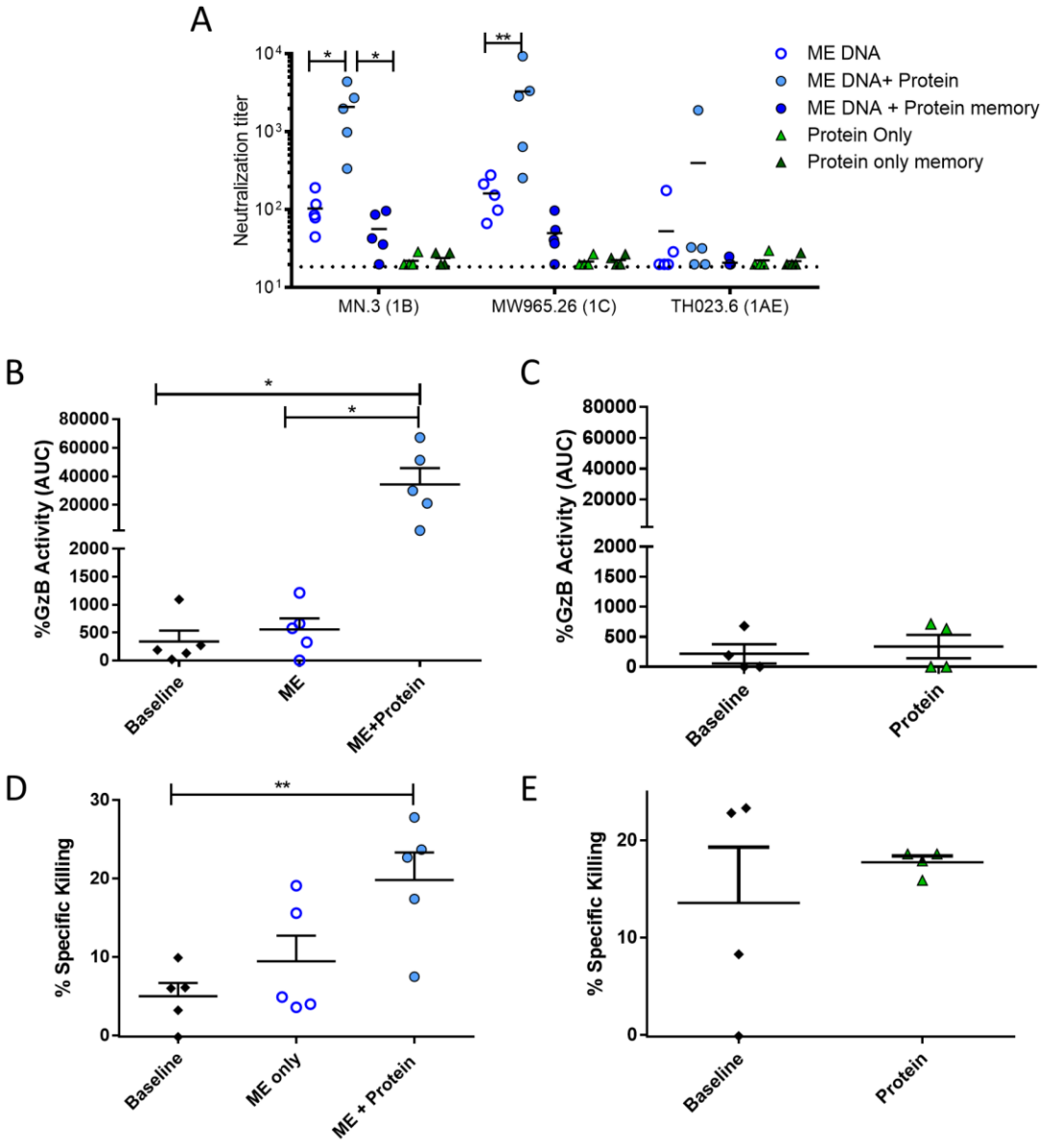

Figure 2.8: ME DNA prime-protein boost induces functional antibodies capable of neutralization and ADCC. (***a***) Neutralizing antibodies were measured two weeks after the ME DNA prime, ME DNA prime-protein boost and into memory. Neutralization titers were also determined for protein only immunizations two weeks after vaccination and into memory. ADCC was analyzed two weeks following the ME DNA prime, ME

DNA prime-protein boost or protein boost alone. The maximal % specific (***b***) and area under the curve (AUC) (***c***) Granzyme B activity was determined using the GTL assay with SF162 coated targets. Background (pre-immunization) activity was subtracted from each measurement. The maximal % specific killing (***d***) and AUC specific killing (***e***) were determined using the luciferase assay with infected $CD4^+$ target cells. The values represent percent specific killing after baseline correction. Bars represent mean with error bars representing standard error. Significance determined using modified ANOVA (*$p<0.05$, **$p<0.01$).

CHAPTER 3: EXTREME POLYVALENCY INDUCES POTENT CROSS-CLADE CELLULAR AND HUMORAL RESPONSES IN SMALL ANIMALS AND NON-HUMAN PRIMATES

3.1. Introduction

A major obstacle to vaccine development is the diversity of HIV-1 and creating an immunogen that can produce responses which will be broad enough to encompass the global or even regional diversity of the virus. As demonstrated in the above chapter, consensus immunogens have displayed considerable potential in driving T cell responses which exhibit cross-clade reactivity when compared to wild-type HIV immunogens (323, 324, 352-355). However, this coverage is limited to cellular responses and fails to induce a potent and broad neutralizing antibody response. Recently, it has been reported that guinea pigs vaccinated with transmitted founder gp140 Envelope proteins were able to induce low but broad neutralizing antibodies to both tier 1 and tier 2 viruses (356). This general induction of coverage may be ideal for a priming immunization, establishing a response which can be boosted with the addition of either chronic or consensus Envelopes.

Given the above requirement, DNA vaccination may be the optimal platform for a successful HIV vaccine. Advances in technology including codon and RNA optimization as well as electroporation can induce anti-HIV cellular responses to levels comparable with viral vectors (31). Also, this platform would allow for the expression of full-length gp160 protein and could allow for the presentation of the native trimer to the immune system. Cryo-EM structures of Envelopes have highlighted the differences between

gp120 and gp140 structures and the potential for off-target effects if the proper immunogen is not provided (357-359). DNA vaccination also allows for multiple difference plasmids to be delivered simultaneously, increasing the coverage of the immunization.

Though DNA vaccines against HIV can induce potent cellular immunity, antibody titers have remained low, are limited in functional antibody titers, and usually require a boost. Thus, we looked to expand the ability of the DNA platform to induce both binding and neutralizing antibodies. In addition to the consensus immunogens which we have previously published on, we have created a panel of 26 plasmids expressing acute or early primary gp160s (360-362). Each plasmid was immunogenicity in mice, inducing either cellular, humoral or both responses. Formulation studies were performed to ensure that delivery of multiple plasmids to a single site did not damp the vaccine induced response. We determined that delivery of up to 6 plasmids into a single site increased humoral responses over delivering each plasmid to a separate site. After down selection of a combination of plasmids in rabbits, the most potent inducer of neutralization was moved into non-human primates (NHP). NHPs immunized with a combination of 14 different Env plasmids developed strong cellular response against heterologous consensus clade A, B and C peptides. Additionally, strong binding titers were induced which remained elevated into memory time points. These NHPs developed a range of binding to V1/V2 scaffold proteins, suggesting a potential binding epitope. Tier 1 neutralization titers were observed across time, and after the final immunization, we were able to detect low neutralization titers against SF162P3 (tier 2 Env).

Additionally, with DNA vaccination alone, we were able to induce potent antibody-dependent cellular cytotoxicity (ADCC) against multiple gp120 and gp140 coated targets. To our knowledge, this is one of the first times that ADCC has been induced with only DNA vaccination. Thus the induction of strong cellular as well as humoral responses, which have limited but detectable functionality, suggest that DNA vaccines composed of acute/ early HIV Envs could be the ideal priming immunizations. Further expansion of these responses could be observed with the use of a protein boost composed of acute or chronic Envs.

3.2. Materials and methods

Envelope immunogens: Plasmids expressing codon and RNA optimized HIV-1 Envelope glycoproteins (gp160) were made synthetically using OptimumGene® Codon optimization analysis (GenScript, Piscataway, NJ). Inserts were then cloned into the pVAX (Invitrogen, Carlsbad, CA) backbone using either BamHI/ XhoI or BamHI/EcoRI cloning sites. Each insert was under the control of the cytomegalovirus immediate-early promoter. A description of each of the inserts can be found in **Table 3.1**.

Expression of plasmids: Each plasmid was tested *in vitro* for proper expression. Briefly, HEK 293T cells (ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlas, Ft. Collins, CO) and 1% penicillin and streptomycin (Thermo Fisher Scientific). Twenty-four hours before transfection, $7.5x10^5$ cells were plated in 1.5mls of media in a six well dish. Each plasmid was used in a separate transfection with pVax empty backbone serving as a negative control. Transfection was performed using

NeoFectin transfection reagent (NeoScientific, Cambridge, MA) following manufacturer's protocol. Forty-eight hours after transfection, cells were collected and washed with PBS and lysed using Cell Signaling lysis buffer (Cell Signaling, Danvers, MA) modified with EDTA-free protease inhibitor (Roche, Basel, Switzerland). Bradford assay was used to quantify protein concentration of lysate following manufacturer's protocol (BioRad, Hercules, CA). The normalized lysate was then run on a NuPAGE® 12% Tris-Acetate gel and transferred to a PVDF membrane following manufacturer's protocol (Thermo Fisher Scientific). After 1 hour blocking with LI-COR Odyssey blocking buffer (LI-COR, Lincoln, Nebraska), membranes were probed overnight with a 1:1000 dilution of human 2G12 antibody (ImmuneTechnologies Corp, New York, NY) and 1:5000 dilution of mouse anti-human β-actin (Sigma-Aldrich, St. Louis, MO) as a loading control. After washing with PBS-Tween, 1:10,000 dilution of secondary goat anti-human IRdye 680 and goat anti-mouse IRdye 800CW (LI-COR) antibodies were added in blocking buffer supplemented with 0.1% Tween and 0.01% SDS (Sigma-Aldrich). Membranes were probed for 1 hour at room temperature followed by washing with PBS-Tween and PBS. Membranes were then scanned using LI-COR Odyssey CXL.

Immunization of Mice: All mice were housed in compliance with the NIH and University of Pennsylvania Institutional Animal Care and Use Committee guidelines. To test for immunogenicity, 6-8 week old C57Bl/6 mice (Jackson Laboratories, Bar Harbor ME) were immunized with 25ug of each plasmid followed by *in vivo* electroporation (EP) using the CELLECTA® 3P adaptive constant current electroporation device (Inovio Pharmaceuticals, Plymouth Meeting, PA) as previously described (323). Mice were

immunized three times at 2-week intervals and sacrificed one week after final vaccination to assess vaccine-induced immune responses.

Immunization of Guinea Pigs for Formulation Study: All guinea pigs were housed and handled according to the standards of the Institutional Animal Care and Use Committee (IACUC) at BioTox Sciences (San Diego, CA). Female Hartley guinea pigs (300-350 grams) were immunized with 100ug of DNA intradermal mantoux injection every three weeks with *in vivo* EP as described above. Six clade A plasmids were delivered to six separate sites or formulated together and spread across six different sites. Each guinea pig received the same total amount of DNA, volume of injection and sites of immunization. Blood was collected for analysis before every vaccination.

Immunization of Guinea Pigs for *in vivo* analysis: To differentiate each of the Envelopes, three tags were added via plasmid mutagenesis (Genscript): pQ168ENVe2-his, pQ23ENV17-flag, pDu151.2-cMyc. All tags were added to the C-terminus of the protein. Two female Hartley guinea pigs (300-350 grams) were injected with 16.5ug of each plasmid (50ug of total DNA) formulated together and injected ID using a mantoux injection. The area was then immediately electroporated using the ELGEN-SEP 4x4 array (3 pulses at 25V, pulse length 100msec, pulse delay 200msec). Guinea pigs were then euthanized 24 hours after treatment, and the vaccinated skin was harvested. The skin biopsies were fixed by immersion in 4% paraformaldehyde (Sigma-Aldrich) for 12hr at 4ºC. After washing with PBS, biopsies were immersed in 15% sucrose solution followed by immersion in 30% sucrose. The biopsies were then embedded in O.C.T compound (Fisher Scientific) and snap frozen. The skin was then sectioned in cryostat at a thickness

of 15µm, placed on a glass slide and stored at -80ºC. Sections were then incubated with BSA-Histology buffer (0.5% (v/v) Triton-X, 3% (w/v) BSA in 1x PBS) for 30 min at room temp. Primary antibodies were then added to each section and incubated for 2 hours at room temp. Primary antibodies include: Goat anti-FLAG (1:1000 QED Bioscience, San Diego, CA); mouse anti-HIS (1:200 Abcam, Cambridge, UK) and rabbit anti-myc (1:100, Abcam). After washing with PBS, the first round of secondary antibodies were added in BSA-Histology buffer. Following washing with PBS, sections were incubated with a second round of secondary antibodies. Round one included: donkey anti-goat IgG – AF488 (1:200 Abcam) and donkey anti-rabbit IgG- AF55 (1:200 LifeTechnologies). The second round included goat anti-mouse- AF647 (1:200 Invitrogen). Sections were washed again and mounted with DAPI-Fluoromount (Fisher Scientific) and covered with a coverslip. Sections were imaged with Olympus BX51 Fluorescent Microscope, QImaging Retiga3000 camera and QImaging software.

Immunization of Rabbits: All rabbits were housed and handled according to the standards of the Institutional Animal Care and Use Committee (IACUC) at BioTox Sciences (San Diego, CA). Female New Zealand white rabbits (1900 grams) were immunized using 100ug/ plasmid of DNA intradermal every three weeks with *in vivo* EP as described above. All plasmids were formulated together and injected into multiple sites (3-6 depending on the number of plasmids). Each site received 100ug of mixed DNA in a 100ul mantoux injection. Blood was collected for analysis before every vaccination.

Immunization of Non-human primates: Four Indian rhesus macaques were housed at Bioqual (Rockville MD) according to the standards of the American Association for Accreditation of Laboratory Animal Care, and all animal protocols were IACUC approved. All animals received six vaccinations: the first four were administered intradermally, and the last two were administered intramuscularly. The first and second vaccination on weeks 0 and 6 were a combination of six clade A primary Envelopes (1.0 mgs each), formulated together and delivered to 6 separate sites. The third immunization delivered on week 12 was a combination of three clade B Envelopes (1.0 mgs each), formulated together and administered to three different sites. The four immunization delivered on week 18 was a combination of five clade B Envelopes (1.0 mgs each), formulated together and administered to five different sites. The fifth and six vaccination were given on weeks 44 and 81, composed of all 14 Envelopes (1.0 mgs each) formulated together and delivered to a single site. All DNA deliveries were followed by *in vivo* EP with the constant current CELLECTRA® device (Inovio Pharmaceuticals, Plymouth Meeting, PA) with three pulses at 0.5A constant current, a 52ms pulse length and 1s rest between pulses.

Blood Collection: Animals were bled two weeks following each immunization (weeks 2, 8, 14, 20, 46, 83) and at memory time points (weeks 32, 43, 68, 81). Blood (15ml at each time point) was collected in EDTA tubes, and peripheral blood mononuclear cells (PBMCs) were isolated using the standard Ficoll-Hypaque procedure with Accuspin tubes (Sigma-Aldrich). An additional 10ml was collected into clot tubes for serum collection.

Mouse IFN-gamma Enzyme-linked immunospot assay (ELISpot): Ninety-six well filter plates (Millipore, Billerica, MA) were coated with anti-IFN-γ capture antibody (R&D, Minneapolis, MN) overnight at 4^{o} C. Spleens were isolated from mice one week after final immunization. After processing the spleens as previously described (323), $2x10^{5}$ cells were added to the blocked plates. Cells were stimulated with overlapping 15mer peptide pools for consensus clade A, B, or C gp160 (5ug/ml per peptide). Media alone and concanavalin A (Sigma-Aldrich) were used as negative and positive controls respectively. After 18hrs of stimulation, the plates were washed, and secondary detection antibody (R&D) was added for 24hrs at 4^{o} C. Plates were then washed and developed using the ELISpot Blue Color Module (Millipore) per the manufacturer's protocol. Plates were then scanned and counted using CTL-ImmunoSpot® S6 FluoroSpot plate reader (CTL, Shaker Heights, OH).

Mouse serum binding using enzyme-linked immunosorbent assay (ELISA): Before sacrificing, serum from mice was collected to determine the vaccine-induced humoral responses. Maxisorp 96 well plates (Thermo Fisher Scientific) were coated with 1ug/ml of consensus clade A, B, or C gp120; consensus clade A, B, or C gp140; or HXBC2 gp41 (clade B) (Immune Technology Corp.) in PBS and stored at 4^{o} C overnight. After blocking with 10% fetal bovine serum (FBS) in PBS for one hour, mouse serum was diluted 1:50 in 1% FBS in PBST (0.1% Tween). After one hour at room temperature and washing, secondary goat anti-mouse HRP-labeled antibody (Santa Cruz Biotechnology, Dallas, TX) was used at a 1:5000 dilution. Plates were washed and developed for 5 minutes using SimgaFast OPD tablets (Sigma-Aldrich) and stopped with 100ul of 2N

sulfuric acid (Sigma-Aldrich). The OD450nm was determined using the Promega GloMax plate reader (Promega, Madison, WI).

Endpoint binding titer ELISA: Maxisorp 96 well plates (Thermo Fisher Scientific) were coated with 1ug/ml of 92RW020, SF162, or ZM197M (Immune Technology Corp) and incubated overnight at 4^{o} C. Plates were blocked as described above for 1 hour at room temperature. Plates were then washed again and incubated with specific guinea pig, rabbit or NHP sera diluted with 1% FBS in 1xPBS + 0.02% Tween-20 for one hour at room temperature. Dilutions started at 1:50 and then a four-fold dilution was performed. After washing, plates were incubated with dilutions of horseradish peroxidase-conjugated goat anti-guinea pig (1:2000) or donkey anti-rabbit (1:5000) IgG (Santa Cruz Biotech) or goat anti-NHP (1:5000) (Southern Biotech, Birmingham, AL) for one hour at room temperature. The plates were developed and read as described above. Endpoint titers were determined as previously reported (45). Briefly, the upper prediction limit of Envelope-specific IgG antibodies was calculated using the Student t distribution. The upper prediction limit was defined as the standard deviation multiplied by a factor based on the number of naïve controls and a 95% confidence interval. Endpoint titer was the lowest dilution that remained above the upper prediction limit.

Avidity Index ELISA: Plates were coated with 1ug/ml of either 92RW020 (clade A), Sf162 (clade B) and ZM197 (clade C) gp120 (Immune Technology, New York, NY) in PBS. After blocking, guinea pig or NHP serum was diluted 1:100 or 1:500 (respectively) in 1% FBS in PBS-T. Each sample was run in quadruplicate where half of the wells were treated, and half were untreated. After one hour incubation, plates were washed five

times with PBS-T. Half of the wells for each sample were incubated with a denaturing reagent, 8M urea, for 5 minutes while the others were incubated with PBS. Plates were washed and incubated with goat anti-guinea pig IgG HRP (1:2000) (Sana Cruz Biotech) or mouse anti-NHP IgG HRP (1:5000) (Southern Biotech, Birmingham, AL) in 1% FBS in PBS-T. Plates were then developed as described above and OD450 values were obtained. The avidity index was determined by dividing the OD450 values of the treated by the untreated and multiplying by 100.

Neutralization: Neutralization was determined using the previously described TZM-bl based assay (304). The 50% inhibitory dose (ID_{50}) titer was determined as the serum dilution that caused a 50% reduction in the RLU compared to the level of the virus control after subtraction of the cell control background.

Rhesus IFN-gamma ELISpot: To determine cellular responses, **i**nterferon-gamma (IFN-γ) ELISpots (MabTech, Stockholm Sweden) were performed following manufacturer's protocols. Isolated PBMCs were stimulated overnight in the presence of either specific peptide antigens (Consensus clade A and B Envelope peptides (NIH AIDS Research & Reagent Program, Germantown, MD), R10 (negative control), or anti-CD3 (positive control). All samples were run in triplicate. Spot-forming units were determined using the CTL-ImmunoSpot® S6 FluoroSpot plate reader.

Intracellular staining of PBMCs: Intracellular staining of PBMCs was performed as previously described (61). Briefly, after isolation, PBMCs (1-2 x 10^6) were stimulated with pools of either consensus clade A, B or C peptides for 6h in a 96 well U-bottom

plate. Each peptide pool contained approximately 1μg of each peptide. Media only (R10) and PMA (0.1μg/ml) and ionomycin (0.5μg/ml) (BD Bioscience, San Jose, CA) were used as negative and positive controls respectively. All stimulations were performed in the presence of Golgi stop/ Golgi plug^{TM} (1:500 dilution BD Biosciences) and anti-CD107a (PE cy7 clone H4A3 BD Bioscience). After stimulation, cells were washed with PBS and stained with violet amine-reactive dye Live/Dead stain (Life Technologies, Carlsbad, CA) for 5min followed by surface staining for 30min at room temperature. Surface stain included CD4 (PECy5.5 clone S3.5 Invitrogen), CD8 (BV650 clone SK1 Biolegend, San Diego), CD95 (PE cy 5 clone DX2, Biolegend), CD28 (BV510 clone CD28.2 Biolegend) and dump channel antibodies CD14 (Pacific Blue clone M5E2 Biolegend) and CD16 (Pacific Blue clone 3G8 Biolegend). Cells were washed with PBS and fixed/permeabilized with BD Cytofix/Cytoperm (BD Biosciences) for 15min at room temperature. Following washing with BD Perm/Wash buffer, cells were stained with intracellular antibodies for 1hr at room temperature. Intracellular stain included CD3 (APC-Cy7, clone SP34-2 BD Bioscience), IL-2 (PE clone Mq1-17H12, Biolegend), IFN-γ (APC, clone B27 Biolegend), and TNF-α (PE-Cy7 clone Mab11, Biolegend). Cells were analyzed using a modified BD LSR II (BD Biosciences) and analysis performed with FlowJo 9.2 (Tree Star, Ashland, OR).

Binding antibody multiplex assay (BAMA): To further determine binding to various gp120s, gp140s, and V1/V2 scaffold proteins, a customized multiplex binding assay was used as previously described (146, 211). Proteins were a gracious gift from Drs. Hua-Xin Liao, Bart Haynes and Shan Lu. Serum from week 20 (post ID), week 46 (post-IM 1) and

week 83 (post-IM 2) were tested at six 5-fold serial dilutions starting at 1:80. The area under the curve (AUC) was calculated using GraphPad Prism.

Antibody-dependent cellular cytotoxicity (ADCC): ADCC activity against various Env coated target cells was measured using the ADCC-GranToxiLux (GTL) assay as previously described (330). Briefly, target cells were CEM.NKR$_{CCR5}$ cells (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: CEM.NKR-CCR5 from Dr. Alexandra Trkola) coated with recombinant HIV-1 gp120 against WITO (B), JR-FL (B) and 92UG037.1 (A) or gp140 1086 (C). Effector cells were PBMC isolated from an HIV-1 seronegative human donor heterozygous for 158F/V polymorphic variants of Fcγ receptor 3A. NHP serum was tested at baseline, week 20 (2 weeks post 4th ID immunization), week 46 (2-week post 1st IM boost), and week 83 (2 weeks post 2nd IM boost). Serum samples were tested using 4-fold serial dilutions ranging from 1:100 to 1:102,400. ADCC titers were calculated as the dilution at which responses were greater than or equal to 8% GzB expression.

Statistics: Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc. La Jolla, CA). Analysis among groups was performed using an independent T-test and a Mann-Whitney test depending on the normalcy of data when two groups were being compared and an ANOVA when three groups were being compared. A p-value less than 0.05 was considered statistically significant.

3.3. Results

Construction and design of primary isolate HIV-1 Envelopes and in vitro expression

A panel of plasmids expressing RNA and codon optimized HIV-1 gp160 primary Envelopes from clade A, B, and C were constructed using the pVAX backbone. All sequences were obtained from GenBank using the accession numbers listed in **Table 3.1**. Envelope sequences were isolated from patients that ranged in disease progress from acute/early transmitted isolates to Fiebig stage VI (360-362). To confirm expression of each plasmid, Western blot analysis was performed on transfected 293T lysate. All plasmids expressed and were detected by the neutralizing antibody 2G12 (**Figure 3.1**).

Immunogenicity of primary HIV-1 Env plasmids in mice

To ensure that each plasmid was immunogenic, C57Bl/6 mice were immunized with 25ug of each plasmid three times at 2-week intervals. One week after final immunization, cellular and humoral responses were determined against consensus clade A, B, and C. All plasmids induced either a cellular or humoral responses; however there was variation between different plasmids (**Figure 3.2**). For example, the highest cellular response as assessed by IFN-γ spot forming units (SFU) was plasmid A5 (Q23ENV17) (over 2000 SFU), and the lowest was plasmid C9 (Du156.12) (<100 SFU but above background) (**Figure 3.2*a*)**. Additionally, the regions of the antigen which stimulate T cell responses differ across plasmids. Cellular responses induced by clade A Envs tended to be more reactive to the N-terminus peptides (pool 1) whereas responses to clade B and C Env were spread across the protein (**Figure 3.2*a***). Humoral responses induced by these plasmids were also determined using consensus clade A, B, and C gp120 and gp140

proteins as well as HXBC2 gp41 (**Figure 3.2*b-d***). Similar to the cellular responses, a wide range of binding reactivity across the plasmids was observed. Surprisingly, certain plasmids like B2 (REJO4541.67), B4 (TRJO4551.58), C1 (CAP45.2.00.G3), and C5 (ZM233M.PB6) which induced strong cellular responses, did not induce any humoral responses against consensus proteins. This could potentially be due to the lack of consensus proteins expressing the binding epitope; the binding epitope induced by each plasmid was conformational; or a lack of overall humoral responses. In contrast, there were plasmids which induce both strong humoral and cellular responses like A6 (Q259d2.17), B1 (WITO4160.33), B5 (CAAN5342.A2), C7 (ZM214M.PL15), and C11 (Du172.17).

Formulation of plasmids affects the strength of the response

We next wanted to determine if multiple plasmids expressing the clade A primary Envs could be formulated together and delivered to increase the breadth of antibody responses. However, questions arose as to if there would be antigen competition between the groups of Envelopes and thus, two vaccination regimens were performed: one where all of the plasmids were formulated together and another were each plasmid was given in a separate site. Guinea pigs were immunized four times with 100ug of each plasmid ID followed by electroporation (**Figure 3.3*a***). The total amount of DNA for each immunization was the same across both groups (600ug total – 100ug/plasmid), and the route and electroporation protocol were the same. The only difference was whether or not the plasmids were immunized separately or mixed. Endpoint binding titers to the same primary gp120s were used to determine the induction of humoral responses. Though at

the end of the vaccination (week 12) binding titers between the mixed vs. separate were similar, the induction of humoral responses was quicker in the mixed group than in the separate group (**Figure 3.3*b***). Avidity of humoral responses was assessed at week 12 to determine if there was any difference between the two vaccination groups (**Figure 3.3*c*)**. The avidity index to 92RW020, SF162, and ZM197 were all slightly higher, though not significantly different, in the guinea pigs which received the mixed formulation. Also, post final vaccination neutralization titers were slightly, though not significantly, higher in the mix vs. separate group for three different tier 1 viruses (MN.3, SF162, and THO23.6) (**Figure 3.3*d***). This data suggest that mixing the Envelopes together did not dampen the humoral responses but instead, increased the initial seroconversion rate and induced more superior functional antibody titers. Due to this and the ease mixed formulation provides for vaccine administration, all further studies were performed in this fashion.

Multiple Env plasmids were expressed in the same cells within the skin

To determine if multiple Envelopes were being expressed in the same cell, tags were added to three different plasmids to efficiently detect each Envelope. Three tags were added to the C-terminus of three existing constructs using plasmid mutagenesis. The three constructs were pQ168ENVe2-HIS, pQ23ENV17-FLAG, pDu151.2-MYC and all expressed *in vitro* (data not shown). Two guinea pigs were injected with 16.5ug of each plasmid formulated together and delivered to the dermis followed by electroporation. Expression of all constructs can be detected after 24 hours after injection (**Figure 3.4*a***).

Importantly, there was an overlap of fluorescent signal in multiple cells (**Figure 3.4*b***). This suggests that multiple constructs were being expressed in a single cell.

Groups of 6 Env plasmids induce strong humoral responses in rabbits

To further investigate the use of small groups of primary Envelopes, groups of four rabbits were immunized with six plasmids expressing either clade A, clade B or clade C Envs (**Figure 3.5*a***). All plasmids (100ug/plasmid) were formulated together and delivered to six sites ID followed by electroporation. Binding titers against clade A (92RW020), clade B (SF162) and clade C (ZM197) were assessed for each group of immunized rabbits over time (**Figure 3.5*b-d***). After a single immunization, half of the animals immunized with clade C Envs seroconverted to clade A, B, and C gp120 proteins (**Figure 3.5*d***). By the second immunization, all animals immunized with clade B and C Envs seroconverted to all gp120s (**Figure 3.5*c-d***). Humoral responses in the rabbits immunized with clade A Envs took slightly longer than with clade B and C combinations but eventually did induce strong binding titers to all three gp120s (**Figure 3.5*b***) Humoral responses were boosted by each immunization, reaching peak titers three weeks after final immunizations. Even though the animals were immunized with only a single clade, all rabbits induce strong cross-clade binding titers. In fact, the clade C immunized rabbits had the highest binding titer responses to the clade B (SF162) gp120 protein. Overall, formulating multiple primary transmitter founder or acute Envelopes together in a single formulation induced strong cross-clade binding titers.

Increased diversity within group expanded antibody responses

To investigate whether the results seen in the single clade immunizations could be further expanded upon, two different groups of plasmids were used each containing two clade A, B, and C primary gp160 Envelopes. Four rabbits were immunized with combination 1 (pA1, A2, B1, B4, C4, C8) twice followed by combination 2 (pA3, A4, B6, B7, C2, C3) (**Figure 3.6*a***). The plasmids were all formulated together per different combination with 100ug (600ug total) of DNA construct used per immunization, delivered ID followed by electroporation. The mean diversity within the groups was 22.0% and 21.0% respectively. The mean diversity between the groups was 20.6%. Once again after two immunizations, there was potent induction of binding titers against primary clade A, B, and C gp120s (**Figure 3.6*b***). Neutralization titers were assessed over time against tier 1 viruses (MN.3, MW965.26, and Q23ENV17) (**Figure 3.6*e***). The highest neutralization titers were observed against MW965.26 on weeks 9 and 12. Limited responses were detected against MN.3 with no responses induced against Q23ENV17. The combination of plasmids expressing two clade A, B, and C gp160s did induce potent binding titers but limited neutralization breadth.

"Clouds" with limited diversity expanded the neutralization breadth of sera

We next wanted to investigate if limiting the diversity within a "cloud" could enhance responses. Using the same six clade A plasmids (pA1-A6) as a priming dose, four rabbits were immunized with additional "clouds" or groups of plasmids which were more limited in diversity and remained within clades (**Figure 3.6*a***). The intra-cloud diversity ranged from 12.4-16.4%, and inter-cloud was consistently around 20%. Each

immunization was between 500ug - 600ug of total DNA (100ug of each plasmid) mixed and administered ID to five or six separate sites followed by electroporation. This limited intra-cloud diversity regimen did not disrupt the ability to induce potent cross-clade binding titers against the three primary isolate gp120 compared to the previous formulation (**Figure 3.6*c***). There was a consistent boosting of titers after every immunization, with the highest binding titers obtained after the final immunization at week 12. Neutralization titers demonstrated stronger kinetics of induction and higher titers compared to group 4 (A, B, C mixed) (**Figure 3.6*e***). In comparison to group 4 (A, B, C mixed), group 5 induced responses to MW965, MN.3 and Q23ENV17 after the second immunization and continued to increase after the final immunization. The ability to induce this robust of a response by DNA alone has yet to be seen and could lend itself well to further expansion by boosting with a different platform.

Highest induction of robust antibody responses in rabbits primed twice with the same "cloud"

The final group of rabbits explored if multiple priming immunizations with the same cloud could increase vaccine-induced responses. This would allow for the immune system to potentially honing in on specific epitopes which would later be expanded by boosting with additional clouds. Rabbits were immunized twice with the clade A plasmids (pA1-A6) and boosted with two different groups of primarily clade B immunogens (**Figure 3.6*a***). The intra-cloud diversity ranged from 13.3-14.3% and the inter-cloud diversity between 14-17.6%. Thus, this regimen has the lowest diversity between the clouds compared to the other two combinations. This low intra-cloud

diversity did not limit the responses, as potent binding titers were induced in all animals after two immunizations (**Figure 3.6*d***). The highest and quickest induction of neutralization was observed for this group, with the most powerful response happening after the final immunization (**Figure 3.6*e***). Also, sera from two rabbits were able to neutralize more isolates at higher IC50 concentrations than groups 4 and 5 (**Table 3.2**). This includes hard to neutralize tier 2 viruses where only one virus (Ce1176_A3) was not able to be neutralized. Thus, priming rabbits with two immunizations of the same group of plasmids focuses the immune system in a way that allows for effective induction of broadly binding and neutralizing antibodies.

Non-human primates immunized with "clouds" of primary Envelopes induced potent cellular responses

To further characterize the vaccine-induced responses produced by the most potent regimen, four rhesus macaques (RhMs) were immunized with a similar vaccine regimen (**Figure 3.7*a***). On weeks 0, 6, 12 and 18, the NHP received a mixture of different Envelopes (1mg/plasmid) formulated together and delivered ID followed by electroporation. To further expand the vaccine-induced responses, at weeks 44 and 81, all animals received all of the Envelopes from vaccination 1-4 (1mg/plasmid) delivered IM at a single site followed by electroporation. Cellular and humoral responses were followed two weeks after each vaccination. After only a single immunization, IFN-γ spot forming units (SFU) were detected against consensus clades A and B peptides (**Figure 3.7*b***). These responses were not boosted with the second or third immunization of the priming cloud but were expanded upon after the fourth immunization. After the final ID

immunization, the average total IFN-γ SFU was around 500 SFU with an even distribution of reactivity between clade A and B (range 100- 1,500 SFU) (**Figure 3.8 a**). Though there was contraction into the memory phase (weeks 32 and 43), cellular responses were still detected against consensus clade A and B almost six months (week 43) after final ID immunization (**Figure 3.7*c***). After the first IM boosting immunization at week 44, cellular responses expanded greatly to levels over quadruple the amount seen after final ID immunization. Over eight months after IM immunization (week 81), cellular responses contracted but remained around the levels observed after final ID immunization. Upon second IM boost, cellular responses again expanded above those detected after the previous IM immunization with IFN-γ SFU averaging around 7000 (responses varying from 4000- 10,000 SFU) (**Figure 3.8*b***). These responses were extremely high, especially since they were against unmatched peptides. Also, since consensus peptides were used, this suggests that these small "clouds" of immunogens induced potent cellular responses against conserved regions within the Envelope. This could be important for the induction of cytotoxic T cells as well as provide broad CD4 T cell help.

To further explore the cellular responses induced by the primary Envelope cloud immunization, intracellular cytokine staining was performed using consensus clade A, B and C peptides. CD8 T cell responses after ID immunization (week 20) primarily expressed IL-2 and TNF-α with limited IFN-γ production (**Figure 3.7*d***). Each IM immunization increased the percent of CD8 T cells expressing IFN-γ. TNF-α production further increased after the final IM immunization (week 83). In contrast, the IL-2

production observed after final ID immunization was not boosted by both IM immunizations and levels after final IM immunization were the same as after final ID immunization. CD4 T cell responses were also assessed against clade A, B and C peptides (**Figure 3.7*e***). The percent of CD4 T cells expressing IFN-γ and IL-2 was relatively the same after the ID immunization (week 20) with a lower percentage of CD4 T cells expressing TNF-α. Similar to CD8 T cells, the proportion of CD4 T cells secreting IL-2 remained relatively consistent across time with slight waning at each memory time point. However, after the first IM immunization, there was a sharp increase in CD4 T cells secreting IFN-γ. Similar boost was not observed after the second immunization. Expression of TNF-α remained consistent into memory after ID immunization, was boosted by the first and second IM immunization. Importantly, similar to ELISpots, we observed potent cytokine secretion after stimulation with cross-clade consensus peptides. Though these NHPs were only immunized with clade A and B primary Envs, cellular responses against consensus clade C peptides were detected at similar levels to clade B responses.

Binding and functional antibodies induced using primary Env DNA vaccination

The primary Envelope cloud immunization also induced potent humoral responses. After a single immunization, two out of eight RhMs seroconvert to clade A, B and C gp120 proteins (**Figure 3.9*a***). After the final ID immunization, all animals had strong endpoint binding titers against the primary Envelopes averaging above 10^4. Similar to cellular responses, binding titers also contracted down in the memory phase but remain high (average above 10^3) six-month post last ID immunization (week 43).

Also similar to cellular responses, after the IM boost, binding titers reached levels higher than after ID immunization with the average binding titer above 10^5. These responses are also slightly boosted after a second IM immunization to levels reaching 10^6. Strong avidity indexes of around 0.8 were induced after the second ID immunization (**Figure 3.9*b***). However, subsequent ID immunization did not improve the avidity index. The first IM boost increased the avidity index across all three gp120 proteins with minimal to no increase in avidity after the second IM immunization. To further explore the binding capacity of the humoral responses induced, binding to consensus and primary gp120 and gp140s was determined using binding antibody multiplex assay (BAMA) (**Figure 3.9*c***). Strong binding titers against clade A, B, C and AE Envs were detected with the highest responses obtained after the first IM immunization. The strongest binding response was detected against the primary isolate gp140 Env 1086c, with almost three-fold higher area under the curve (AUC) binding compared to other Envs. V1/V2 binding against multiple different gp70 scaffolds was also assessed (**Figure 3.9*d***). Interestingly there were three binding patterns to V1/V2 scaffolds which emerged. The first was binding kinetics similar to that which was observed in the binding to the whole protein with induction by the final ID immunization, peak after first IM immunization and similar levels after the second IM immunization (**Figure 3.9*d*** bottom graph). The second pattern was the induction of binding after ID immunization but no boosting after each IM immunization (**Figure 3.9*d*** top graph – TT31P and TV1.21). The final pattern was limited to no induction of binding (**Figure 3.9*d*** top graph – RHPA4259 and 62357). These differences in binding patterns could help suggest a potential target epitope.

In addition to binding titers, the vaccination regimen also induces functional antibodies. Using only DNA vaccination, we were able to get cross-clade neutralization titers against a diversity of tier 1 viruses (**Figure 3.10*a***). After ID immunization, neutralization titers for MN.3, MW965 and SF162 average above or around 10^2. After the first IM boost, levels were increased to above 10^3 for MN.3 and MW965 and just below 10^3 for SF162. After the second IM boost, we did not see levels increase above those observed after the initial IM boost. In fact, for MN.3, MW965, and SF162, the levels were lower and usually averaged around the same titers as those seen after the ID immunizations. However, levels against SF162P4 IMC were detected and importantly, there were limited but low neutralization titers induced against the tier 2 virus SF163P3 after final IM immunization (**Figure 3.10*b***). Since the role of antibodies with ADCC capabilities has been suggestive in protection against HIV-1 infection (RV144 correlates analysis), we also tested ADCC activity against targets coated with 1086c (gp140), WITO (gp120), JR-FL (gp120) and 92UG037.1 (gp120) (**Figure 3.10*c***). Similar to V1/V2 binding, three different patterns of ADCC induction emerge. The first displays similar kinetics to BAMA, V1/V2 binding pattern 1 and neutralization titers with peak titers induced post 1^{st} IM immunization which was not further boosted after the 2^{nd} IM (1086c and JR-FL). The second pattern was observed with WITO coated targets where the strongest response was observed after the ID immunizations. If the one outlier was removed from the analysis, these responses are maintained with the first IM immunization but slightly decline with the second. The third pattern was observed with 92UG037.1 where only 1 or 2 NHPs were able to induce low ADCC activity against the

target cells. Differences between these three Env could again suggest differences in binding epitopes and induction of certain humoral responses after each immunization. Interestingly, the AUC determined by the binding antibody multiplex assay and ADCC titers against 1086c correlated (Spearman r=0.8909 p= 0.0005) (**Figure 3.10*e***). However, similar correlations were not found for WITO, JR-FL, and 92UG037.1 (**Figure 3.11**). These data supports the use of primary transmitter founder Envelopes deliver in small "cloud" immunizations for the induction of potent cellular and humoral responses.

3.4. Discussion

An effective HIV-1 vaccine will likely need to induce both cellular and humoral responses. Previously, DNA vaccines have been able to induce potent cellular responses but lacked humoral responses. Advances in plasmid optimizations, formulation, and delivery, have significantly increased DNA vaccine's ability to induce humoral responses. Here, we explore the ability to use combinations of full-length gp160 Envs which were isolated during the early/ acute phase of infection (360-362). All inserts were immunogenic in mice, displaying a range of cellular and humoral responses. Interestingly, there was not a consistent pool of peptides which was dominated across all antigens. Instead for clades B and C inserts, cellular immune responses were detected across the entire antigen. We observe similar breadth of responses using our consensus antigens (324). In contrast, the majority of clade A Env inserts induced very strong responses against the N-terminus (pool 1) and fewer responses across the rest of the protein. This could be due to the heterogous nature of the peptide used or a dominant epitope at the N-terminus of the protein. Additionally, this dominance could be mouse

specific since when a combination of these plasmids was administered into NHPs, responses to all four peptide pools is evident; however, pool 1 still dominates (**Figure 3.7** and **3.8**). Furthermore, we currently do not know if different peptide pool reactivities were dominated by CD4 or CD8 T cell responses. Additional studies to further understand why certain Env induce balanced cellular and humoral responses to the entire antigen while others produce responses to only certain regions is underway.

The ability to induce protective responses against multiple serotypes is evident in the Influenza, Human Papillomavirus, and Pneumococcus vaccines (19, 153, 247, 248, 363). Due to the breadth of HIV diversity, is it likely that multiple antigens will need to be formulated into a single injection for ease of delivery. Regarding humoral responses, within this study, we demonstrated that up to six plasmids could be combined and lead to strong humoral responses than when delivered to individual sites. Though none of these differences (kinetics, endpoint binding titers, avidity or neutralization) were significantly different, the ability to formulate multiple plasmids together far outweighs delivering them to separate sites. Using immunofluorescence and tagged constructs, we also show that up to three constructs were detected in the same cell. This leads to the possibility of heterotrimers, which have been previously shown to induce stronger neutralization titers compared to homotrimers of Env proteins (364, 365). In theory, the diversity within these heterotrimers could focus the immune response on conserved epitopes. Importantly, it is within these conserved regions of HIV-1 that broadly neutralizing antibodies targets (167). However, these regions tend to be much less immunogenicity than the variable loops and thus are more difficult to target. We are continuing to follow up on whether or

not DNA encoded gp160s can form *in vivo* heterotrimers and if so, how does the percent diversity between the Envs affect this ability.

In addition to exploring multiple different combinations of HIV Envs, we also used different sites for delivery. Due to advances in electroporation technology, different tissues can be targeted including the traditional, intradermal and intramuscular (64). Within this study, NHP were vaccinated with four ID immunizations followed by two IM boost. These two sites have different cellular composition and thus could produce unique vaccine-induced responses. We observed a single ID immunization induced strong cellular responses and seroconversion in 50% of the animals (**Figure 3.7*a*, 3.9*a***). Interestingly, even though cellular responses did not boost with the 2nd and 3rd ID immunization, we observed continual improvement in humoral responses. However, though binding titers improve over the ID immunizations, the avidity of these antibodies remains fairly consistent only increasing upon IM immunization (**Figure 3.9 *a*-*b***). We observed a sharp increase in cellular responses after the first IM immunization, with both CD4 and CD8 T cells expressing IFN-γ. This boost in responses was also observed in both binding and functional antibody titers (**Figure 3.9** and **3.10**). Unexpectedly, these functional antibody titers did not further increase after the second IM immunization but instead were at levels similar to after the ID immunizations. Following the second IM immunization, CD8 T cells dominated IFN-γ production with a decrease in CD4 T cell production compared to after the 1st IM immunization (**Figure 3.7 *d*-*e***). Here we demonstrate for the first time that ID DNA immunization can be further expanded by IM immunization. The ability to understand how the different sites of immunization skew the

immune response and how boosting can affect memory cell activation is imperative for DNA vaccine development.

Overall, the work within this chapter builds on the success we have previously seen with our consensus immunogens (323, 352). We have developed numerous different plasmids expressing consensus, chronic and acute/ early Envs. We demonstrated that guinea pigs and rabbits exposed to groups of immunogens could induce strong binding titers to heterologous Envs and different clouds of plasmids can influence the kinetics of tier 1 neutralization induction. Additionally, combinations of 14 different Env plasmids were able to induce strong cellular and humoral responses. Importantly, these humoral responses were functional after only DNA vaccination. Determining what combination of Envs produces the strongest and broadest responses is imperative for the HIV vaccine field.

Tables

Name	Insert	Clade	Tier	Accession #	Transmission	Stage
A1	Q769ENVd22	A	2	AF407158	FSW	acute early
A2	Q168ENVe2	A	2	AF407148	FSW	acute early
A3	Q842ENVd12	A	2	AF407160	FSW	acute early
A4	Q461ENVe2	A	2	AF407156	FSW	acute early
A5	Q23ENV17	A	2	AF004885	FSW	Fiebig IV
A6	Q259d2.17	A	2	AF407152	FSW	acute early
B1	WITO4160.33	B	2	AY835451	F-M	Fiebig II
B2	REJO4541.67	B	2	AY835449	F-M	Fiebig II
B3	RHPA4259.7	B	2	AY835447		Fiebig < V
B4	TRJO4551.58	B	3	AY835450	M-M	Fiebig II
B5	CAAN5342.A2	B	2	AY835452	M-M	
B6	PVO.4	B	3	AY83544	M-M	Fiebig III
B7	TRO.11	B	2	AY835445	M-M	Fiebig III
B8	AC10.0.29	B	2	AY835446	M-M	Fiebig III
B9	QHO692.42	B	2	AY835439	F-M	Fiebig V
C1	Cap45.2.00.G3	C	2	DQ435682	FSW	
C2	Cap210.2.00.E8	C	2	DQ435683	FSW	
C3	Du422.1	C	2	DQ411854	FSW	Fiebig V
C4	ZM53M.PB12	C	2	AY423984	F-M	
C5	ZM233M.PB6	C	2	DQ388517	F-M	
C6	ZM249M.PL1	C	2	DQ388514	F-M	
C7	ZM214M.PL15	C	2	DQ388516	F-M	
C8	Du123.6	C	2	DQ411850	FSW	Fiebig VI
C9	Du151.2	C	2	DQ411851	FSW	Fiebig V
C10	Du156.12	C	2	DQ411852	FSW	Fiebig <IV
C11	Du172.17	C	2	DQ411853	FSW	Fiebig VI

Table 3.1: Characteristics of acute/ early primary Envs. For ease, each plasmid is denoted by the clade letter followed by a number throughout the chapter. All inserts were RNA and codon optimized and encoded for the full gp160 Env protein.

			ID50 in Tzmbl Cells						ID50 in A3R5.7 Cells		
			RHPA4258.7 Tier 2 Clade B	TRO.11 Tier 2 Clade B	Ce1176_A3 Tier 2 Clade C	BF1266.431 a Tier 2 Clade C	Q842.d12 Tier 2 Clade A	C2101.c01 Tier 2 Clade AE	RHPA Tier 2 Clade B	REJO Tier 2 Clade B	CM235-2 Tier 2 Clade AE
Group	**Animal**	**Bleed Week**									
Group 4	1	Week 0	<20	<20	<20	<20	<20	<20	23	<20	42
		Week 12	<20	<20	<20	<20	<20	<20	**363**	<20	34
	2	Week 0	<20	<20	<20	<20	<20	<20	<20	<20	36
		Week 12	<20	<20	<20	<20	<20	<20	**435**	<20	97
Group 5	1	Week 0	<20	21	<20	<20	<20	<20	40	<20	62
		Week 12	**154**	36	<20	**143**	**288**	**45**	**139**	**438**	**294**
	2	Week 0	<20	<20	<20	<20	<20	<20	31	<20	47
		Week 12	**47**	<20	<20	**50**	**100**	<20	**109**	**83**	110
Group 6	1	Week 0	<20	<20	<20	<20	<20	<20	24	<20	74
		Week 12	**214**	**54**	**21**	**228**	**387**	**84**	**404**	**749**	**915**
	2	Week 0	<20	<20	<20	<20	<20	<20	31	<20	<20
		Week 12	**310**	**57**	**26**	**364**	**716**	**109**	**270**	**329**	**389**

Table 3.2: Serum neutralization titers against a panel of tier 2 viruses from the top two rabbits from groups 4, 5, and 6. The two rabbits with the strongest binding titers were tested for neutralization against a panel of Tier 2 viruses. Colors represent the strength of neutralization with green between baseline to 100, yellow 100-200, red 200-500 and deep red great than 500.

Figures

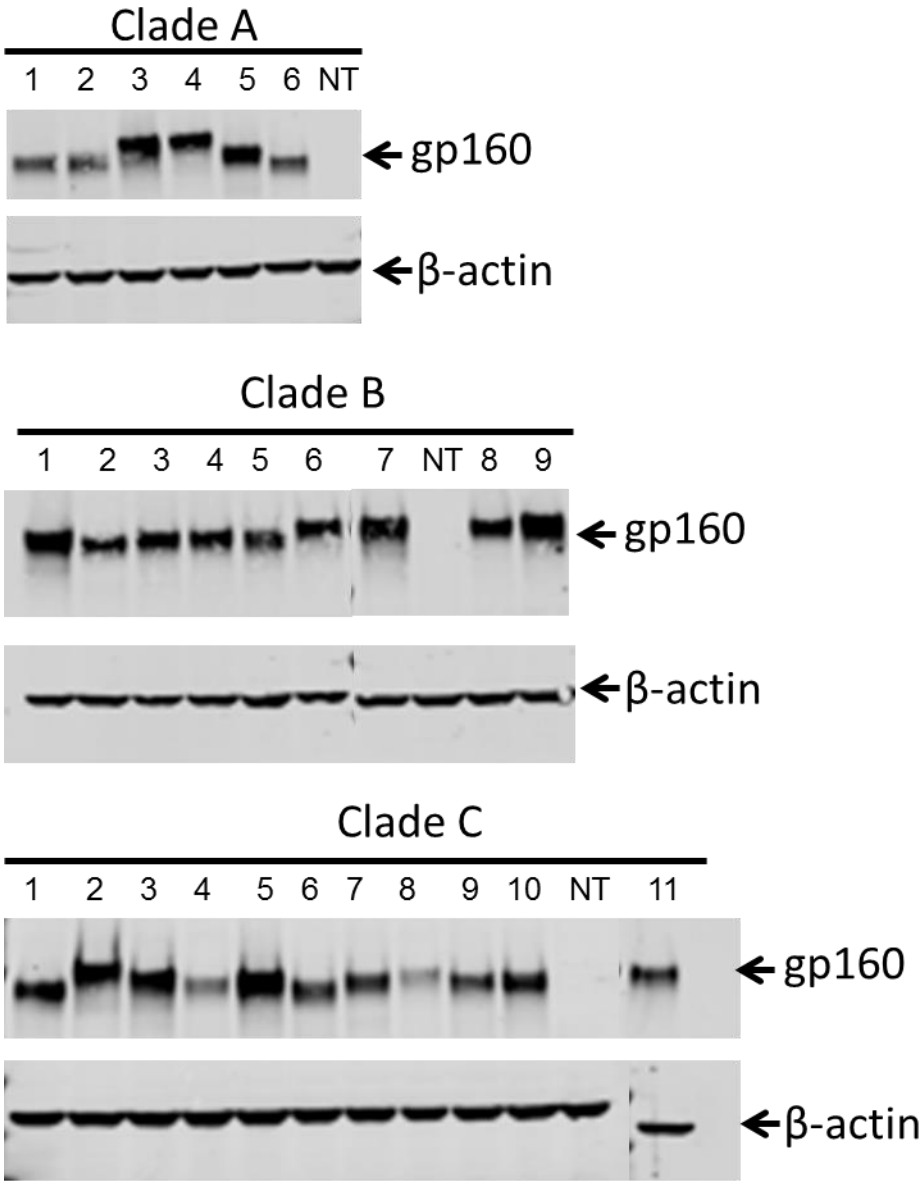

Figure 3.1: *In vitro* expression of primary HIV-1 Env plasmids. 293T cells were transfected with each plasmid. Forty-eight hours later, cell lysate was harvested, and Western blot was performed to determine expression levels. All plasmid express Env detected by the neutralizing antibody 2G12 and the expected length.

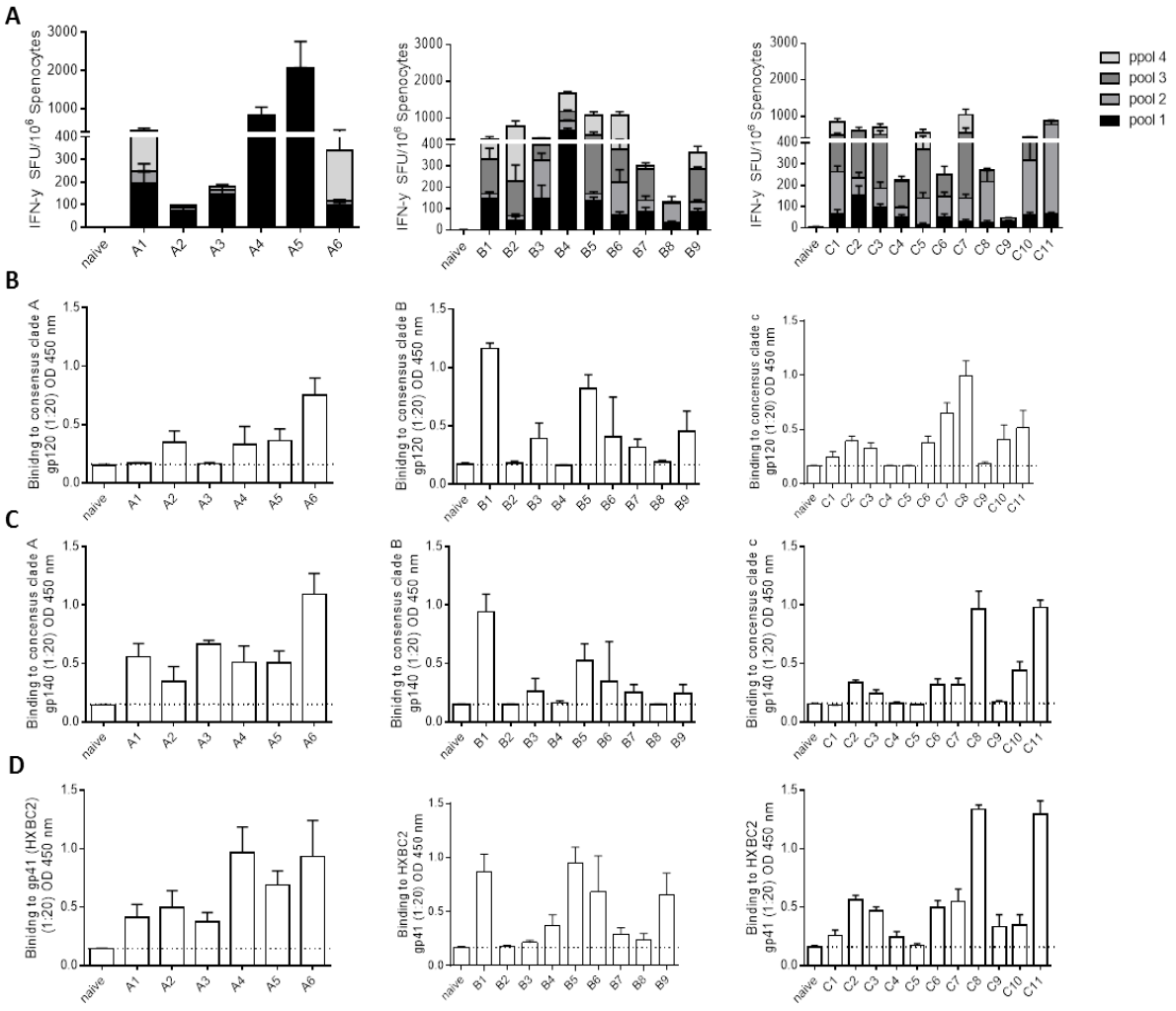

Figure 3.2: Immunogenicity of each plasmid in mice. (***a***) Cellular responses post final vaccination as measured by IFN-γ spot forming units (SFU) after *ex vivo* stimulation of splenocytes with consensus clade A, B or C depending on the clade of the insert. Humoral antibody responses as assessed by binding to consensus clade A, B, or C gp120 (***b***) or gp140 (***c***). Binding to gp41 was also determined (***d***). Dotted line in represents background binding level.

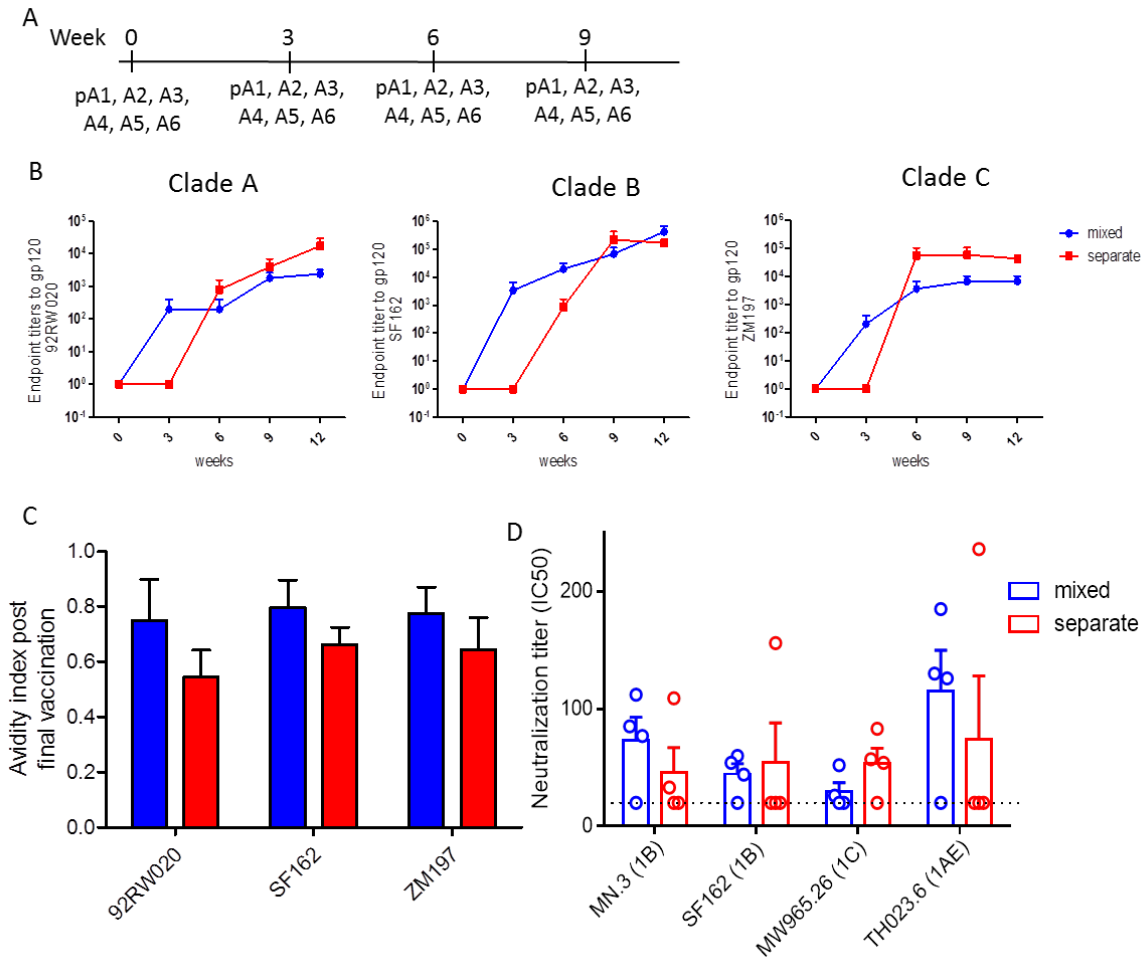

Figure 3.3: Guinea pigs immunized with mixed Envelopes induced stronger and quick humoral responses compared to separate immunization. (***a***) Immunization scheme for guinea pig vaccination with two different groups: one were all of the plasmids were mixed and formulated together and another where each plasmid was delivered into a separate site. (***b***) Binding titers against clade A (92RW020), clade B (SF162) and clade C (ZM197) primary gp120s over time. (***c***) Avidity index of binding to 92RW020, SF162, and ZM197 at week 12. (***d***) Neutralization titers for week 12 serum were determined for a set of tier 1 viruses.

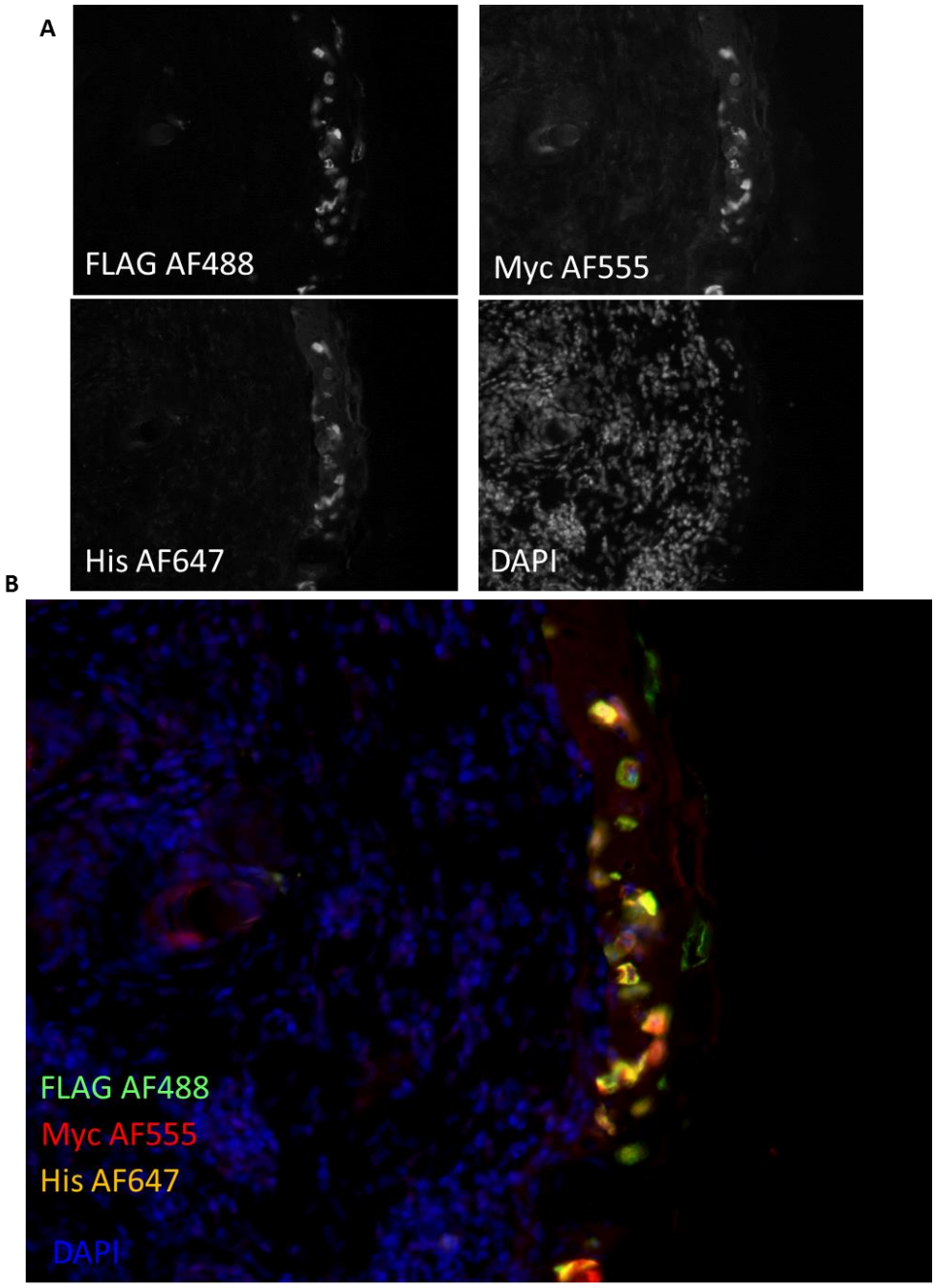

Figure 3.4: Expression of multiple constructs in skin. Guinea pigs were vaccinated intradermally with three constructs expressing a tagged HIV-1 Env construct. After 24 hours, the skin was biopsied and stained for expression of the tags. (***a***) Expression of each

construct can be detected. (***b***) Overlay of each construct demonstrating multiple constructs were expressed from a single cell.

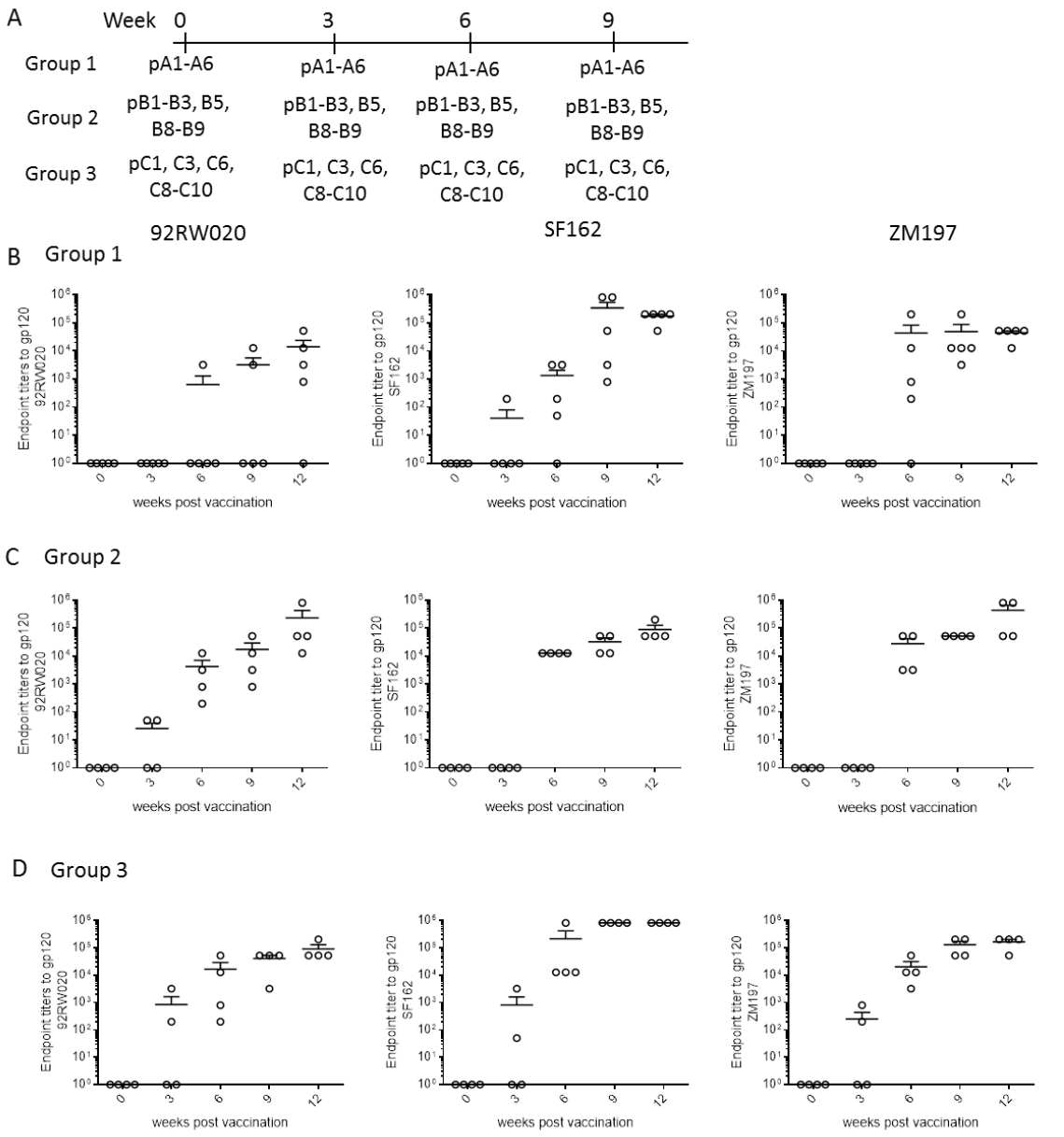

Figure 3.5: Rabbits immunized with mixed clade A, B or C Envelopes induced strong humoral responses. (***a***) Rabbits were immunized with six clade A, B or clade C Env plasmids. All plasmids were formulated together (100ug of each plasmid, 600ug total) and delivered ID followed by electroporation. Binding titers against clade A (92RW020), clade B (SF162) and clade C (ZM197) primary gp120s for clade A (group 1

(***b***)), clade B (group 2 (***c***)) or clade C (group 3 (***d***)) immunized rabbits. Individual titers are denoted in the shapes, geometric mean titers by the horizontal bar and standard error by the bracket.

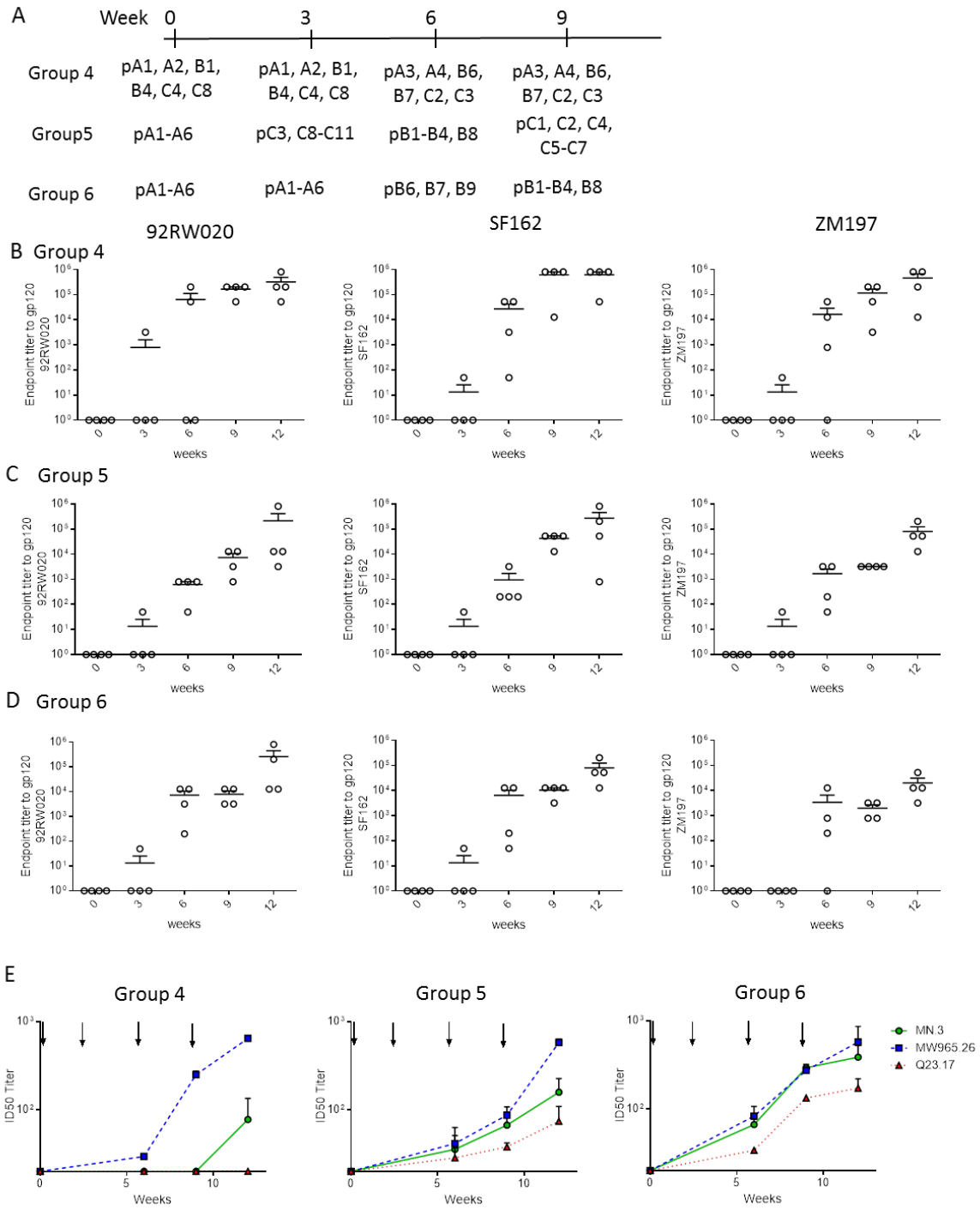

Figure 3.6: Clouds of Envelope plasmids increased functional humoral responses. (***a***) Rabbits were immunized with 3-6 Envelope plasmids formulated together and delivered intradermally followed by EP. Endpoint binding titers over time against

92RW020 (clade A), SF162 (clade B) and ZM197 (clade C) for group 4 (***b***), group 5 (***c***) and group 6 (***d***) immunized rabbits. (***e***) Neutralization titers against tier 1 viruses across time for each immunization group.

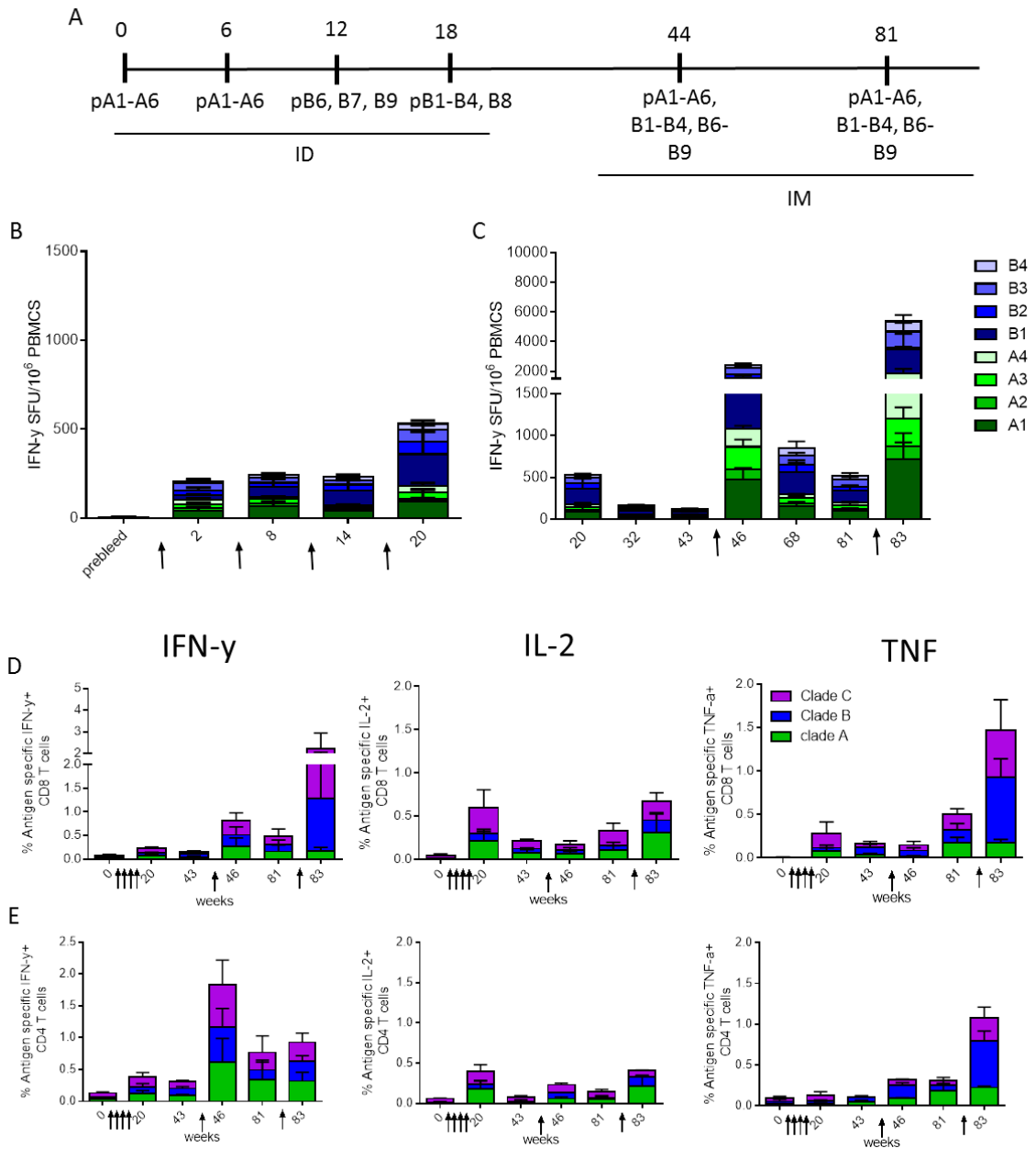

Figure 3.7 Cellular responses induced by clouds of primary HIV-1 Env plasmids in non-human primates. (***a***) Four Indian Rhesus Macaques were immunized with a combination of 14 different plasmids expressing primary HIV-1 Envelopes following a similar immunization protocol as in rabbit group 6 (**Figure 3.6**). IFN-γ ELISpot

responses in peripheral blood mononuclear cells (PBMCs) after overnight stimulation with consensus clade A and B peptides after ID immunizations (***b***) or memory and IM boost (***c***). Cellular responses were also assessed for intracellular cytokine production of IFN-γ, IL-2 and TNF-α after stimulation with consensus clade A, B or C peptides. Cytokine production over the time course of immunizations for CD8 (***d***) or CD4 (***e***) subset of CD3 T cells.

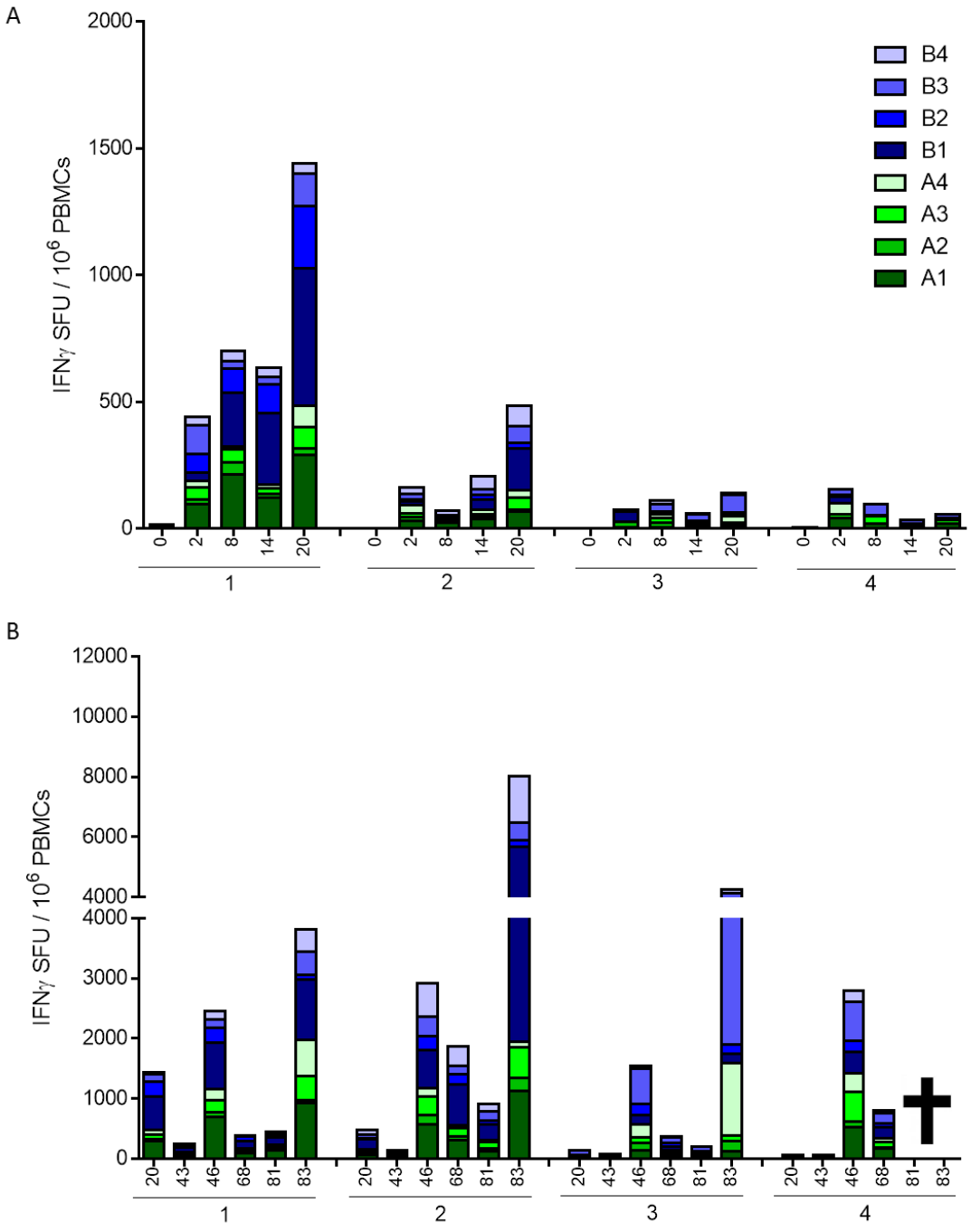

Figure 3.8: Individual ELISpot responses over time. IFN-γ ELISpot responses over time for each NHP after ID immunizations (***a***) or memory and IM boost (***b***). NHP 4 died due to unrelated causes on week 80.

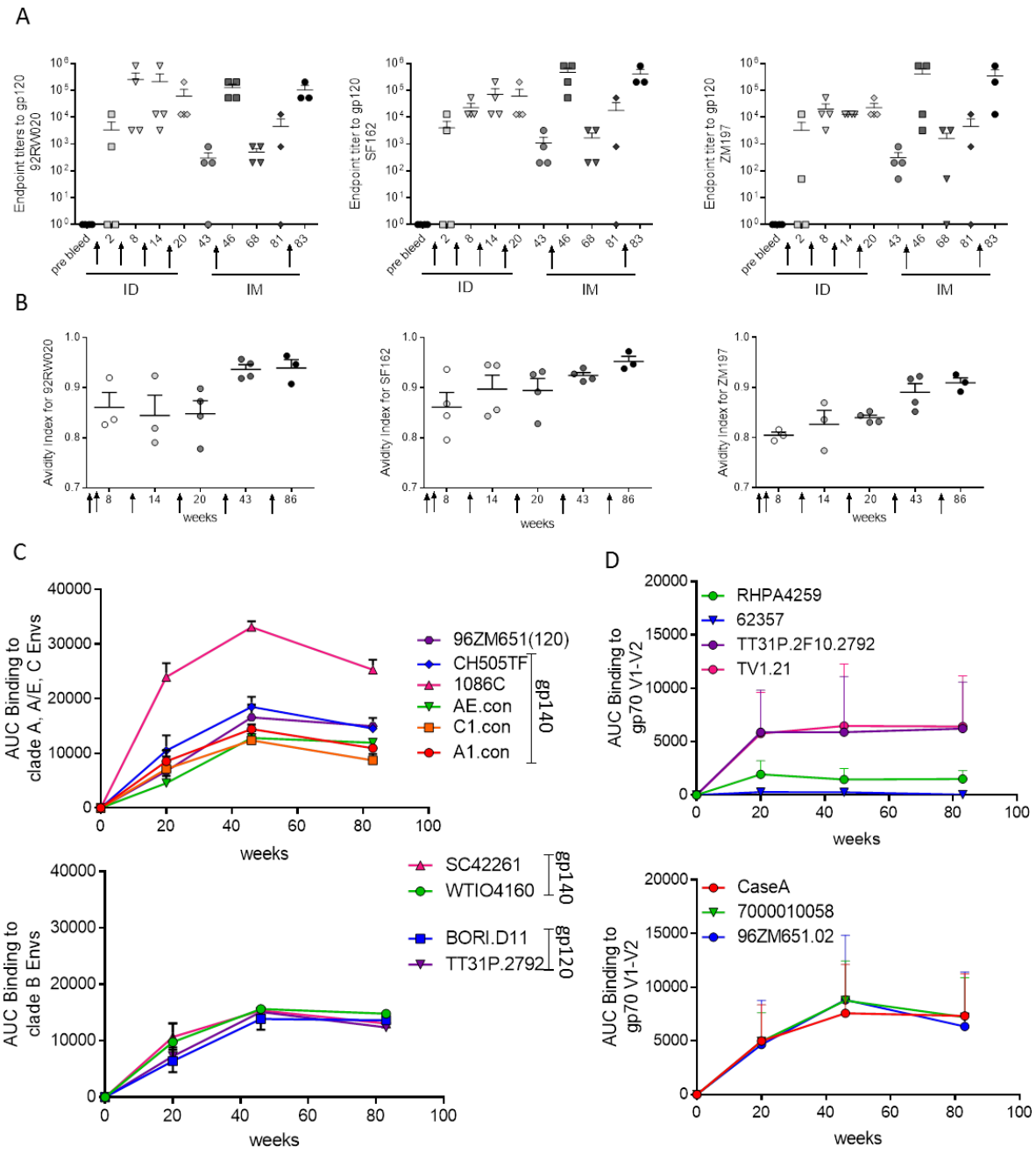

Figure 3.9: Strong humoral binding responses induced by clouds of plasmids expressing primary HIV-1 Envs. (***a***) Endpoint binding titers over time against 92RW020, SF162, and ZM197. (***b***) Avidity index against 92RW020, SF162 and ZM197 after the second, third, fourth ID immunization and each of the IM boost. (***c***) Binding to

consensus and primary gp120/gp140 Envs as assessed by binding antibody multiplex assay (BAMA). (***d***) Antibody binding responses to multiple scaffolded (gp70) V1/V2 after final ID immunization and after each IM boosts.

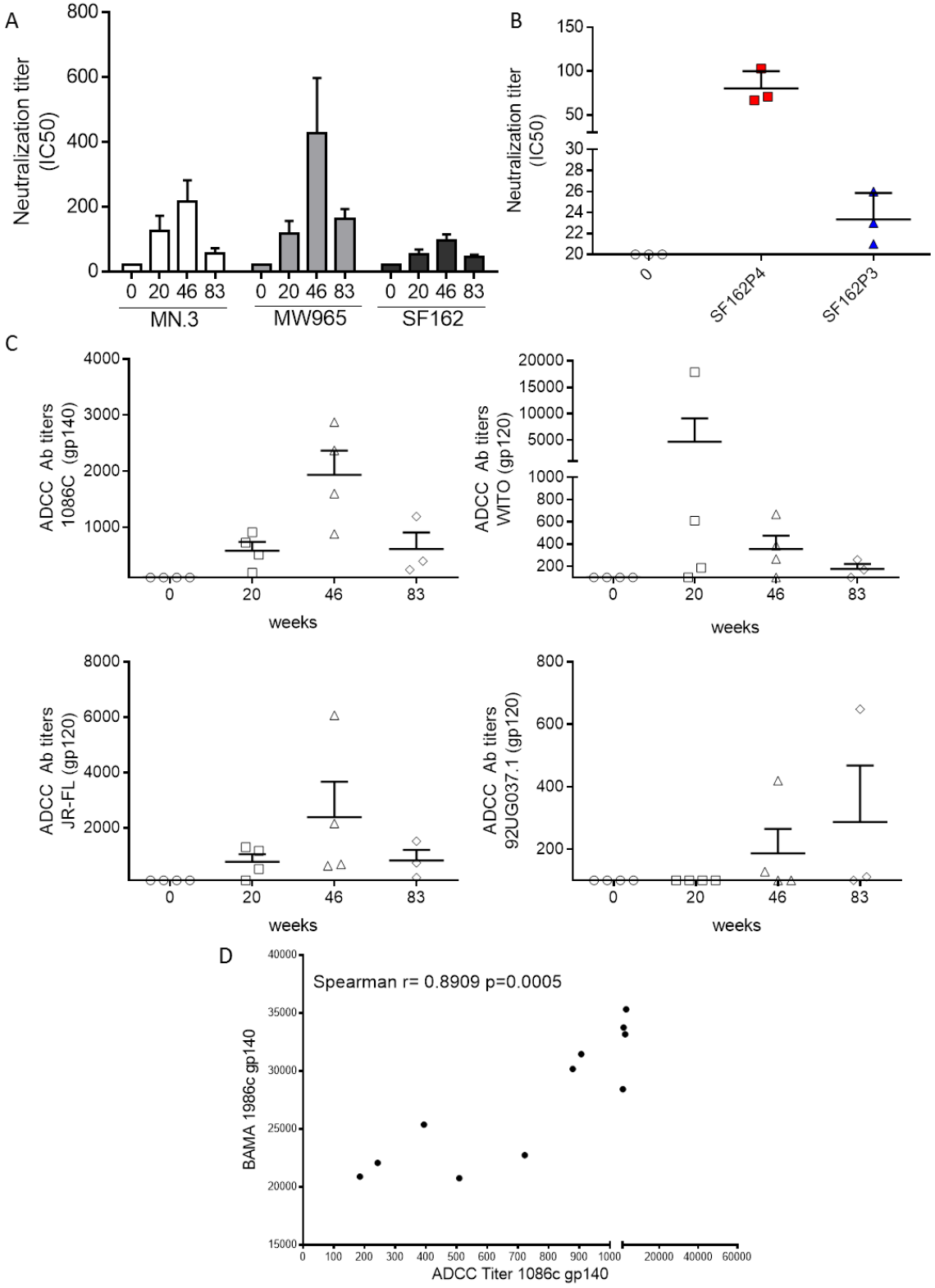
A
Neutralization titer (IC50)
800
600
400
200
0
0 20 46 83
MN.3
0 20 46 83
MW965
0 20 46 83
SF162
B
Neutralization titer (IC50)
150
100
50
30
28
26
24
22
20
0
SF162P4
SF162P3
C
ADCC Ab titers 1086C (gp140)
4000
3000
2000
1000
0
20
46
83
weeks
ADCC Ab titers WITO (gp120)
20000
15000
10000
5000
1000
800
600
400
200
ADCC Ab titers JR-FL (gp120)
8000
6000
4000
2000
ADCC Ab titers 92UG037.1 (gp120)
800
600
400
200
D
Spearman r= 0.8909 p=0.0005
BAMA 1986c gp140
40000
35000
30000
25000
20000
15000
0 100 200 300 400 500 600 700 800 900 1000
20000 40000 60000
ADCC Titer 1086c gp140

Figure 3.10: DNA immunization alone induced functional antibody titers. To further understand the vaccine-induced humoral response induced by the cloud DNA vaccination, both neutralization titers, as well as, ADCC activity were explored over the time course of immunizations. (***a***) Neutralization titers against a panel of tier 1 viruses across time. (***b***) Week 83 serum (two-week post final immunization) was assessed for neutralization capacity against two infectious molecular clones: SF162P4 (tier 1) and SF162P3 (tier 2). (***c***) Antibody-dependent cellular cytotoxicity (ADCC) titers were determined against targets coated with gp140 (1086c) or gp120 (WITO, JR-FL, and 92UG037.1) for serum from weeks 20 (post final ID), week 46 (post 1^{st} IM) and 83 (post 2^{nd} IM). (***d***) There was a strong correlation between binding to 1086c gp140 as assessed by BAMA and ADCC titers against 1086c gp140.

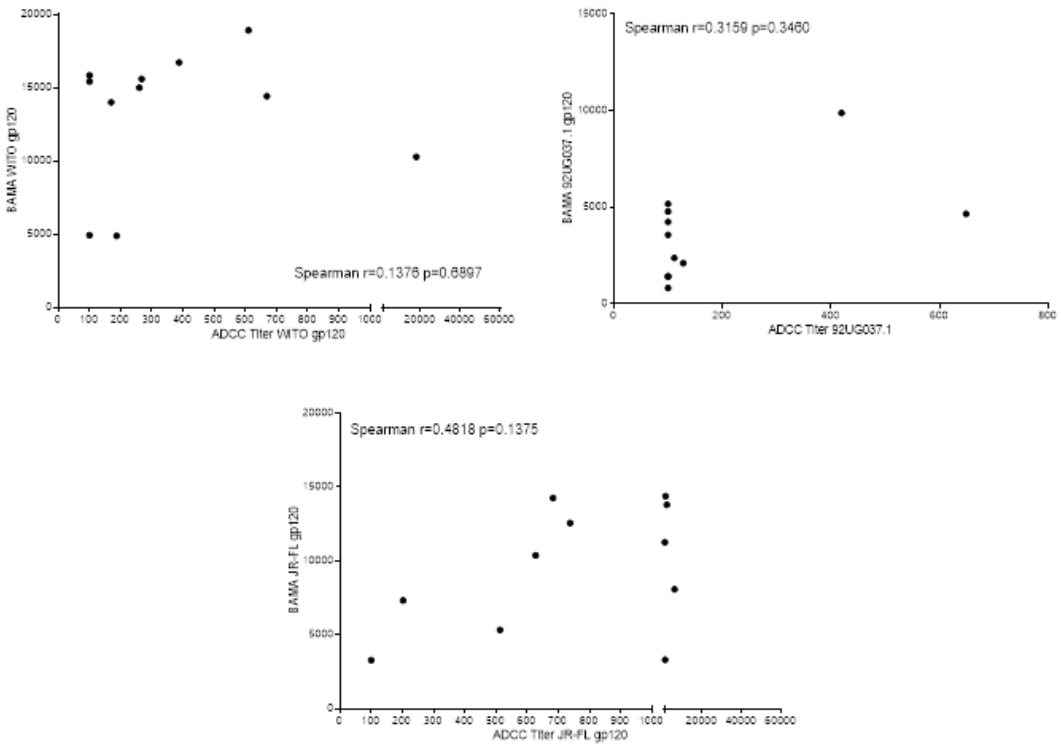

Figure 3.11: No correlation between BAMA binding and ADCC titers for WITO, JR-FL, and 93UG037.1. Contrary to the correlation observed with 1086c, there was no correlation between BAMA binding and ADCC titers for the other three gp120s which were assessed in both assays.

CHAPTER 4: CHEMOKINE ADJUVANTED ELECTROPORATED-DNA VACCINE INDUCES SUBSTANTIAL PROTECTION FROM SIMIAN IMMUNODEFICIENCY VIRUS VAGINAL CHALLENGE

4.1 Introduction

Although a large number of vaccines have been tested, after a 30-year effort, there is still a need for a highly efficacious HIV-1 vaccine. The recent RV144 clinical vaccine trial in Thailand demonstrated that 31% of vaccinated individuals could be protected (208, 211, 366). The need for an effective HIV-1 vaccine to extend positively on these results remains pressing. DNA-based vaccines alone have been shown to induce weak immune responses in non-human primates (NHP) and humans thus limiting their stand-alone utility. However, many technological advances to the platform have recently resulted in improving this platform's performance in the clinic (58, 301). Such advances include using codon and RNA optimization, electroporation, and the use of genetic adjuvants to tailor the immune response (57, 61, 101, 321, 322, 367-371). The potency of plasmid adjuvants for DNA vaccines was recently demonstrated in HVTN080 trial, reporting that the inclusion of pIL-12 (plasmid encoded IL-12) in a DNA + EP formulation in humans increased vaccine-induced responses (58). In this study following three immunizations, 88.9% of vaccinated subjects developed $CD4^+$ or $CD8^+$ responses. However, an effective HIV vaccine will likely need to induce antibody responses (366, 372). The role of antibodies in protection has been supported by the immune correlates analysis of RV144 and in several studies using passive transfer of broadly neutralizing antibodies to NHPs resulting in protection against challenge (180, 211, 212, 217, 335,

373-375). However, these broadly neutralizing antibodies are highly somatically hypermutated with uncommon characteristics such as long CDR3s, calling into question whether a vaccine will be able to induce such antibodies (376).

To increase the magnitude and quality of humoral responses induce by DNA vaccination, we explored the use of mucosal chemokine plasmid adjuvants in combination with an SIV vaccine. Previously, we determined that the CCR10L adjuvants CTACK (cutaneous T-cell attracting chemokine or *CCL27*) and MEC (mucosa-associated epithelial chemokine, or *CCL28*) increase the levels of vaccine-specific mucosal IgA and IgG in small animals (349, 377). The receptor for these two chemokines is CCR10 which is expressed on mucosal and epithelial tissue, allowing for the recirculation and localization of naïve, memory and effector T cells and antibody secreting B cells (378-385). Also, the chemokine TECK (thymus-expressed chemokine or *CCL25*) which binds to CCR9 has been found to be important in T cell homing to the lamina propria and intraepithelium of the small intestine (386-389). Previous studies have also shown that the inclusion of TECK with a DNA vaccine can elevate antigen-specific responses in both the serum and mucosal compartments of mice (390).

We report here that rhesus macaques (RhMs) vaccinated with SIV *gag*, *env*, and *pol* and CCR9L and CCR10L adjuvants delivered by electroporation can be protected from multiple low-dose intravaginal challenges with SIVsmE660. When all vaccine arms were combined, 13 out of 19 animals remained uninfected or displayed aborted infection, controlling the virus to undetectable levels, leading to a total vaccine protection of 68% vs. 14% in control challenged animals ($P = 0.0016$). The highest protection was seen in

the DNA + CCR10L group with an 89% protection rate (P = 0.0003) with 6 of 9 RhMs displaying aborted infection and two RhMs remaining uninfected. The inclusion of mucosal chemokine plasmid adjuvants improved challenge outcomes by over two-fold compared to DNA alone and suggests that further study of novel immune adjuvanted vaccines are of importance.

4.2 Materials and Methods

Study Design: Groups of female rhesus macaques (*Macaca mulatta*) of Indian origin (n = 5 per group) were immunized at weeks 0, 6, 12, 18 and 48 with 1.5 mg per construct of pSIVmac *pol*, consensus pSIVsm *env* and 3.0 mg pSIVsm *gag* without adjuvant. Adjuvanted groups included rhCCL25 (n = 5), rhCCL27 (n = 5) or rhCCL28 (n = 5), at 0.5 mg. DNA was formulated in sterile water with 1% wt/wt poly-L-glutamate sodium salt and delivered in two separate sites followed by *in vivo* electroporation using the CELLECTRA® device (Inovio Pharmaceuticals, Inc.; Plymouth Meeting, PA). An additional 14 animals were treated with water followed by EP and served as a naïve control. RhMs with protective MHC allele mamu A01* were evenly distributed to not bias results. TRIM5α analysis was performed after challenge and did not appear to have a major impact on the overall challenge outcome (**Table 4.1**). One animal from the CCR10L immunized group died before challenge due to unrelated causes and was not included in any of the analysis.

Animal husbandry and specimen collection schedule: RhMs were housed at Tulane National Primate Research Center in accordance with the standards of the American

Association for Accreditation of Laboratory Animal Care. Animals were allowed to acclimate for at least 30 days before any immunization. All protocols were approved by Tulane National Primate Center Animal Care and Use Committee.

Collection of whole blood from rhesus macaques: Animals were anesthetized with ketamine (0.1 ml/kg) or tiletamine/zolazepam (0.06–0.10 ml/kg). Blood samples were collected from the femoral vein using the Sarstedt S-Monovette collection system (Sarstedt; Nümbrecht, Germany). Peripheral blood mononuclear cells (PBMC) were isolated by standard Ficoll-hypaque centrifugation.

Collection of vaginal wash from rhesus macaques: After administration of anesthesia, an appropriate-sized feeding tube was carefully introduced into the vaginal vault. A syringe containing 2 ml saline was attached to the feeding tube and used to instill and aspirate the saline from the vaginal vault. The sample was transferred to a sterile conical tube placed on ice, centrifuged at 800g and the supernatant divided into small aliquots and stored at -80^{o}C until assayed. The pellet of cells was also stored at -80^{o}C until assayed, however, due to low recovery of antigen-specific T-cells, these were of poor quality and low in numbers which were not useful for analysis. No vaginal biopsies were collected during this study due to the concern of scarring which could affect challenge outcome.

Rhesus IFN-gamma Enzyme-linked immunospot assay (ELISpot): Interferon-gamma (IFN-γ) ELISpot was performed as previously described for macaque (391) to determine antigen-specific (IFN-γ) secreting cells from immunized animals. Cells were stimulated

overnight in the presence of either specific peptide antigens (SIV-1mac239 Gag or Pol (NIH AIDS Research & Reagent Program, Germantown, MD) and SIVsmE660 Env (Invitrogen), R10 (negative control), or Concanavalin A (positive control).

Detection of SIV-specific antibodies: The presence of antibodies specific for SIV antigens was semiquantitatively determined by WB. The IgA and IgG SIV-specific antibodies from sera and genital secretions were analyzed using SIV western blot strips from ZeptoMetrix Corp (Buffalo, NY). Strips were incubation overnight with dilutions of sera or vaginal secretions normalized to ~0.5 µg IgG or IgA/ strip. The WB strips were developed with affinity purified alkaline phosphatase-conjugated goat anti-monkey IgA and with peroxidase-conjugated goat anti-monkey IgG reagents (Rockland Immunochemicals, Pottstown, PA). The reactivity of samples with particular SIV antigens was visualized after the addition of alkaline phosphatase (Bio-Rad, Hercules, CA) and peroxidase (Sigma, St. Louis, MO) substrates. The densities of relevant bands of assay samples were measured using an AlphaImager 3400 (Alpha Inotech Corp, San Leandro, CA). According to the intensity of the resulting blue and red bands to a particular SIV antigen, arbitrary values ranging from 0 to 4 were ascribed to each sample. For SIV Envelope-specific antibodies endpoint titers were determined as previously reported(328).

V1/V2 mapping: V1/V2 mapping was performed by using peptides for the V1/V2 region of SIVsmE660 Envelope region. Nunc MaxiSorp (Rochester, NY) plates were coated with approximately 1 µg/ml pooled peptides. Plates were blocked with 10% fetal bovine serum followed by washing in 0.1% polysorbate 20 in PBS. Serum was diluted 1:50.

Plates were washed, and an anti-monkey IgG HRP secondary antibody (SouthernBiotech, Birmingham, AL) was added. Plates were washed and developed using the Sigmafast OPD substrate (Sigma). Values are reported as the OD read at 450 nm.

Determination of neutralizing antibody titers: Neutralizing antibody responses against tier 1 SIVsmE660.11 were measured using luciferase-based virus neutralization assays with TZM-bl cells as previously described (392).

Antibodies for PBMC flow cytometry: Surface stain monoclonal antibodies (mAbs) include: anti-CD4 [L200], anti-CD49d α4 integrin [9F10] and anti-CD95 [DX2] (BD Biosciences, San Jose, CA); anti-CD14 [TUK4], anti-CD20 [HI47] and LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen, Grand Island, NY); anti-CD28 [CD28.2] (Beckman Coulter, Pasadena, CA); anti-CD8 [2ST8.5H7] (Custom, mAb from Serotec, conjugation kit from Invitrogen). Intracellular stain mAbs include: anti-CD3 [SP34-2] and anti-TNF-α [MAb11] (BD Biosciences); anti-IFN-γ [4S.B3] and anti-IL-2 [MQ1-17H12] (Biolegend, San Diego CA).

Flow cytometry staining protocol for PBMCs: PBMCs were isolated from RhMs and cryopreserved. Samples were thawed and stimulated overnight (18 hours) in R10 at $2x10^6$ cells/mL with (i) SIVmac239 peptide pools specific for Gag or Pol, R10 (negative) or Staphylococcal Enterotoxin B (SEB, positive). 1ul/mL GolgiPlug (brefeldin A) and 0.7ul/mL GolgiStop (monensin) (BD Biosciences) were added 1 hour after stimulation began. Cells were then stained as previously described[13].

Intravaginal challenge of rhesus macaques: All 28 animals were intravaginally

challenged with 500 $TCID_{50}$ SIVsmE660 prepared in the laboratory of Dr. Phil Johnson (Children's Hospital of Pennsylvania) twice a week for two weeks. The $TCID_{50}$ of this stock was re-titered in CEMx174 cells at the time of challenge and was 4000 $TCID_{50}$. The dose was chosen to mimic early HIV infection. Depo-Provera was not used during the challenge to increase the RhMs ability to become infected. Blood samples were collected twice weekly for six weeks, weekly for two weeks, and then monthly to day 190 after challenge to monitor plasma viral load. TRIM5α analysis was performed and did not affect challenge outcome. Additional blood and tissue samples were collected at days 14, 28, and 56 after challenge and processed as described for the pre-challenge samples. RhMs were defined as aborted infections if the viral loads remained below the level of detection for the remainder of the study (6 months post challenge). Time to viral control was determined as the number of days after initial infection to the first day viral loads were undetectable or 150 days if progressively infected.

SIV viral RNA quantitation: SIV viral RNA was quantitated using a procedure described previously (393, 394).

Statistical analysis: Data are presented as the mean ± S.E.M or median as specified in the figure legends based off of the normalcy of the data as calculated from triplicate wells from each experimental group. The statistical difference between immunization groups was assessed by using Mann-Whitney test, modified ANOVA test or Fisher exact test. Comparisons between samples with a P value <0.05 were considered to be statistically different and therefore significant.

4.3 Results

Inclusion of mucosal chemokine adjuvants induces robust cellular responses to all antigens

In this study, we vaccinated four groups of animals consisting of five female RhMs with pSIVmac239 *pol* and pSIV sooty mangabey consensus *envelope* and *gag* vaccine alone or in combination with CCR9L *pCCL25* or CCR10Ls *pCCL28* or *pCCL27* at weeks 0, 6, 12, 18 and boosted at week 48. We also vaccinated 14 female rhesus macaques with water followed by EP and termed this group "naïve" control animals (**Figure 4.1*a***). The consensus immunogens were developed as previously described using multiple sooty mangabey SIV sequences (324, 326, 395). The homology of the SIVsm Envelope to isolates from SIVsmE660 swarm ranges from 94-97% (**Figure 4.1*b***). Compared to pre-vaccination levels (**Figure 4.2*a***), after four immunizations, all RhMs showed robust cellular responses against all vaccine immunogens (**Figure 4.2*b***). RhMs immunized with CCR9L chemokine had significantly higher total amount of IFN-γ secreting PBMCs compared to DNA only ($P < 0.01$) which was predominately $CD8^+$ T cell driven (**Figure 4.3**). All animals demonstrated good recall responses after the final immunization to all included vaccine antigens (**Figure 4.2*c***). Cellular responses were further investigated two weeks after final immunization to determine if there were differences in cytokine profiles between the groups. Peripheral blood mononuclear cells (PBMCs) were stimulated with Gag and Pol peptides followed by intracellular cytokine staining. The inclusion of CCR9L adjuvant increased the amount of antigen-specific $CD8^+$ T cells secreting IFN-γ, TNF-α, and IL-2 (**Figure 4.2*d***). The addition of CCR10L

adjuvants only marginally affected $CD8^+$ T cells, with the largest difference in TNF-α and IL-2 expression, compared to the DNA only group (**Figure 4.2*d***). Polyfunctionality for both $CD4^+$ and $CD8^+$ T cells were also assessed after final vaccination (**Figure 4.4**), displaying limited differences in the number of cells secreting all three cytokines but increased differences in populations secreting either two or a single cytokine.

Inclusion of mucosal chemokine adjuvants increases humoral responses in sera and secretions

Since both CCR9L and CCR10L adjuvants have previously been shown to increase humoral responses (349, 377, 390), we investigated the vaccine-induced antibody production in serum and vaginal washes. We confirmed that the addition of CCR10L adjuvant enhanced vaccine-specific IgA above the levels induced by DNA alone vaccination. These results were obtained by measuring Gag (p27)- and Env (gp160)-specific IgA in the serum and vaginal wash by ELISA and western blot (WB) two weeks after final vaccination. The use of CCR10L adjuvant resulted in significant elevation in serum IgA levels against p27 antigen ($P<0.05$ compared to both DNA only and CCR9L) and against gp160 ($P < 0.05$ compared to DNA only) measured as WB band intensity (**Figure 4.5a**). Additionally, there was a trend for increased serum IgA against gp160 observed in RhMs vaccinated with CCR9L adjuvant. However, there were no significant differences in the levels of serum SIV-specific IgA Envelope ELISA binding titers between groups (**Figure 4.5*e***). Serum IgG revealed similar strong WB band intensities against p27 and gp160 in the CCR10L adjuvant group (**Figure 4.5*b***). Moreover, endpoint titers of serum IgG antibodies to gp140 also tended to be elevated in

CCR10L adjuvanted RhMs (**Figure 4.5*f***). Although the values are not significant, due to large variability, the p27 and gp160 IgA binding antibodies were elevated in vaginal secretions of animals receiving CCR10L adjuvants (**Figure 4.5*c***). Only 3 out of 5 RhMs receiving the DNA vaccine exhibited measurable IgA responses averaging 0.53 WB band intensity units. In contrast, 7 out of 9 animals receiving CCR10L adjuvants had measurable p27 IgA responses, with an average WB band intensity of 1.6. In the CCR9L adjuvant group, 4 out of 5 animals had measurable p27 IgA responses with an average WB band intensity of 1.1. Likewise, there were no detectable vaginal gp160 IgA responses in DNA-vaccinated animals, whereas 4 out of 9 animals receiving CCR10L adjuvanted vaccine had gp160-specific responses. Neither CCR9L nor CCR10L adjuvants appeared to have much of an effect on vaginal IgG responses compared to DNA only immunized group (**Figure 4.5*d***).

To further characterize potentially protective vaccine-induced humoral responses, we measured V1/V2 binding using a linear peptide pool ELISA and the neutralizing antibody titers using the standard TZM-bl assay. The consensus SIVsmE660 vaccine induced V1/V2 binding antibodies, but V1/V2 binding seemed to be only slightly enhanced by the addition of CCR9L or CCR10L adjuvants (**Figure 4.5*g***). Serum IgG binding to linear peptides against V3 and gp41 were also investigated, but there was no induction of responses to these regions by the vaccine (data not show). We also observed neutralizing titers present against the tier 1 SIVsmE660.11 pseudotyped virus; however, there were no significant differences in neutralization titer between groups, with CCR9L vaccinated animals showing a slight increase compared to the other groups (**Figure 4.5*h***).

Inclusion of CCR9L and CCR10L adjuvants enhance protection against challenge

To assess the impact of vaccine induce responses and acquisition of SIV, we performed a repeat intravaginal challenge with 500 $TCID_{50}$ (median tissue culture infective dose) SIVsmE660 that had been previously titered for vaginal challenge. Fourteen vaccine-naïve animals were included as challenge controls. Following challenge, we observed that 12 out of 14 vaccine-naïve RhMs became infected, and all animals exhibited an acute peak of viremia of 10^6 to 10^8 viral copies per ml and setpoint from 10^4 to 10^6 viral copies per ml (**Figure 4.6*a***). Two vaccine-naïve animals did not become infected with a baseline percent protection of 14.2. Grouping all vaccinated animals together, 13 out of 19 display either no infection or aborted infection corresponding to 68% protection (**Figure 4.6*b***) which is highly significant (P = 0.0016 compared to naïve). When animals were divided into their corresponding vaccine regimens, there was a large difference in challenge outcome. Two out of five DNA only vaccinated RhMs remained uninfected, leading to 40% protection (P= 0.23 compared to naïve) (**Figure 4.6*c***). This protection trended higher in the CCR9L vaccinated animals, in which three out of five RhMs were protected, corresponding to 60% protection (P = 0.06 compared to naïve) (**Figure 4.6*d***). The challenge outcomes for animals immunized with CCR10L adjuvanted vaccine were noticeably different; 2 out of 9 RhMs remained uninfected, and 1 out of 9 displayed progressive infection. The remaining 6 out of 9 animals had aborted infections, exhibiting brief viremia that rapidly declined to below detectable levels, resulting in 89% protection which is highly significant (P = 0.003 compared to naïve) (**Figure 4.6*e***).

Following challenge, we also observed significant vaccine effects on viral parameters. Compared with vaccine-naïve animals, there was a significant decrease in peak viral load in all vaccinated animals ($P<0.05$) (**Figure 4.7*a***), specifically in the CCR10L adjuvanted group ($P<0.05$) (**Figure 4.7*b***). A more dramatic adjuvant effect was observed when analyzing the time to viral control. There was a trend towards decrease time to viral control in all vaccinated compared to naïve animals (**Figure 4.7*c***). This was further emphasized when each group was analyzed. RhMs immunized with CCR10L adjuvants showed a significant decrease in days to viral control when compared to naïve ($P<0.001$), DNA only ($P<0.001$) and CCR9L chemokine adjuvant ($P< 0.001$) with an average time to control of 38.7 days (**Figure 4.7*d***). Viral loads for all uninfected and aborted infections remained below detection thru the end of the study, six months post-challenge (**Figure 4.6**).

Differential induction of vaginal IgA and IgG antibodies could influence outcome of vaccination

To further understand how differential induction of antibody isotypes could influence the challenge outcome, data analysis was performed for animals grouped according to their disease progression. Specifically, “uninfected” animals were defined as having no detectable viral loads through challenge follow up; “aborted infection” for the animals which were infected but controlled viremia to undetectable levels; and finally “progressive infection” for the animals with measurable viral loads throughout the study. Humoral responses were followed two months post challenge, a time chosen to be after the peak of viral infection. In the uninfected animals, the vaginal and serum IgA and IgG

antibodies specific for Envelope remained unchanged after challenge, suggesting that these animals remained truly uninfected (**Figure 4.8*a*-*b***). However, animals with aborted or progressive infections had significant increases in Envelope binding antibody titers in both systemic and mucosal compartments (**Figure 4.8*a*-*b***). In both abortive and progressive animals, vaginal antibody endpoint binding titers to Envelope increased almost 4000-fold for IgA and 30,000-fold for IgG after infection. Within the serum compartment, these increases were 50,000-fold and 1,000,000-fold for IgA and IgG respectively. Additionally, serum neutralizing titers of antibodies did not change after challenge for uninfected RhMs but did increase significantly for both aborted and progressively infected RhMs (**Figure 4.8*c***).

To determine whether potential correlates of immunity exist for RhMs which remained uninfected or displayed aborted infection, we analyzed responses two weeks after final immunization. Due to the limited number of animals in each outcome group, the study analysis was not powered to detect small changes in antibody levels and thus there was no significant difference when evaluating individual groups. However, there were some trends of importance: including differences in the induction of vaginal IgA and IgG to viral proteins (**Figure 4.9*a*–*b***). Specifically, RhMs with progressive infection only exhibited vaginal IgA and IgG antibodies to Gag (p27) whereas RhMs which remained uninfected or aborted infection displayed vaginal IgA and IgG antibodies to Envelope, Gag, and Pol. For all proteins except Gag, RhMs with aborted infection exhibited the highest levels of IgA and IgG. Within the serum, all challenge outcome groups induced binding IgA and IgG antibodies to all vaccine antigens (**Figure 4.9*c*-*d***).

Binding titers of serum antibodies to Envelope (gp160) did not show any difference between the groups (**Figure 4.9*e-f***). Uninfected animals exhibited the highest level of SIVsmE660.11 neutralizing antibody titers followed by the abortively infected group (**Figure 4.9*g***). When investigating the V1/V2 linear epitope binding response, both uninfected and abortively infected RhMs showed higher responses than the progressively infected animals (**Figure 4.9*h***). The number of IFN-γ secreting T cells were similar in all outcomes indicating that peripheral T cell responses did not appear to contribute to challenge outcome (**Figure 4.9*i***). Total $CD4^+$ or $CD8^+$ T cells secreting cytokines after 4^{th} and 5^{th} immunization also did not appear to correlate with challenge outcome or peak viral loads (**Figure 4.10*a-b***). Taken together, the data from this pilot study suggest that the presence of mucosal IgA and IgG and neutralization titers inversely correlate with levels of SIV infection and likely contributes to prevention of infection.

4.4 Discussion

A strength of the DNA vaccine platform is its ability to combine plasmids encoding cytokines and chemokines as part of the vaccine formulation, which were able to specifically influence the immune responses towards the desired outcome (57, 58, 101, 113, 349, 350, 369, 377, 390, 396). In this study, we demonstrated that the addition of immune plasmid adjuvants encoding mucosal chemokines could increase the effectiveness of a DNA vaccine against an SIV challenge. Macaques immunized with CCR10L adjuvanted vaccine demonstrated 89% protection with 6 of 9 displaying aborted infection. These animals did not exhibit positive viral loads through the end of the study, corresponding to 6 months post-challenge follow-up. Within the CCR10L immunized

animals, only 2 out of 9 animals remained uninfected compared to 3 out of 5 for CCR9L vaccinated animals and 2 out of 5 in the DNA only immunization, suggesting different possible mechanisms of protection between the vaccinated groups. The majority of control in the CCR10L immunize animals occurs after the virus has already disseminated whereas the control in the CCR9L immunized animals blocks the establishment of infection or dissemination into the peripheral blood. Future studies investigating these differences in control could shed light on the development of an efficacy HIV-1 vaccine.

In addition to the use of a highly novel gene adjuvant, this study has many other innovative factors. These include the use of adaptive electroporation to drive increased transfection efficiency and *in vivo* expression of antigen. Within this study, we see strong protection against challenge with the use of a DNA only immunization regimen. A strength of DNA vaccination continues to be the induction of strong cellular responses but limited to no antibody responses. Due to this, we have continued to focus on increasing DNA vaccine's ability to drive systemic and compartmentalized antibody responses while trying to maintain cellular responses. Within this study, we were able to induce both strong cellular and humoral responses using only DNA without the possible serological complications of viral vectors or live attenuated vaccines. There have been few studies which have looked at the ability of DNA vaccination to induce mucosal responses and in many cases, the addition of a heterologous boost is required (320, 397-399). However, within this study using only DNA, we see 15 out of 19 RhMs inducing mucosal responses as measured by WB band intensity units against either Envelope or Gag. Additionally, the constructs used in this study were not matched to the SIVsmE660

swarm and demonstrate the ability of a synthetic consensus immunogens to drive cross-reactive and broad responses that can impact viral infection. The viral challenge was specifically titered for vaginal challenge, mimicking early infection from male to female while yielding a high rate of infection in naïve. Another novelty of the study is the strength of looking at both the serum and mucosal responses. The ability to induce responses in both compartments will likely be important for future HIV vaccines. Within the study, we see that what is observed in the serum does not necessarily predict what is observed in the vaginal mucosa.

Though correlate analysis is difficult with smaller animal groups, we do see some trends. Compared to other platforms such as the CMV vectors, which show increase abortive infection after peak viral load (400-402), we do not observe differences in the assayed T cell responses induced between groups. Instead, all difference appeared to be related to humoral responses. As expected, uninfected RhMs have the highest titers of neutralizing antibodies to SIVsmE660.11 isolate. Subsequent analysis of RV144 trial indicated that antibodies to the V1/V2 loops of HIV Envelope correlated with a lower risk of HIV infection (211, 334). Following this, RhMs which remained uninfected and abortively infected had a higher level of serum IgG binding to the V1/V2 region of SIVsmE660 peptides compared to progressively infected animals. In contrast to RV144, there was no difference in serum IgA binding titers to Envelope (gp140) across all groups or a correlation between vaccine-induced $CD4^+$ T cells and challenge outcome and control. These results suggest the need to further investigate the relationship between vaginal IgA and IgG antibodies in HIV protection.

Though all of the differences in immune responses detected were related to humoral responses, this does not eliminate the potential for cellular responses to play a role in protection after vaccination and additional study in this regard is warranted. We have reported in a trial with the HVTN, that pIL-12 can increase the number of vaccine responders in humans receiving an HIV DNA vaccine delivered by EP (58). A future study to compare pIL-12 alone or in combination with mucosal adjuvants in this model would be informative. Additionally, the chemokine adjuvant's effects on resident effector cells at the mucosa is also important. The presence of effector memory T cells at the initial mucosal sites of infection could allow for abortive infection to occur. Previous studies in mice have suggested that the use of the mucosal chemokine adjuvants was able to upregulate the number of cells positive for either the CCR10 or CCR9 receptor at the site of vaccination (349, 377, 390). We are continuing to investigate how these cells leave the muscle and migrate to mucosal sites where they become effector cells.

Within this study, we report an overall protection rate of 68% in all vaccinated RhMs against an SIVsmE660 swarm mucosal challenge vs. a control rate of 14%. Within the study, there is a significant increase in protection in the CCR10L-adjuvanted animals, displaying 89%. These levels of protection from chronic progressive infection are significant and thus warrant further investigation. By including different chemokine and cytokine adjuvants including mucosal chemokines, DNA vaccines appear to specifically focus the immune response to enhance protection. Such a mechanism is of clear clinical relevance for HIV vaccine studies.

Tables

		Uninfected	Abortive	Progressive
Mamu	A*01 +	4	3	8
	A*01 -	5	3	10
TRIM5	Strong/ moderate	9	6	14
	Weak	0	0	2

Table 4.1: Genotype analysis of RhMs and challenge outcome. Genotype analysis was performed on each RhM to ensure it did not affect challenge outcome. RhMs were grouped based on challenge outcome for all groups including naïve RhMs. Two RhMs in the naïve group did not have TRIM5α analysis performed on them and have not been included in the table.

Figures

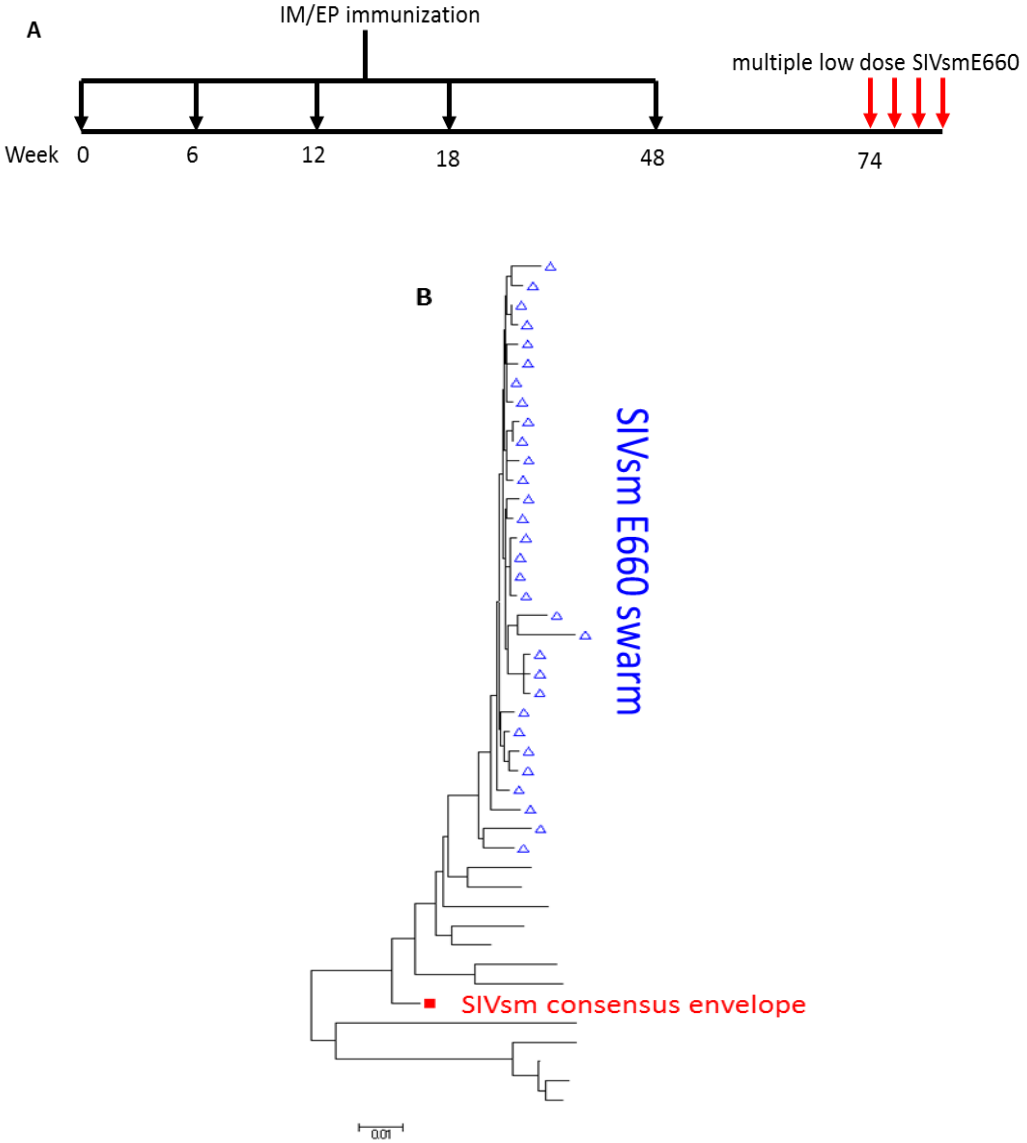

Figure 4.1: Genetic diversity of constructs and immunization schedule. (***a***) Schematic of the immunization and challenge regimens. RhMs were immunized on weeks 0, 6, 12, 18 and 48. After a 26 week rest, RhMs were challenged with multiple low dose SIVsmE660 swarm. (***b***) Genetic tree of SIV sooty mangabey Envelopes. The consensus e*nvelope* was made using multiple different SIVsm e*nvelopes*. The blue open triangles

how sequences from the SIVsmE660 swarm stock used in the experiment. The red square represents the consensus Envelope used for immunization.

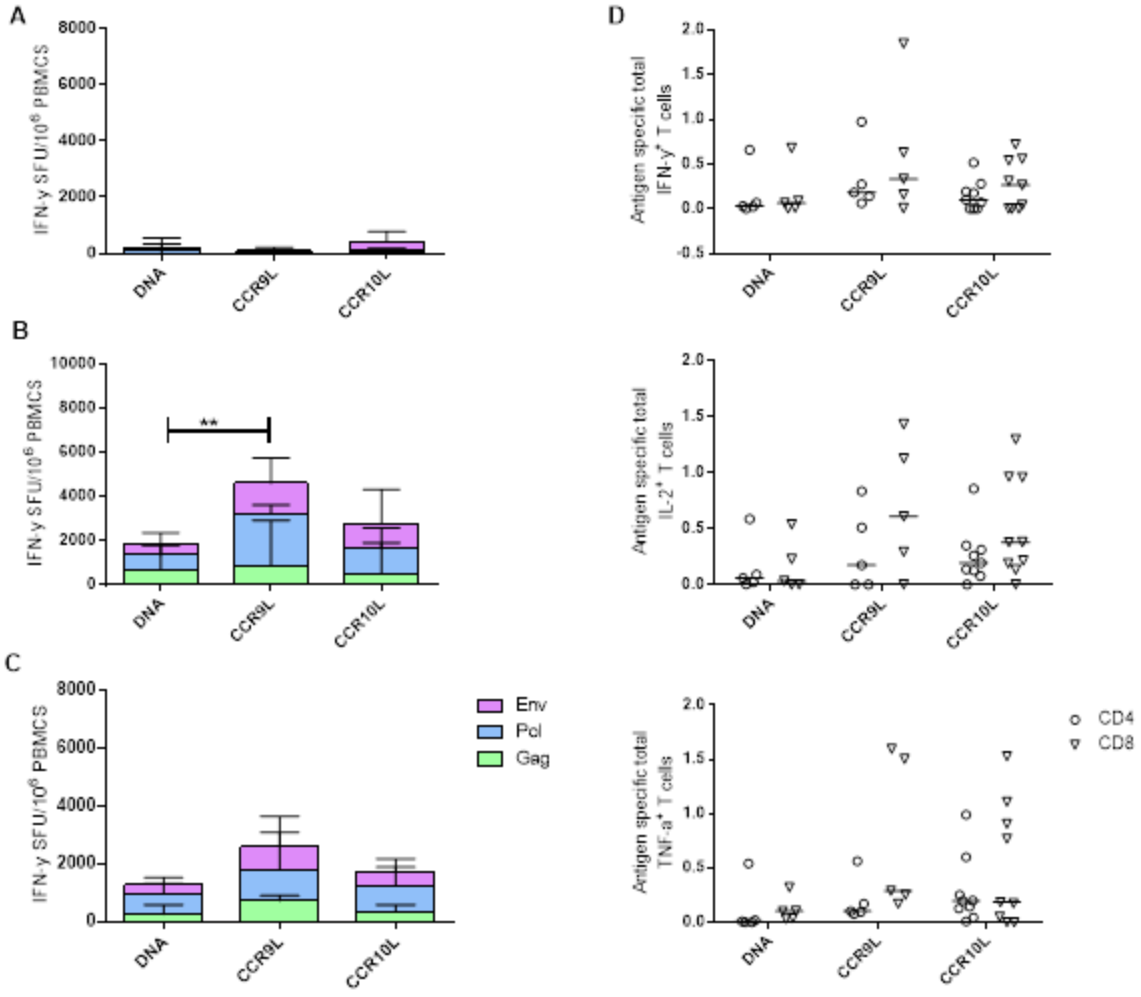

Figure 4.2: **Cellular response induced by vaccination.** Interferon-gamma secreting cells against Gag (green) Pol (blue) and Env (purple) were enumerated by ELISpot assay. Responses were measured at week 0 (***a***) week 20 after 4th vaccination (***b***) and week 53 recall / final immunization (***c***). Intracellular cytokine staining was performed on cells stimulated with peptides from Gag and Pol and totaled for CD4 and CD8 at week 53(***d***).

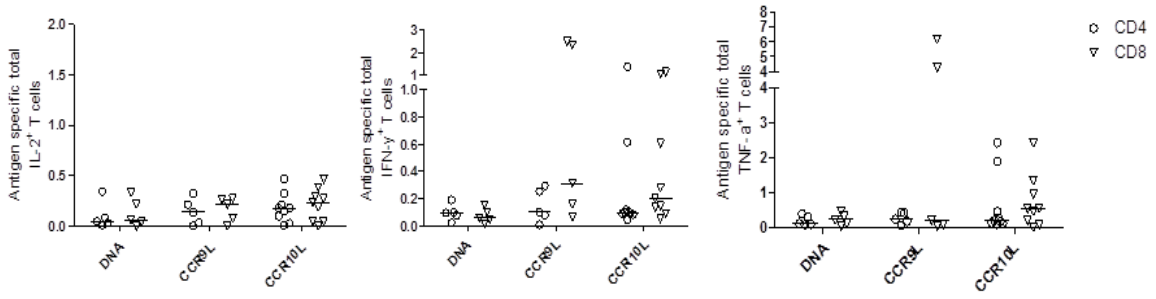

Figure 4.3: Cytokine production from CD4$^+$ and CD8$^+$ T cells after 4th immunization. Intracellular cytokine staining was performed on PBMCs isolated at week 21 and stimulated with peptides from Gag and Pol and totaled for CD4 (circles) and CD8 (triangles). Bars indicate median

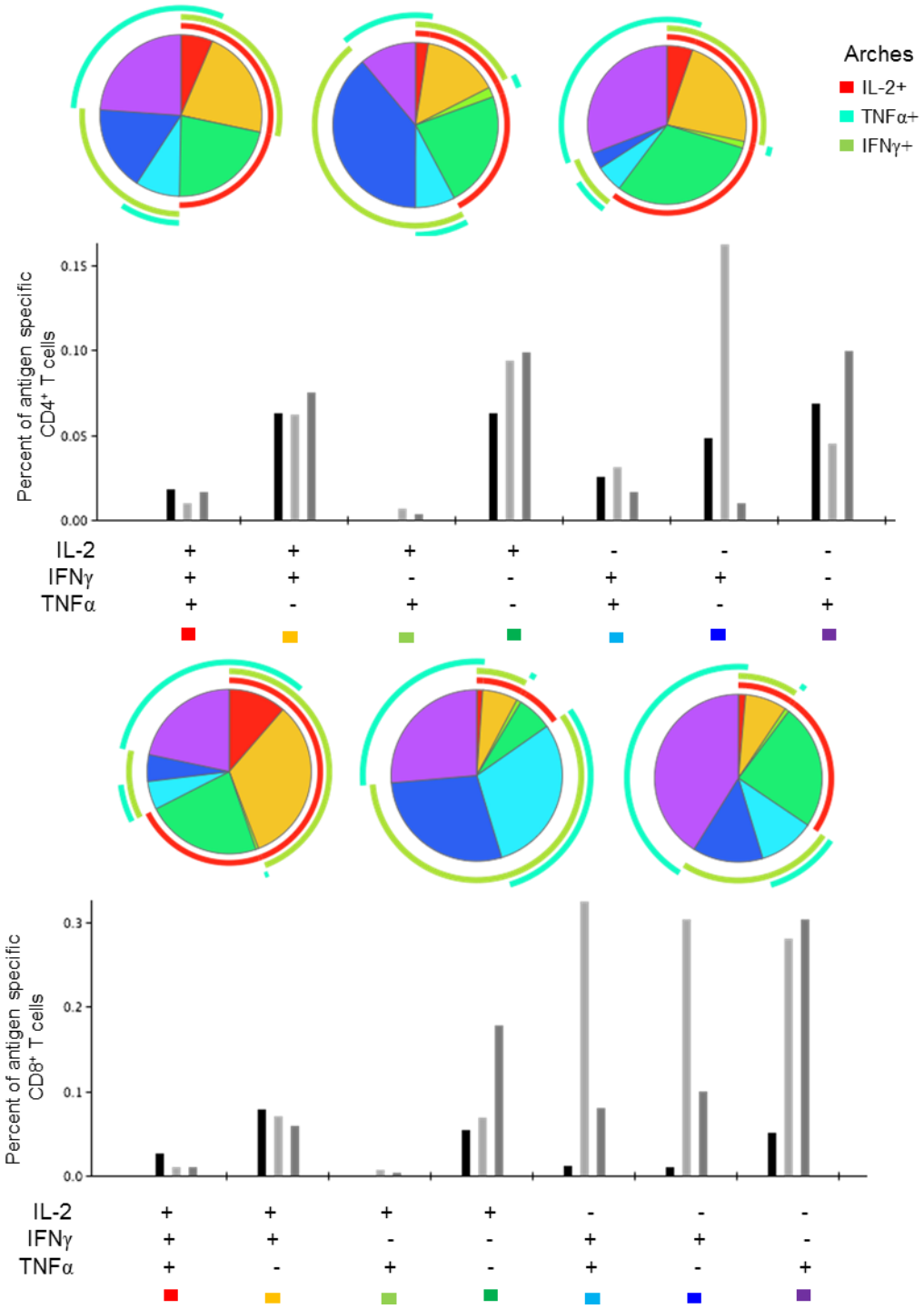

Figure 4.4: Polyfunctional T cell responses induced by DNA vaccination. Polyfunctionality of PBMCs isolated at week 53 was determined for both CD4 (top) and CD8 (bottom) against Gag and Pol

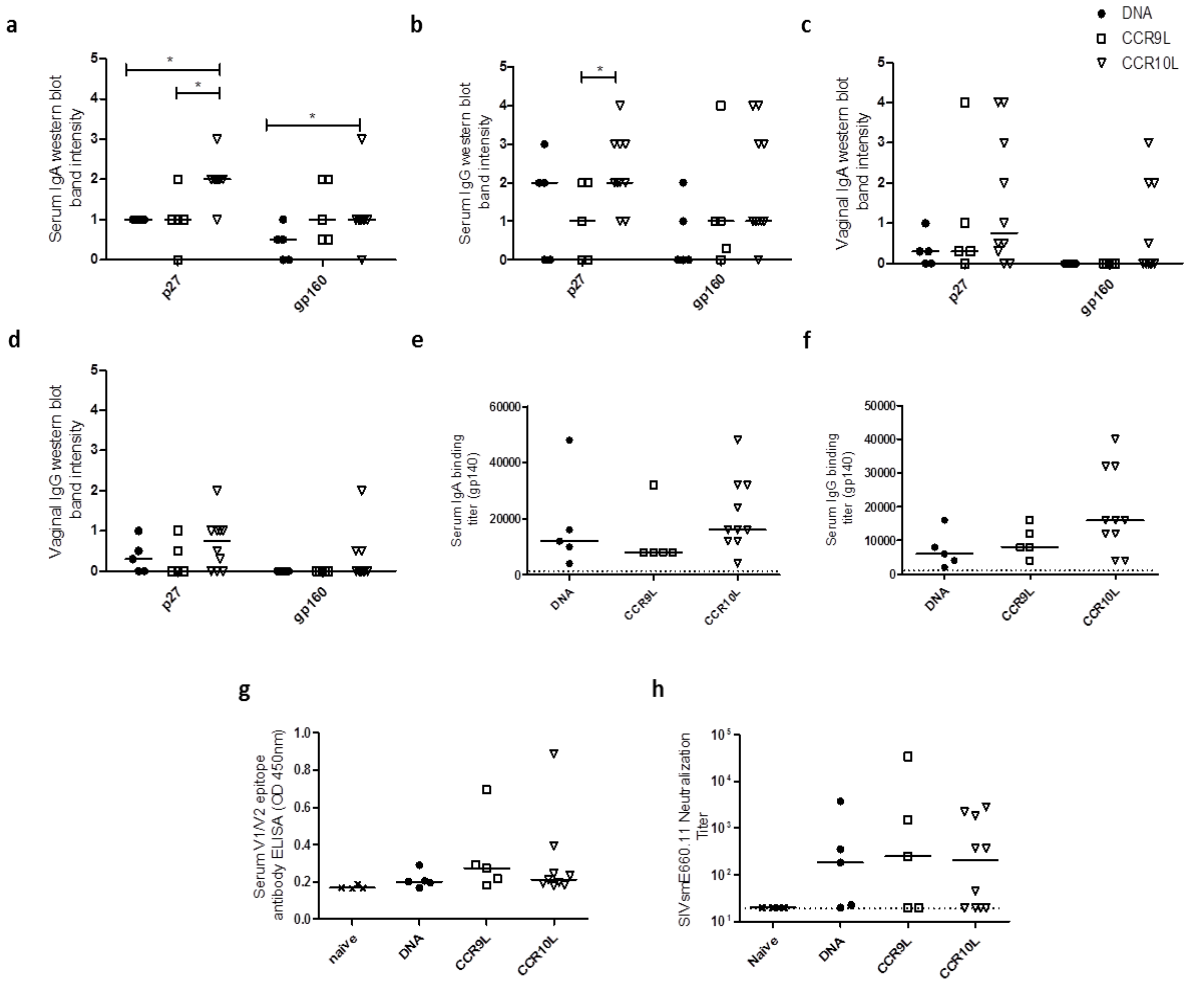

Figure 4.5: Chemokine adjuvants enhance the vaccine-induced humoral response. Antibody responses were measured at week 50 (2 weeks after final vaccination). Serum IgA (***a***) and IgG (***b***) antibodies specific to Gag (p27) and Envelope (gp160) expressed as WB band intensities. Serum IgA against p27 was elevated in the group received CCR10L compared to DNA only ($P < 0.05$) and CCR9L ($P<0.05$). IgA (***c***) and IgG (***d***) against Gag (p27) and Envelope (gp160) measured in Ig-normalized vaginal secretions and expressed as WB band intensities. Serum IgA (***e***) and IgG (***f***) antibodies against gp140 Envelope protein expressed as ELISA endpoint titers. Serum IgG antibody binding to 15mer

peptides spanning the V1/V2 region of Envelope (***g***). SIVsmE660.11 serum antibody neutralizing titers measured by TZM-bl assay (***h***). Dashed lines denote the limit of detection for respective assay. Bars indicate median. The *P*-values reported were calculated using the Mann-Whitney test. * indicates a $P < 0.05$**.**

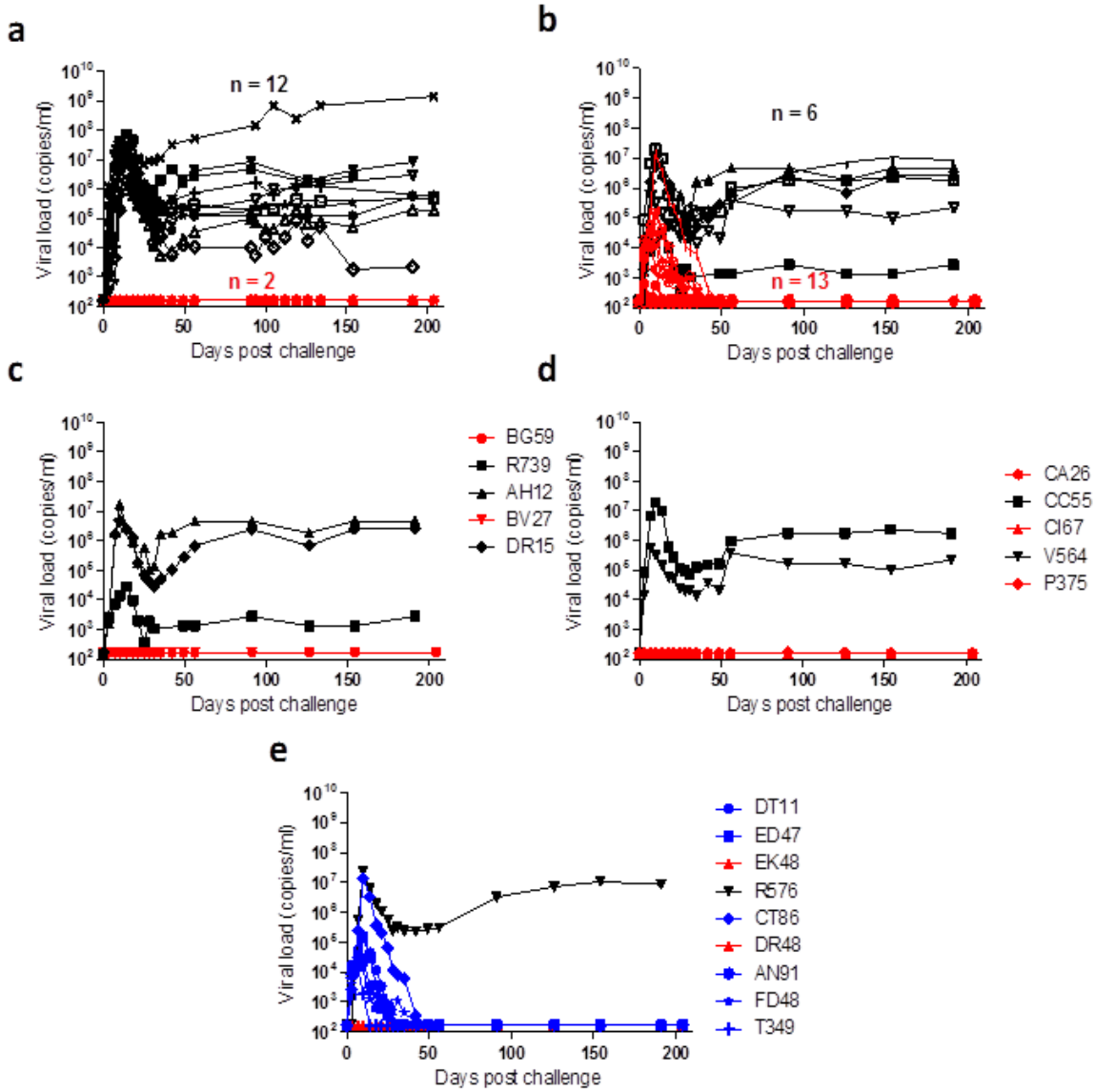

Figure 4.6: DNA vaccination and chemokine adjuvants improve the challenge outcome. Animals were intravaginally challenged twice a week with SIVsmE660 26 weeks after the final booster vaccination and the viral loads were determined. The color black indicates animals with progressive infection, blue with abortive infection, and red the uninfected animals. Viral load in (***a***) vaccine-naïve animals (n=14); (***b***) all vaccinated

animals (n=19); (***c***) in DNA only vaccinated animals (n=5); (***d***) in CCR9L vaccinated animals; and (***e***) in CCR10L vaccinated animals (n=9).

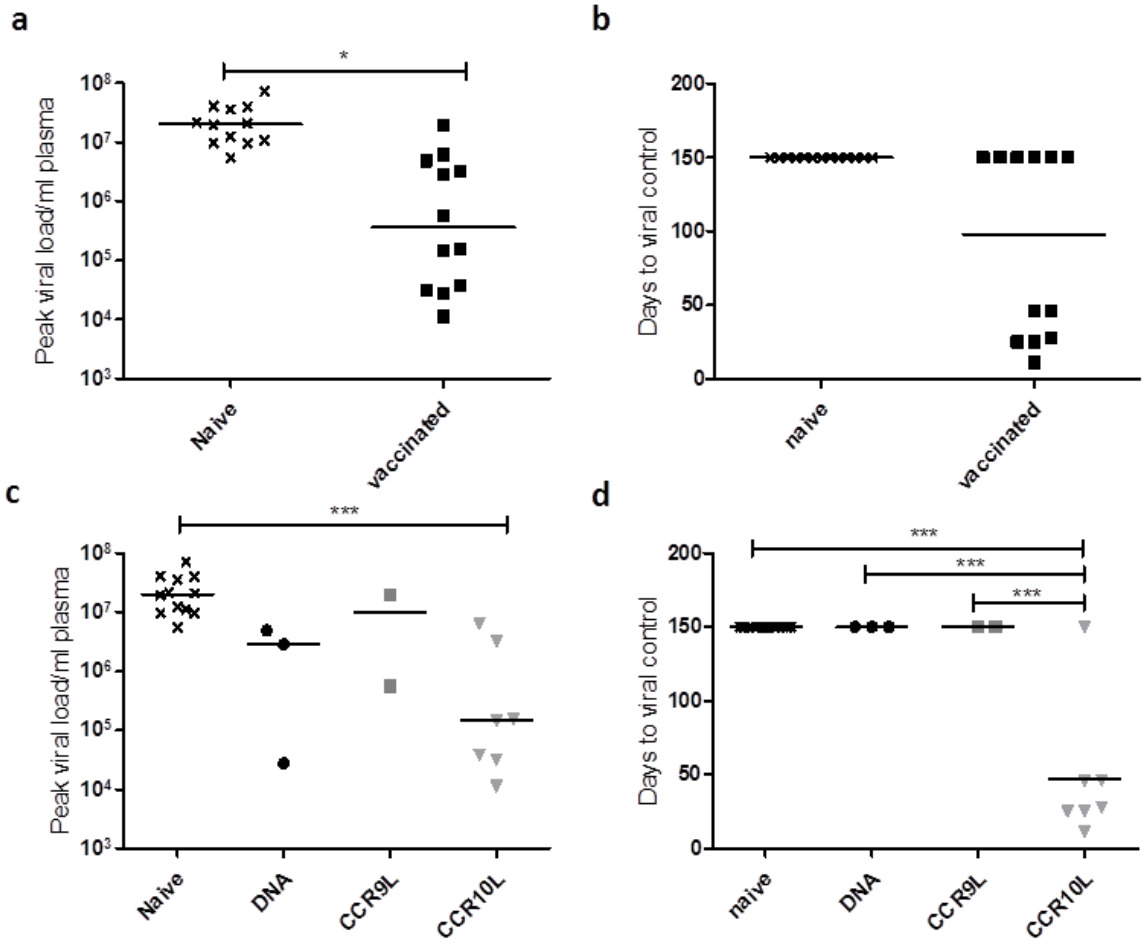

Figure 4.7: CCR10L adjuvanted vaccine improved the challenge outcome characteristics. (***a***) Peak viral loads in animals that became infected. The peak of viral load was significantly (P<0.05) reduced in vaccinated compared to naïve animals. (***b***) Peak viral loads of each group of vaccinated animals. Peak viral loads was significantly reduced (*P*<0.05) in RhMs receiving CCR10L adjuvanted vaccine compared to naïve animals. (***c***) The number of days until viremia reached undetectable levels in infected RhMs. Animals in which viremia was never controlled were scored as day 150. Animals receiving CCR10L adjuvant had a shorter time to control of viremia compared to animals

receiving CCR9L adjuvanted vaccine ($P<0.001$), DNA only ($P<0.001$) and naïve animals ($P<0.001$). Bars indicates mean. The *P*-values reported were calculated using the Student-T test for (***a***) and a modified ANOVA for (***b***) and (***c***).

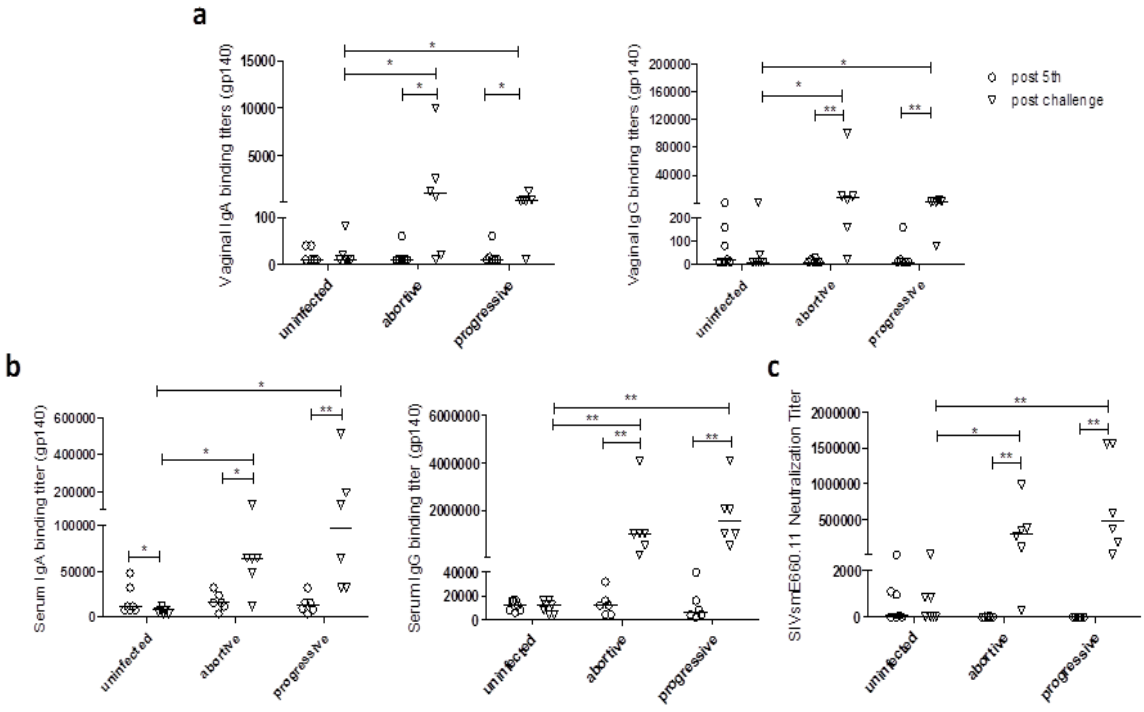

Figure 4.8: Differential induction of humoral responses post challenge. Humoral responses were monitored after last immunization and at two months post challenge. Serum IgA and IgG specific for gp140 Envelope glycoprotein in vaginal secretions (***a***) and serum (***b***) expressed by ELISA endpoint titers. Neutralization titers against SIVsmE660.11 after final vaccination and at two months post challenge (***c***). RhMs were assigned to either uninfected, aborted or progressively infected groups based on the challenge outcome. Bars indicate median. The *P*-values reported were calculated using the Mann-Whitney test. * indicates a $P < 0.05$ and ** indicates a $P < 0.01$.

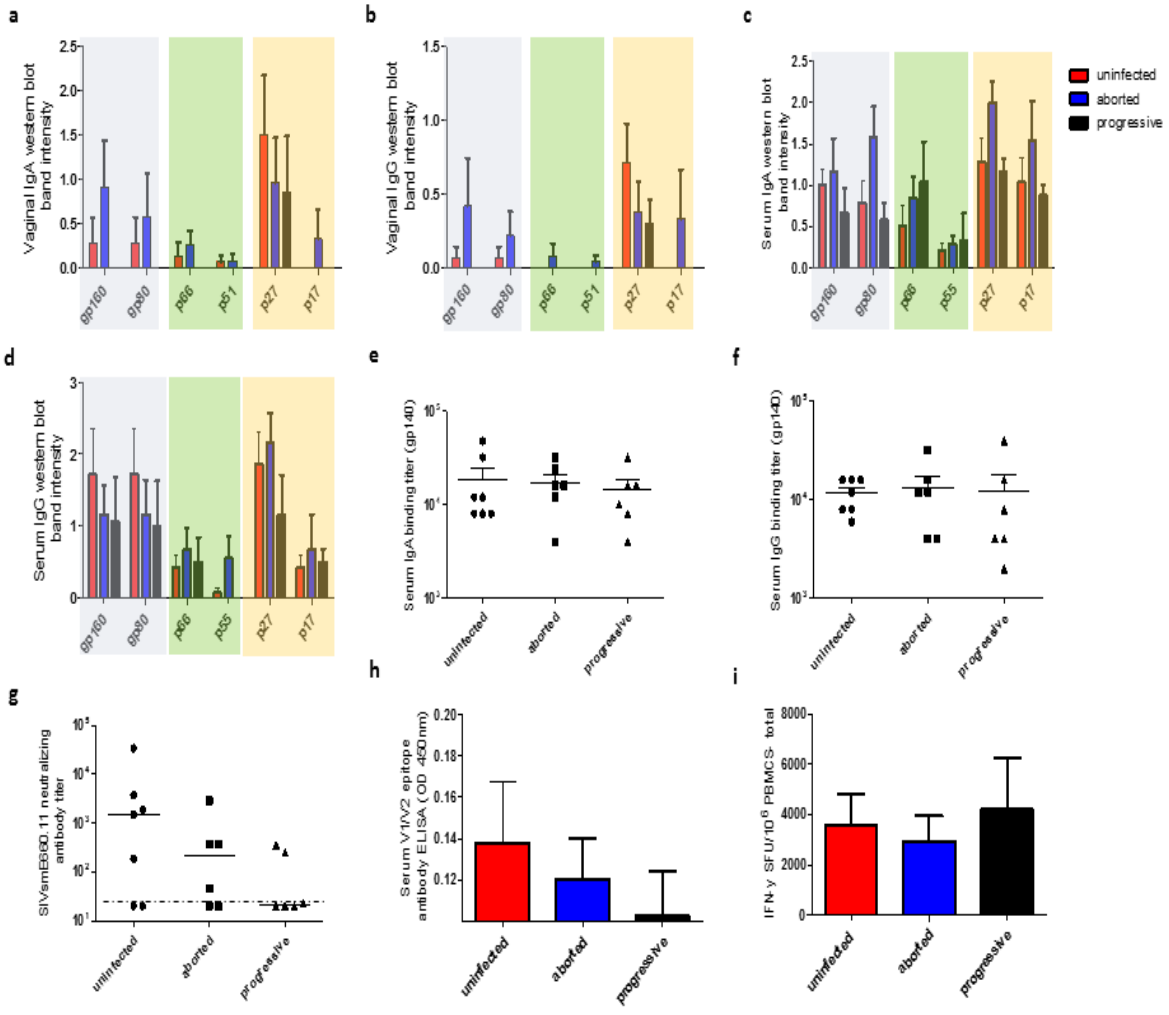

Figure 4.9: Correlates analysis of humoral and cellular responses. Antigen-specific antibody and IFN-γ responses were measured two weeks following the final vaccination. Vaccinated animals were grouped according to their challenge outcome in uninfected (n=7) (red), aborted (n=6) (blue) and progressive infection (n=6) (black) groups. Vaginal IgA (***a***) and IgG (***b***) antibodies specific for different HIV proteins, expressed as intensity of WB bands. Both vaginal IgA and IgG antibodies were elevated in uninfected and aborted infection compared to progressively infected animals. Serum IgA (***c***) and IgG (***d***) for different HIV proteins, expressed as WB band intensity. Serum IgA (***e***) and IgG (***f***)

against SIV gp140, expressed as ELISA endpoint titers. Antibody neutralizing titers (***g***) against SIVsmE660.11 isolate were elevated in uninfected animals compared to abortive and progressive infection. Serum IgG (***h***) antibodies binding to V1/V2 were elevated in uninfected, compared to progressively infected animals. Total IFN-γ (**i**) responses to Gag, Pol, and Env by peripheral blood mononuclear cells (PBMCs). The number of IFN-γ secreting cells was determined by ELISpot assay and expressed as spot forming units (SFU). Dashed lines denote the limit of detection for respective assay. Bars indicates mean with s.e.m.

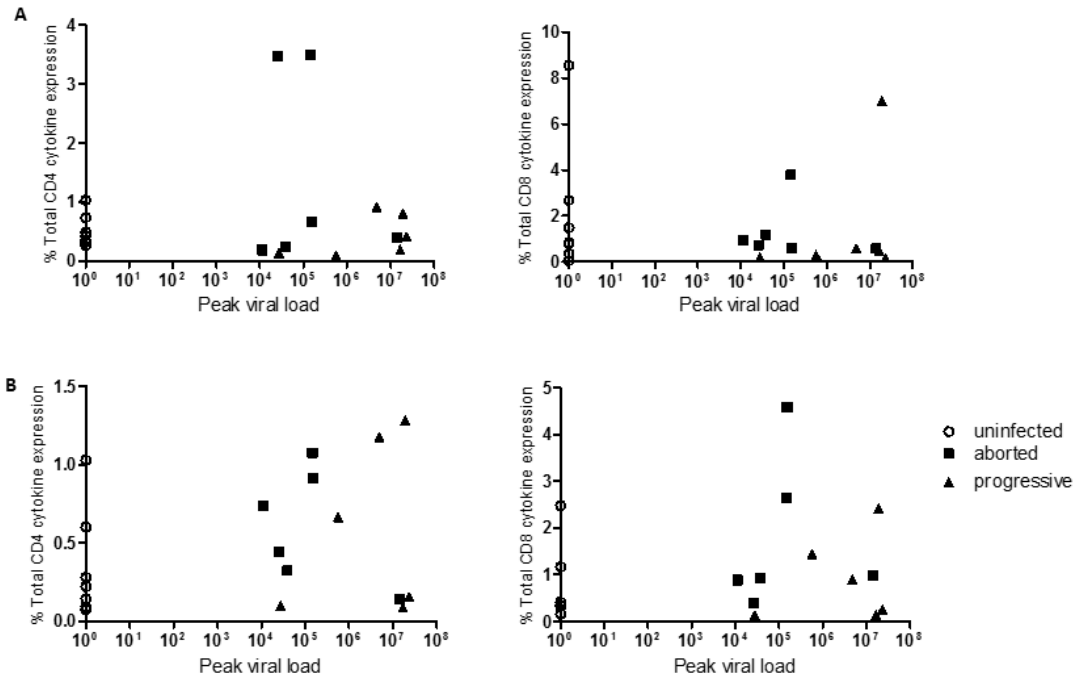

Figure 4.10: Peripheral cellular responses do not correlate to challenge outcome or peak viral loads. The percent of $CD4^+$ (left) and $CD8^+$ (right) T cells which secreted cytokines after stimulated with Gag and Pol peptides was determined two weeks after 4th immunization (***a***) and two weeks after final immunization (***b***) and plotted against peak viral load. There is not a strong correlation between cellular responses, peak viral loads or challenge outcomes.

CHAPTER 5: A NOVEL SYNTHETIC CD40L PLASMID ADJUVANT GENERATES UNIQUE ANTI-HPV DNA VACCINE INDUCED RESPONSES THAT IMPACT TUMOR GROWTH

5.1. Introduction

Recently, there have been numerous successes in immunocology, further increasing the focus on targeting the immune system to control cancer malignancies. The importance of CD8 T cells in controlling tumor growth has been well established in mouse models and further supported by correlates found in humans (403-407). However, there is continued need to develop novel strategies to increase immune responses against cancer antigens. Vaccination lends itself well to inducing both cellular and humoral responses against tumor-associated antigens (TAAs), driving the production of antigen-specific cytotoxic CD8 T cells (122, 408). In many cases, though, these T cells are not enough to control or eliminate tumor growth. The use of adjuvants to increase or tailor vaccine-induced responses could improve the generation of T cells and increase the efficacy of the vaccine (88, 93).

Due to its role in both T and B cell development, CD40 ligand (CD40L) is an ideal vaccine adjuvant candidate (409, 410). CD40L is a type II membrane protein and part of the TNF superfamily of molecules (411). It is expressed on epithelial and smooth muscle cells and is upregulated on CD4 T cells upon activation or stimulation (412). Its binding partner, CD40, is mainly expressed on antigen presenting cells (APCs), CD8 T cells and B cells (413, 414). CD40L binds to CD40 on APCs, leading to the licensing of the APC, increased maturation and antigen presentation as well as increased production

of IL-12 and other pro-inflammatory cytokines (410, 415, 416). Additionally, CD40-CD40L interaction on B cells leads to antibody class switching and increased proliferation (409). Because of CD40L's role in acting as a switch of the innate-adaptive immune response, there have been many studies to explore its potential as a vaccine adjuvant. Several studies used CD40L fused to either viral vectors or proteins to target the antigen of interest to APCs (417-425). Others have used CD40L encoded on a DNA plasmid and injected together with a plasmid encoding the antigen (109, 426-431). Many of these studies observed increases in responses with the addition of CD40L, dominant by the enhancement of the CD8 T compartment.

We wanted to explore further the adjuvanting properties of CD40L by exploring its different isoforms. Naturally, CD40L can be membrane bound or cleaved into a soluble form (412). Within this study, we sought to directly compare an uncleavable membrane-bound form (UC), a wild-type form (WT) and a soluble form (S) of CD40L encoded as an immunoadjuvant for a DNA vaccine. These optimized adjuvant plasmids were co-delivered with a plasmid expressing the oncogenic proteins E6 and E7 of HPV type 16 (300) followed by *in vivo* electroporation (EP). After two vaccinations, we observed significantly higher CD8 and CD4 T cell responses when the soluble (pS-CD40L) was included as well as slight increases in antibody responses. Similar responses were not observed with the pUC- or pWT-CD40L constructs. Upon further investigation, these CD8 T cells peaked 11 days after first vaccination when pS-CD40L was included and were boosted after the second immunization. Contrary to previous reports, these CD8 T cell responses were still partially dependent on CD4 T cell help. Additionally, the trend

in responses observed after final vaccination were maintained into memory and were dependent on delivery to the same local site. Each of the CD40L constructs alone was not able to confer protection from a tumor challenge. However, when pS-CD40L was included with the HPV DNA vaccine, there was significant tumor control and clearance compared to DNA alone or DNA plus pUC- or pWT-CD40L. The inclusion of pS-CD40L increases both systemic as well as tumor infiltrating antigen-specific CD8 T cells in tumor-bearing mice compared to vaccine alone or naïve mice. Additionally, when pS-C40L was added, the ratio of antigen-specific CD8 T cells to CD4 Tregs in the tumor was significantly higher compared to vaccine alone or naïve. These results demonstrate that soluble but not membrane-bound CD40L can adjuvant a DNA vaccine and leads to increase vaccine efficacy in a therapeutic tumor model.

5.2. Materials and Methods

DNA constructs: The sequence for mouse CD40 ligand was obtained from Uniprot (P27548). To make the uncleavable (UC) form, the amino acids around the cleavage site (111-112) were deleted. For the soluble form (S), the amino acids from the cleave site to the C-terminus were included. All inserts were RNA and codon optimized and cloned into the pVax backbone (Genscript Piscataway, NJ). The plasmid expressing HPV 16 E6 and E7 was used as previously described (300).

Transfection and *in vitro* expression of plasmid: Transfection and *in vitro* expression

To confirm expression of the CD40L constructs, mouse myoblast cell line C2C12 cells (ATCC, Manassas, VA) were transfected with each plasmid using Lipofectamin 3000 (ThermoFisher Scientific) following the manufactures protocol. Media and cell lysate

was collected at 24, 48 and 72 hours after transfection. Cells were lysed using a mixture of cOmplet, mini, EDTA-free protease inhibitor (Roche, Basel, Switzerland) with Cell Signaling Cell lysis buffer (Danvers, MA). To test for expression, cell lysate was run on a 12% Bis-Tris NuPAGE gel (ThermoFisher Scientific) followed by transfer to PVDF membrane (Millipore). The membrane was blocked for 1 hour at room temperature with LiCor PBS blocking buffer (Lincoln, NE) and was probed overnight with 1:1000 dilution of anti-mouse CD40L (R&D Systems, Minneapolis, MN) and 1:5000 dilution anti β-actin (ThermoFisher Scientific) as a loading control in LiCor blocking buffer. After washing with 0.1% PBS-Tween (PBST), the membrane was probed with 1:15,000 dilution of IRDye® 680RD goat anti-mouse and IRDye® 800CW donkey anti-goat secondary antibodies (Licor). The membrane was then washed and developed on the Licor Odessey CxL. Expression was also quantified using an anti-mouse CD40L quantitative ELISA kit (Boster Biological, Pleasanton, CA) following manufactures protocol.

Animals: All mice were housed in compliance with the NIH, the University of Pennsylvania Institutional Animal Care and Use Committee guidelines, and the Wistar Institutional Animal Care and Use Committee. Six to eight week old female C57/BL6 mice were purchased from Jackson Laboratory.

Animal Immunizations: Mice were immunized with 5ug of HPV16 E6/E7 plasmid with or without 15ug of the different forms of plasmid CD40L (pCD40L). All DNA was formulated in water. Mice were injected intramuscularly (IM) in the tibialis anterior muscle followed by electroporation (EP) using the CELLECTRA® 3P (Inovio Pharmaceuticals, Plymouth Meeting, PA) as previously described (432).

Isolation of peripheral blood mononuclear cells (PBMCs): Blood from mice was collected in tubes containing 4% sodium citrate (Sigma-Aldrich, St. Louis, MO) to prevent clotting. Histopaque 1083 (Sigma-Aldrich) was then layered under the blood. After spinning to create the gradient, cells from the buffy coat were collected and used for tetramer analysis.

ELISA

Before sacrificing, serum was collected to determine the vaccine-induced humoral responses. Maxisorp 96 well plates (ThermoFisher Scientific) were coated with 1ug/ml of E7 protein (ProteinX Lab, San Diego, CA) in PBS and stored at 4 degrees overnight. ELISAs were performed as previously described with the primary mouse serum diluted 1:50 followed by four-fold dilutions to create a dilution curve. Secondary goat anti-mouse HRP-labeled antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a 1:5000 dilution. Plates were developed for 5 minutes using the Sigma Fast OPD tables and stopped with 2N sulfuric acid. Endpoint binding titers were calculated as previously described.

CD4 depletion: Mice were administered 100ug of anti-mouse CD4 antibody (clone GK1.5 BioXCell, Lebanon, NH) formulated in PBS intraparietal (IP) one day before first vaccination followed by every three days after that. Successful depletion was confirmed by flow cytometry.

ELISpot assay: Ninety-six well filter plates (Millipore, Billerica, MA) were coated with anti-IFN-γ capture antibody (R&D, Minneapolis, MN) overnight at 4^{o} C. Spleens were isolated from mice at various time points depending on the study. After processing the

spleens as previously described, $2x10^5$ cells were added to blocked plates. Cells were stimulated with overlapping 15mer peptide pools for either E6 or E7 (5ug/ml per peptide) (Genscript). Media alone and concanavalin A (Sigma-Aldrich) were used as negative and positive controls respectively. After 18hrs of stimulation, the plates were washed, and secondary detection antibody (R&D) was added for 24hrs at 4ºC. Plates were then washed and developed using the ELISpot Blue Color Module (Millipore) per the manufacturer's protocol. Plates were then scanned and counted using the ImmunoSpot® S6 FluoroSpot ELISpot plate reader (CTL, Shaker Heights, OH).

Flow cytometry: For intracellular cytokine staining, $2x10^6$ splenocytes were stimulated in the presence of protein transport inhibitor, GolgiStop™ GolgiPlug™ (BD Bioscience, San Jose, CA) with the same peptide pools as the ELISpots. Media alone and phorbol 12-myristate 13-acetate (PMA) and ionomycin (BD Biosciences) stimulations were used as negative and positive controls respectively. To test for degranulation of cells, anti-CD107a antibody (FITC; clone 1D4B; Biolegend, San Diego, CA) was also added during stimulation. After 6hrs, cells were washed and stained with LIVE/DEAD violet (Invitrogen, Carlsbad, CA). Surface staining was then added containing anti-CD4 (BV510; clone RM4-5; Biolegend), anti-CD8 (APC-Cy7; clone 53-6.7; Biolegend), and anti-CD44 (Alexa Fluor 700; clone IM7; Biolegend). After 30 minute incubation, cells were spun, washed, and fixed using the FoxP3/Transcription factor fixation/permeabilization kit (eBioscience, San Diego, CA) following manufacturer's protocol. Intracellular staining was then prepared using anti-IFNγ (APC; clone ZMG1.2;

Biolegend), anti-TNFα (BV605; clone MP60ZT22; Biolegend), anti-IL2 (PE/Cy7; clone JES6-5H4; Biolegend), and anti-CD3 (PE/Cy5; clone145-2C11; eBiosciences).

For tetramer staining, isolated PBMCs were washed and staining using LIVE/DEAD violet. The surface stain was then added containing anti-CD8 (APC-Cy7; clone 53-6.7; Biolegend), anti-CD44 (Alexa Fluor 700; clone IM7; Biolegend) and the iTAg tetramer H-2D^{b} HPV16E7 tetramer (RAHYNIVTF) (PE; MBL. Woburn, MA). Cells were then fixed using BD stabilizing fixative (BD Bioscience).

All data was collected on a modified LSRII flow cytometer (BD Bioscience) followed by analysis with FlowJo software (Tree Star, Ashland, OR).

Tumor cell line: The TC.1 cell line expressing the HPV E6 and E7 proteins were given by Dr. Yvonne Paterson of the University of Pennsylvania. Cells were cultured in Dulbecco's modified Eagle media (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (Atlas Biologicals, Ft. Collins, CO). Cells were passaged one time before implantation.

Tumor challenge studies: Groups of 10 mice were subcutaneously implanted with $5x10^4$ TC.1 cells on the right flank of the mouse. One week after implantation, mice were vaccinated (IM) with 5ug of HPV16 E6/E7 DNA only, HPV16 E6/E7 DNA only in combination with 15ug of pCD40L, or with 15ug of pCD40L alone followed by *in vivo* EP. Mice received boosters on days 14, 21, and 28. Tumors were monitored and measured with electronic calipers twice weekly. Tumor volume was calculated using $(\pi/6)*(\text{height})*(\text{width})^2$. Per the University of Pennsylvania's and Wistar's Institution

Animal Care and Use Committee, all mice which had tumors between 18-20mms or which became ulcerated were sacrificed.

Isolation of tumor infiltrating lymphocytes (TILs): For all TIL studies, tumors were harvested at day 21 post tumor implant and immediately placed in cold PBS. Lymphocyte isolated was performed as previously described (433). Briefly, tumors were minced in 500µL of digestion mix. The digestion mix consists of 170mg/L Collagenase I, II and IV (ThermoFisher, Waltham, MA), 12.5mg/L DNAse I (Roche, Basel, Switzerland), 25mg/L Elastase (Worthington, Lakewood, NJ) in a 50/50 mixture of Hyclone L-15 Leibowitz Media (ThermoFisher) and RPMI, supplemented with 5% FBS and 1% Penicillin/Streptomycin. Minced tumors were then transferred to a 50mL conical filled with 10mL of digestion mix, and rocked gently for 45 minutes. 10mL of complete RPMI (RPMI + 10% FBS) was added to the tumor digestion mix, and then cells were filtered through a 40µm mesh filter. Cells were then counted and plated for tetramer analysis.

Statistical analysis: Statistical analysis was performed using a one-way modified ANOVA with a Turkey posthoc test for all studies except for the tumor challenge study. For this study, a two-way modified ANOVA was performed. All analysis was performed using GraphPad Prism Software (La Jolla, CA). Horizontal bars represent mean with error bars expressing the standard error.

5.3. Results

Various forms of plasmid CD40L (pCD40L) are expressed in vitro

Naturally, CD40L is expressed as both a membrane-bound form or as a solubilized cleaved version (**Figure 5.1*a***). To address the contributions of each form, three different plasmids were produced. First, the wildtype (WT) form of mouse CD40L contained the entire coding sequence of CD40L. Two additional constructs were also produced, the uncleavable form (UC) expressing only the membrane-bound form of CD40L by deleting the amino acids around the extracellular cleavage site (aa111-112) and a soluble-only form which only encoded for the amino acids post the cleavage site (S) (**Figure 5.1*b***). We transfected the mouse myoblast cell line C2C12 with each plasmid to test for the expression *in vitro*. Western blot analysis of cell lysate revealed that all constructs expressed (**Figure 5.1*c***). We used a quantitative ELISA for mouse CD40L to quantify the expression of each of the constructs over time. As expected, expression of pUC is highest in the cell lysate whereas the pS-CD40L was highest in the cell supernatant (**Figure 5.1*d***).

Soluble CD40L increased CD8 T cell responses against HPV16 E6/E7

To initially test for immunogenicity, groups of 5 C57Bl/6 mice were vaccinated with DNA plasmids expressing the oncogenic proteins of HPV16, E6/E7, with or without the different forms of pCD40L. Ten days after the second vaccination, mice were sacrificed to assess the vaccine-induced responses (**Figure 5.2*a***). The inclusion of pS-CD40L significantly increased the number of IFN-γ spot-forming units (SFU) compared to vaccine alone (**Figure 5.2*b***). To further characterize the cellular responses,

intracellular cytokine staining was performed on splenocytes stimulated with overlapping peptide pools for E6 and E7 (**Figure 5.2*c***). Compared to DNA only vaccination, the inclusion of pS-CD40L significantly increased the percent of CD8 T cells producing IFN-γ, IL-2, and TNF-α ($p<0.001$ for all parameters). These responses were also significantly higher than mice vaccinated with HPV+ either the pWT or pUC form of CD40L ($p<0.001$ for all parameters). Since cytolytic killing function and degranulation is important for CD8 T cells to combat tumor cells, we also looked at the ability of these CD8 T cells to express the degranulation marker CD107a and co-produce IFN-γ. Co-formulation with pS-CD40L significantly increased antigen-specific CD8 T cells which expressed both of these markers compared to HPV only ($p<0.05$) and HPV+ either pWT or pUC-CD40L ($p<0.001$) (**Figure 5.2*b*** lower left graph). The inclusion of pS-CD40L also increases the percent of CD8 T cells which were able to express multiple cytokines and CD107a compared to vaccine alone (**Figure 5.2*d***). Though CD8 T cell responses have been previously found to be the driving force of tumor regression for the HPV16 E6/E7 DNA vaccine, we also investigated if the inclusion of pCD40L could increase CD4 and humoral responses. Antibody responses to HPV E7 protein were slightly increased in mice vaccinated with HPV+ pS-CD40L (**Figure 5.3 *a-b***). All five mice in the HPV+ pS-CD40L seroconverted and had detectable endpoint binding titers compared to 3 out of 5 in the DNA vaccinated alone group. The HPV16 E6/E7 DNA vaccine induces limited CD4 T cell responses. However, upon the inclusion of pS-CD40L, the percent of antigen-specific CD4 T cells producing IL-2 and TNF-α were significantly higher than vaccine alone ($p<0.01$ and $p<0.001$ respectively) (**Figure 5.3*c***). These

responses were also higher than the pWT or pUC form of CD40L. Overall, the inclusion of soluble (pS) CD40L increased both cellular and humoral responses over vaccine alone. Interestingly, the same responses were not observed with the pUC or pWT-CD40L which either maintains or slightly, though not significantly, decrease vaccine-induced responses.

Inclusion of soluble CD40L increased antigen-specific CD8 T cell responses in both the spleen and the periphery

We can investigate antigen-specific responses in both the spleen and the peripheral blood mononuclear cells (PBMCs) using a tetramer which is specific to the dominant epitope in E7 (H-2D^{b} RAHYNIVTF). Ten days after final vaccination, CD8 tetramer specific responses in both the spleen and PBMCs were significantly higher in mice vaccinated with the pS-CD40L and HPV compared to vaccine alone ($p<0.01$ (PBMC) and $p<0.05$ (spleen)) (**Figure 5.4 *a*-*b***). Similar to the CD8 T cell functional responses, the inclusion of pUC- or pWT-CD40L slightly decreased the responses compared to vaccine alone. Due to the ability to follow CD8 T cell responses in the periphery, antigen-specific responses over time were explored. Within seven days after vaccination, low, but detectable tetramer-specific CD8 T cells were observed in the PBMCs in all groups. Surprisingly, 11 days after first vaccination there was a dramatic increase in tetramer-specific CD8 T cells in mice vaccinated with HPV + pS-CD40L (**Figure 5.4*c***). These responses averaged around 17% with some mice having a quarter of all circulating CD8 T cells specific for this epitope. Responses contracted to levels slightly higher than those in vaccine alone by day 21 when a second boosting immunization was given. Within seven days, responses in the vaccine alone group

boosted to levels higher than after the first immunization. Though tetramer specific levels were higher in mice immunized with HPV + pS-CD40L, after the boost compared to those in the vaccine alone, we did not observe levels as high as day 11. Additionally, the kinetics of contraction were slower in the HPV + pS-CD40L compared to DNA alone suggesting that into memory, the increase in CD8 T cells could be maintained.

CD8 T cells induced by day 11 post first vaccination are functional but have limited IL-2 expression

Due to the large peak in tetramer specific responds on day 11, we wanted to see if these antigen-specific T cells were functional. Mice were immunized with either HPV alone or in combination with pCD40L and sacrificed 11 days after first vaccination. The inclusion of pS-CD40L significantly increased antigen-specific CD8 T cells expressing IFN-γ and TNF-α compared to vaccine alone ($p<0.00001$ for both) (**Figure 5.5*a***). Interestingly, this increase in IFN-γ and TNF-α was not mirrored in IL-2 expression. Where DNA alone and HPV + pS-CD40L had similar expression levels. Additionally, a significantly higher percentage of CD8 T cells induced by HPV + pS-CD40L express the combination of CD107a and IFN-γ ($p<0.00001$ compared to vaccine only) (**Figure 5.5*a*** lower left graph). Compared to after first vaccination, there is a decrease in CD8 T cells expressing all four parameters (CD107a, IFN-γ, TNF-α, and IL-2) due in part to the lack of IL-2 expression (**Figure 5.5*b***). Similar to what was detected in the time course study, around 20% of circulating CD8 T cells were tetramer specific when pS-CD40L was included which was significantly higher than with DNA alone (average = 7.9% $p<0.01$) (**Figure 5.5*c*** top graph). We also observe similar findings in the spleen, with the

combination of HPV + pS-CD40L inducing significantly higher amount of tetramer-specific CD8 T cells compared to vaccine alone ($p<0.00001$) and when pWT or pUC CD04L is included ($p<0.00001$) (**Figure 5.5*c*** bottom graph).

Vaccine-induced responses at day 11were partially depended on CD4 T cells

Studies have demonstrated that the addition of CD40L or CD40 monoclonal antibodies could bypass the need for CD4 help (414, 434-439). To address if this was also the case in our vaccine system, we depleted mice of CD4 T cells using an anti-CD4 monoclonal antibody, followed by vaccination at day 0 and day 21 (**Figure 5.6*a***). In mice vaccinated with HPV DNA alone (solid line), the depletion of CD4 T cells (dotted line) dramatically lowers the percent of tetramer-specific CD8 T cells induced in the periphery (**Figure 5.6 *b-c***). After the boost, the increase in tetramer responses in the isotype control treated mice was not observed in the CD4 depleted group. If CD8 T cells responses were dependent on CD4 T cell help, we would hypothesize that when mice were vaccinated with HPV + pS-CD40L, the responses would mimic those of vaccine alone without CD4 T cells. However, though the tetramer specific responses are significantly lower on day 11 in the CD4 depleted mice compared to isotype controls ($p<0.05$), we observed levels similar to those induced by the vaccine alone with intact CD4 T cell help. After the second immunization, the responses in mice depleted of CD4s and vaccinated with HPV + pS-CD40L, contract down but do not boost. These findings suggest that vaccine-induced responses when pS-CD40L was included were partially dependent on CD4 T cell help for initial responses but require it for memory and recall responses.

Addition of pS-CD40L to contralateral site does not increase systemic immune responses

We next wanted to determine if pS-CD40L had systemic or localized adjuvanting effects. Mice were injected with pS-CD40L either formulated together or separated and delivered to the contralateral leg. Mice received two immunizations at three-week intervals followed by EP. Unlike when pS-CD40L was added to the same site, the addition of it to a separate site did not lead to adjuvanting effects (**Figure 5.7**). When pS-CD40L was given at a separate site, expression of cytokines IFN-γ, TNF-α and the co-production of IFN-γ and CD107a are at similar levels compared to DNA alone (**Figure 5.7*a***). Additionally, there is no increase in tetramer-specific CD8 T cells in the spleen or PBMCs between DNA alone vs. pS-CD40L at separate sites (**Figure 5.7*b***). Thus, the adjuvanting effect of pS-CD40L was only observed when co-formulated and delivered to the same local site.

Vaccine-induced memory responses were maintained with the use of soluble pS-CD40L

The ability to induce strong memory responses is important for an efficacious vaccine. To address if the use of pS-CD40L as an immune adjuvant can maintain memory responses, we vaccinated mice two times at three-week intervals and sacrificed the mice two months after final vaccination (**Figure 5.8*a***). We observed responses similar to those found when mice were sacrificed one week after final vaccination, with HPV + pS-CD40L inducing significantly higher percentage of CD8 T cells expressing IFN-γ, TNF-α, and IL-2 compared to vaccine alone ($p<0.01$, $p<0.001$, and $p<0.05$ respectively) or when pUC or pWT CD40L were included (pUC: $p<0.05$, $p<0.001$, $p<0.05$ respectively and pWT: $p<0.01$, $p<0.01$ and n.s. respectively) (**Figure 5.8*b***). For

all vaccination regimens, we see a contraction of CD8 T cells expression IFN-γ but a maintenance in IL-2 and TNF-α expression compared to one week after final vaccination. In both the PBMCs and spleen, there is also a contraction in tetramer-specific CD8 T cell responses, but in both compartments, the use of pS-CD40L increased these levels (**Figure 5.8*c***). Thus, the use of pS-CD40L increased vaccine-induced responses but also maintains them into memory.

The inclusion of soluble CD40L significantly decreased tumor burden compared to vaccine alone and favorably shifts the infiltrating lymphocytes.

Due to the increase in CD8 T cell responses induced by the addition of pS-CD40L, we next explored if these increases could confer better protection in a therapeutic tumor model. First, we investigated if each of the CD40L plasmids alone could increase tumor clearance and control. Groups of 9-10 mice were implanted with $5x10^4$ TC.1 cells which express the E6 and E7 protein and have been previously used to test for vaccine efficacy (300). One week later, mice were vaccinated with each of the CD40L plasmids alone and tumor growth was monitored bi-weekly. None of the constructs alone led to any increase in tumor clearance and control compared to naïve mice (**Figure 5.9 *a-b***). We next explored if the combination with HPV DNA vaccine could increase responses. Groups of 9-10 mice were implanted with TC.1 cells and vaccinated one week later with either HPV alone or in combination with plasmids expressing each of the different CD40Ls (**Figure 5.10*a***). The HPV DNA vaccine alone did confer protection when compared to naïve mice (**Figure 5.10*b***). However, none of these mice were able to clear the tumor, and by the end of study (day 67 post-implantation), only two mice had not

reached the study endpoint. The addition of either pUC or pWT forms of CD40L to the vaccine had limited effect on tumor control compared to HPV alone (tumor volume n.s. compared to vaccine alone for both groups) (**Figure 5.10*b*** and **Figure 5.10*c***). However, when pS-CD40L was included in the vaccine, 50% of mice were able to clear tumors (**Figure 5.10 *b-c***). The inclusion of pS-CD40L led to significantly higher tumor clearance and control compared to vaccine alone ($p<0.001$) or the inclusion of pUC-CD40L and pWT-CD40L ($p<0.001$). Overall, the use of DNA delivered pCD40L alone did not increase tumor control in a therapeutic challenge model. However, when pS-CD40L was included in combination vaccine, mice were able to significantly control tumor growth compared to vaccine alone.

To further explore the vaccine-induced responses and control of tumor growth; we analyzed the tumor infiltration lymphocyte (TIL) population. Groups of 9-10 mice were implanted with TC.1 cells and vaccinated at days 7 and 14 post-implant with HPV DNA alone or HPV + pS-CD40L. On day 21, PBMCs, spleen, and tumors were harvested from the mice and analyzed for vaccine-induced responses (**Figure 5.11*a***). The timing was determined based on the rate of clearance of tumors by day 28 in the previous experiment when mice were immunized with HPV + pS-CD40L. Similar to the immunogenicity data, when pS-CD40L was included, systemic CD8 T cells expressed a significantly higher amount of IFN-γ and TNF-α compared to DNA vaccine alone or naïve mice (**Figure 5.11*b***). In addition, when pS-CD40L is added, antigen-specific CD8 T cells also co-express a significantly higher amount of IFN-γ and CD107a compared to vaccine alone ($p<0.001$) or naïve mice ($p<0.0001$) (**Figure 5.11*b*** bottom left graph). We

also observed significantly higher amount of CD8 T cells which were tetramer positive in both the PBMCs and the spleen with HPV + pS-CD40L (**Figure 5.11*c***). Due to the immune suppressive role of CD54 T regulatory cells, we also explored how vaccination affected this population systemically and in the tumor. The percent of total CD4 T cells which expressed Treg markers (CD25 and FoxP3) was unchanged in both compartments for all treatment regimens (**Figure 5.11*d***). Similar to the systemic immune responses, the inclusion of pS-CD40L significantly increases the percentage of CD45+ CD3+ tumor infiltrating lymphocytes that were positive for both CD8 and the tetramer ($p<0.001$ compared to vaccine alone, $p<0.0001$ compared to naïve) (**Figure 5.11*e***). Though the HPV vaccine was able to significantly increase the infiltration of antigen-specific CD8 T cells compared to naïve ($p<0.001$) the addition of pS-CD40L is still able to enhance these responses. Both vaccine alone and vaccine + pS-CD40L significantly decrease the percent of $CD45^+$ $CD3^+$ T cells that are $CD4^+$, $CD25^+$, $FoxP3^+$ Tregs compared to naïve mice ($p<0.001$ vaccine, $p<0.0001$ HPV + pS-CD40L) (**Figure 5.11*e***). Thus, the ratio of CD8 tetramer positive T cells to CD4 Tregs is favorably skewed towards CD8 T cells in both HPV alone and HPV + pS-CD40L compared to naïve but is highest when pS-CD40L is included (**Figure 5.11*e***). This data supports the earlier findings that pS-CD40L increases CD8 T cells and suggest that these cells are functional and able to traffic to the tumor site.

5.4. Discussion

Over the past decade, there is increased interest in cancer immunotherapies. The ability to harness the power of a person's immune system could limit toxicities and

provide durable and effective control (408). In this study, we demonstrate that a DNA plasmid encoded immune adjuvant can increase the effectiveness of our HPV16 DNA vaccine in mice. The ability to use plasmid encoded immune adjuvants is a strength of DNA vaccination and allows for the enhancement or tailoring of vaccine-induced responses. Here, we used various forms of CD40 ligand, a molecule having important roles in the link between the innate and adaptive immune system. We demonstrate that the inclusion of plasmid encoded soluble CD40L (pS-CD40L) can increase HPV DNA vaccine-induced responses. When pS-CD40L is formulated together with a plasmid encoding HPV16 E6 and E7, we observed increased CD8, CD4, and humoral responses after final vaccination and into memory (**Figure 5.2, 5.3,** and **5.8**). It is important to note that enhancement of antigen-specific CD8 T cell responses was maintained into memory. Studies have shown that when CD40L or CD40mabs are given, APCs can directly stimulate CD8 T cells, bypassing the need for CD4 help (414, 434-439). These stimulated CD8 T cells, however, are quickly lost and are not maintained into memory since the formation of memory CD8 T cells requires CD4 help (440-442). Here we observed that CD8 T cells induced one week after final vaccination display similar cytokine profiles as memory CD8 T cells. This was further supported by the CD4 depletion studies which demonstrated that the quick burst of tetramer-specific CD8 T cells on day 11 is only partially dependent on CD4 T cells as well (**Figure 5.6**). However, the second boost and increase of CD8 T cells observed with pS-D40L were completely dependent on the requirement of CD4 help (**Figure 5.6**). Additionally, we observe that the use of pS-CD40L increased the expression of IL-2 and TNF-α CD4 T cells suggesting that this

plasmid encoded immune adjuvant is influencing CD4 T cells. The maintenance of potent cellular responses is imperative for an effective therapeutic cancer vaccine.

Time course analysis revealed a significant peak in tetramer-specific CD8 T cells on day 11 post first vaccination when pS-CD40L is included (**Figure 5.4**). Though tetramer-specific CD8 T cells are significantly higher after the second immunization, the day 11 peak in response was not replicated to the same level. Further analysis of these CD8 T cell responses on day 11 demonstrated that these CD8 T cells display many markers of effective CD8 T cells including the expression of IFN-γ, CD107a, and TNF-α (**Figure 5.5**). Importantly, IL-2 expression was not increased significantly over any of the other vaccine formulations. Interlukin-2 has an important role in T cell proliferation and memory maintenance (443). Thus, if IL-2 production was increased or maintained in these antigen-specific CD8 T cells, we could observe a prolonged peak. This could be achieved with the combination of other plasmid-encoded immune adjuvants such as pIL-12 (58, 113). CD40L has been shown to increase IL-12 production from APCs and T cells (415), but by combining pIL-12 with pS-CD40L, we could observe a synergistic effect. Additional studies into vaccine responses induced by pS-CD40L vs. pIL-12 or in combined are currently in progress.

Due to its ability to potently induce immune responses, increased levels of sCD40L could lead to high toxicities and off-target effects (444, 445). Notably, within this study, we did not observe any toxicity related to co-administration of any of the pCD40L isotypes. This was further supported by the observation that only when pS-CD40L was co-formulated together and delivered to the same site do we detect enhanced

responses (**Figure 5.7**). When pS-CD40L was given to a distal site, in this case, the contralateral leg, immune responses are the same as vaccine alone. This is an important safety measure, demonstrating that pS-CD40L is only expressed locally and does not appear to be circulating systemically.

Within this study, we also observe significantly higher control of tumor growth when pS-CD40L was given with a suboptimal dose of our HPV DNA vaccine. It is important to note that we must use a suboptimal dose of HPV 16 E6/E7 DNA vaccine, as a full dose will completely control tumor growth to undetectable levels (data not shown). However, at a suboptimal dose, we observe initial control which was then lost after final vaccination. In comparison, when pS-CD40L was added, 50% of mice control the tumor to undetectable levels and this control was maintained throughout the completion of the study (**Figure 5.10**). The other 50% of mice eventually lose control and succumb to the tumor. To further understand the correlate of protection, tumors were harvested at day 21 and assessed for tumor infiltrating lymphocytes (TILs). This time point was selected since day 28 was around the time mice had cleared the tumor. Systemic immune responses detected in tumor-bearing mice showed similar trends as in non-tumor mice with increased cytokine production in CD8 T cells when pS-CD40L is included (**Figure 5.11**). Additionally, there was no difference in the systemic T regulatory cells as defined by $CD4^+$ $CD25^+FoxP3^+$ T cells. However, when we investigated the TILs when pS-CD40L was included, there was a significantly higher percentage of antigen-specific CD8 T cells and a decrease in Tregs compared to vaccine alone or naïve. This inverse in effector to regulatory T cells supports the increase clearance observed with pS-CD40L

addition. However, the clearance was not 100% and thus there is still room for improvement. The loss of initial control observed in all of the groups suggests that these CD8 T cells are either becoming exhausted or are receiving inhibitory signals. The ability to block these inhibitory signals using checkpoint inhibitor blockade has been a significant breakthrough in cancer immunotherapy (446). Combining these blockades with our HPV DNA vaccine with or without pS-CD40L could increase the vaccine effectiveness.

The strength of plasmid encoded immune adjuvants has been demonstrated by the use of pIL-12 in non-human primates and the clinic (57-59, 113). However, there is a need to develop novel DNA adjuvants which could be used alone or in combination with IL-12 to increase vaccine-induced responses. Here, we show the use of novel optimized plasmids encoding various forms of CD40L. We demonstrate that the inclusion of soluble CD40L increases cellular and humoral responses and these responses can increase control in a therapeutic tumor model. Further investigation into pS-CD40L's ability to adjuvant other antigens is imperative for its movement into clinical trials.

Figures

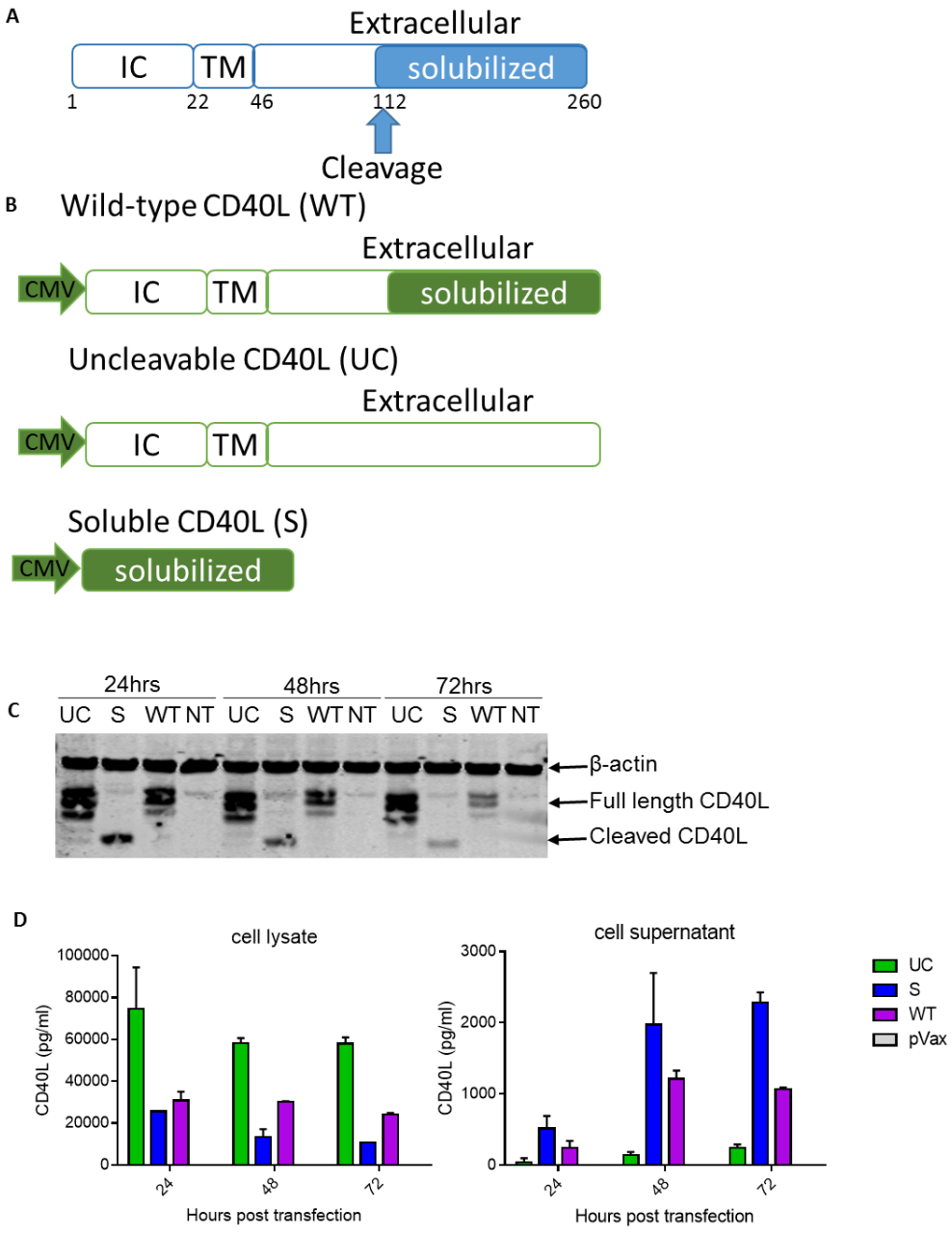

Figure 5.1: Plasmid construction and *in vitro* expression. (***a***) Diagram of annotated naturally expressed CD40L. (***b***) Diagram of each plasmid. (***c***) Western blot expression of plasmid CD40L in transfected cell. (***d***) Quantification of plasmid expressed CD40L in cell lysate and cell supernatant.

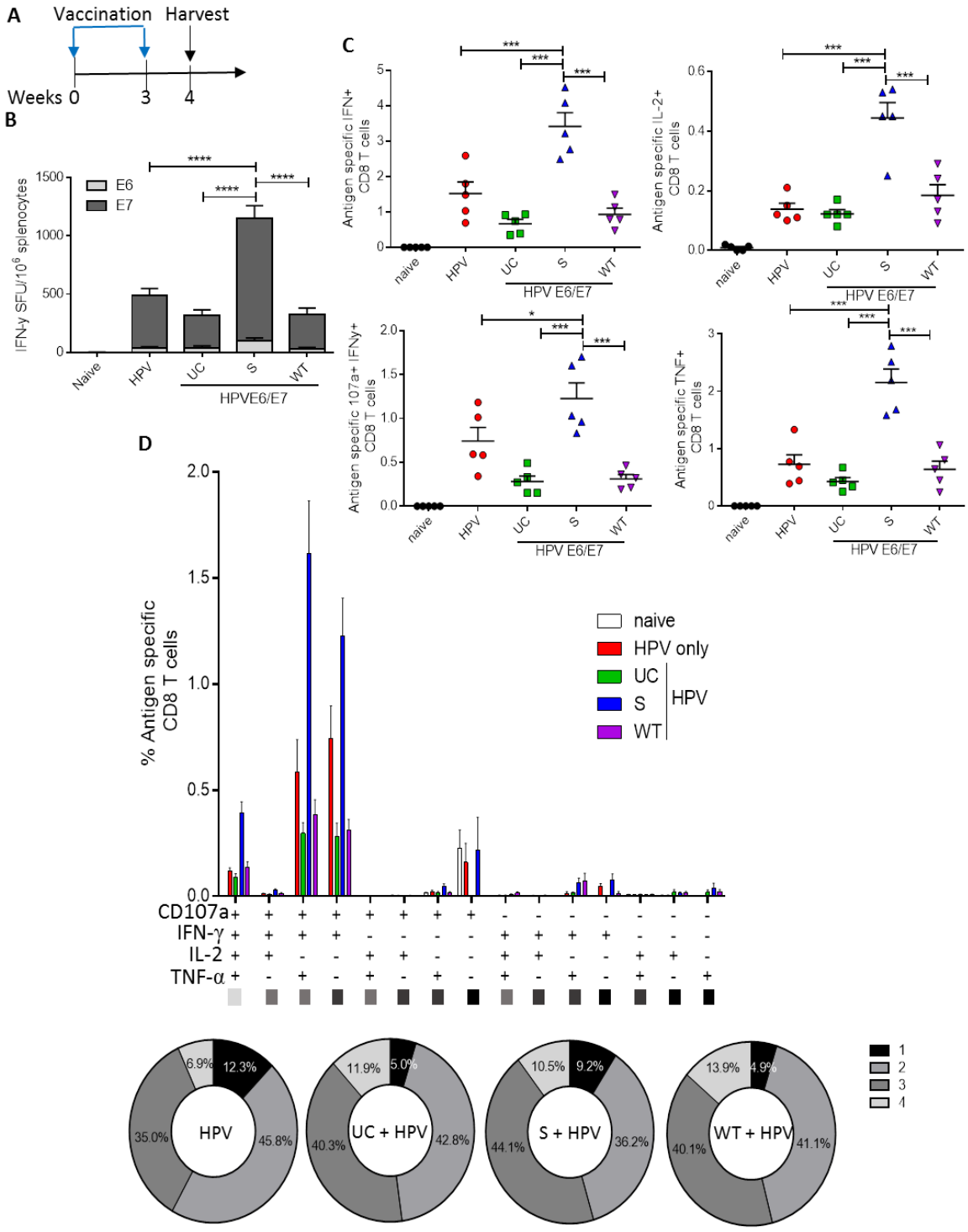

Figure 5.2: Inclusion of soluble CD40L increased antigen-specific CD8 T cells responses. (***a***) Diagram of the experiment. (***b***) IFN-γ ELISpot responses to overlapping

peptides from E6 and E7. (***c***) Intracellular cytokine staining of CD8 T cells after peptide stimulation. (***d***) Polyfunctionality of CD8 T cells responses. Pie charts for the percent of cells expressing 4, 3, 2, or 1 function. A representative of two separate experiments. Significance determined by modified ANOVA *<0.05, **< 0.01 ***< 0.001.

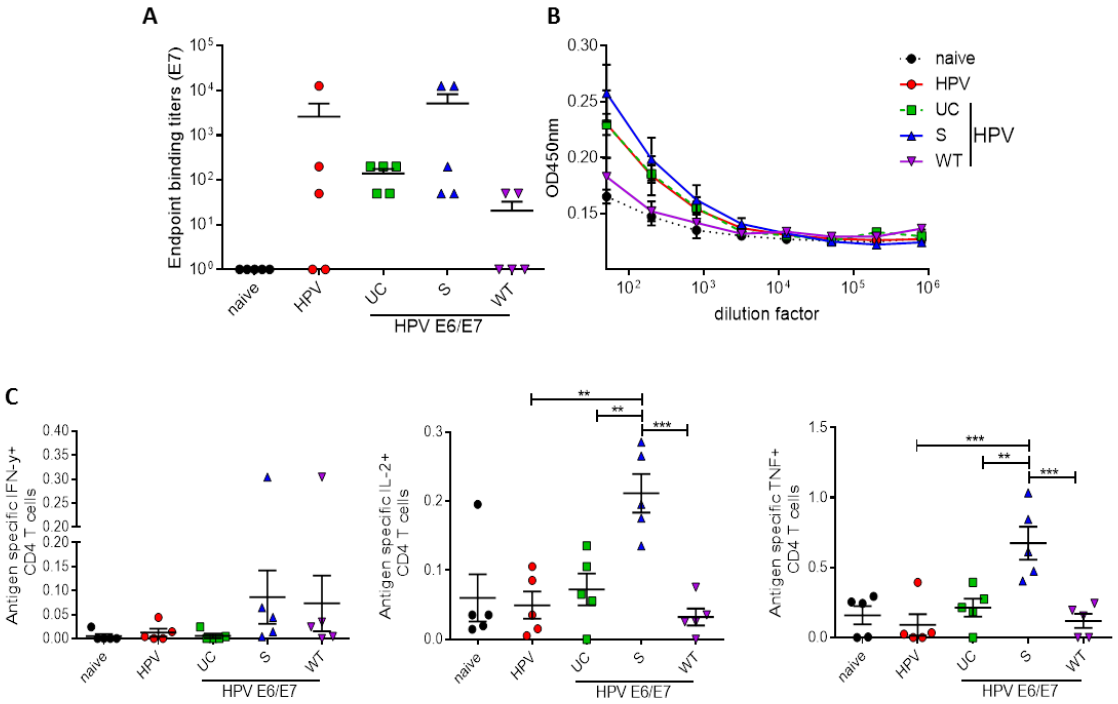

Figure 5.3: Inclusion of pS-CD40L in HPV vaccine increased CD4 T cell and humoral responses. Endpoint binding titers (***a***) and dilution curves (***b***) against E7 protein. (***c***) Intracellular cytokine staining of CD4 T cells after peptide stimulation. Representative of two separate experiments. Significance determined by modified ANOVA *<0.05, **< 0.01 ***< 0.001.

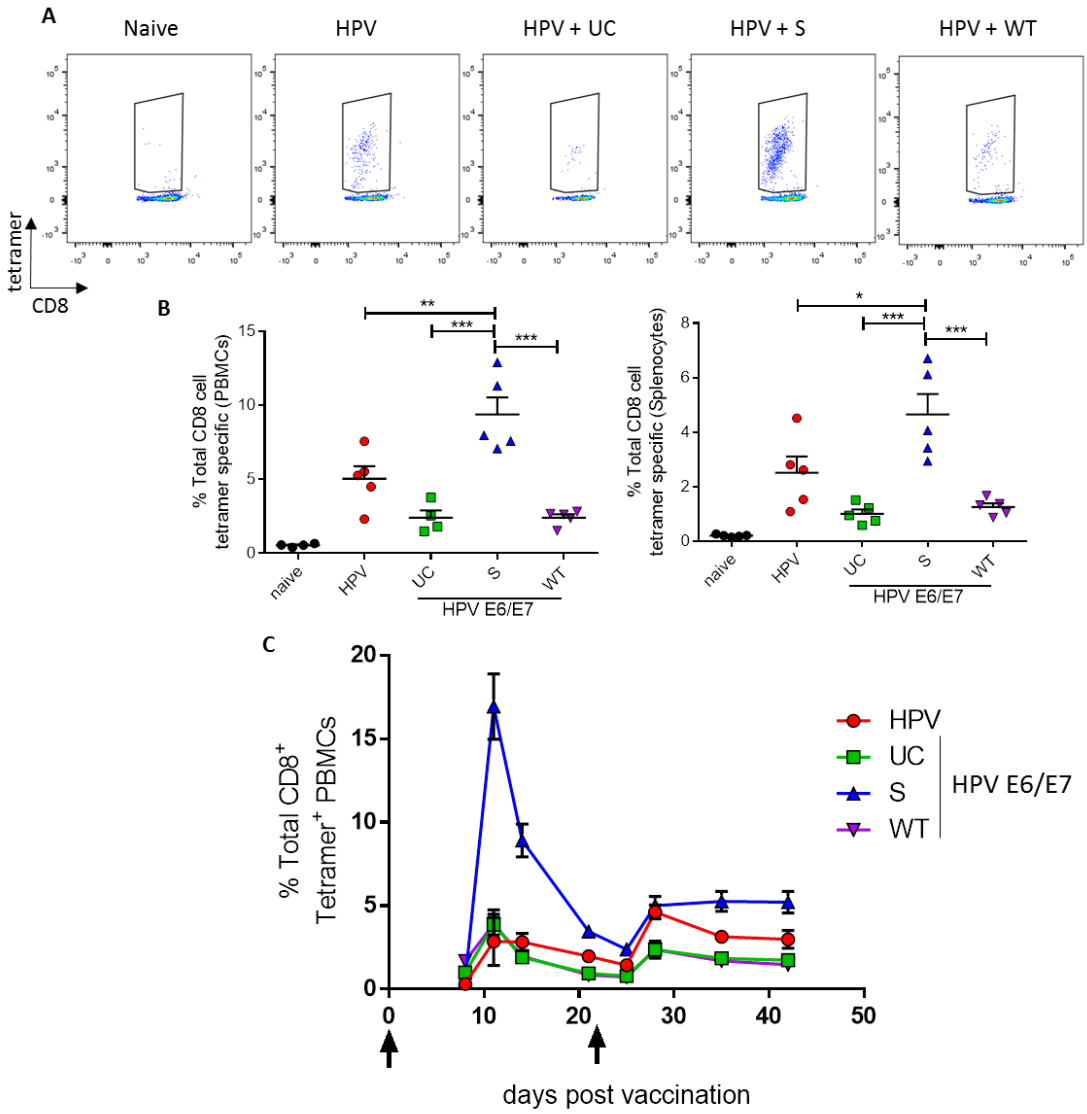

Figure 5.4: Soluble CD40L increased tetramer specific responses in both the spleen and in the periphery. (***a***) Lymphocytes were stained with HPV E7 (H-2D^{b} RAHYNIVTF) tetramer. Representative flow plots of CD8 cells. (***b***) Tetramer specific responses ten days after final vaccination in the spleen and PBMCs. (***c***) Time course analysis of tetramer specific responses in the periphery. Mice were immunized at day 0 and 21 and serially bled to isolate and stain PBMCs for tetramer specific responses. Time

course responses are the combination of three separate experiments. Significance determined by modified ANOVA *<0.05, **< 0.01 ***< 0.001.

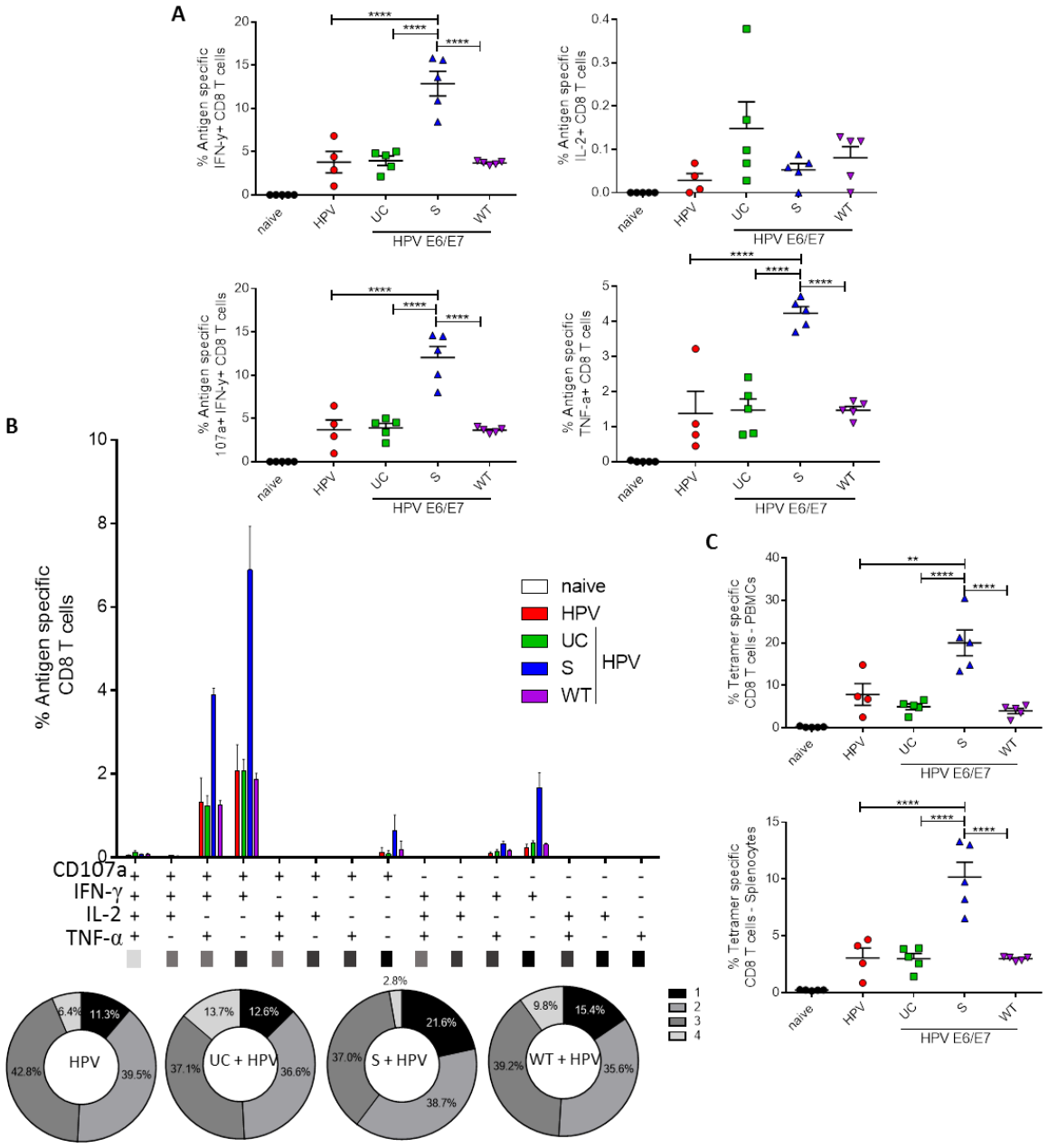

Figure 5.5: CD8 T cell responses 11 days after first vaccination were functional when pS-CD40L was included. Eleven days after first vaccination, mice were scarified and immune responses assessed. (***a***) Intracellular cytokine staining of CD8 T cells stimulated with E6 and E7 peptides. (***b***) Polyfunctionality of CD8 T cell responses as

well as percent of polyfunctionality. (***c***) Tetramer specific responses in both the spleen and in the periphery. Significance determined by modified ANOVA *<0.05, **< 0.01 ***< 0.001 ****<0.0001.

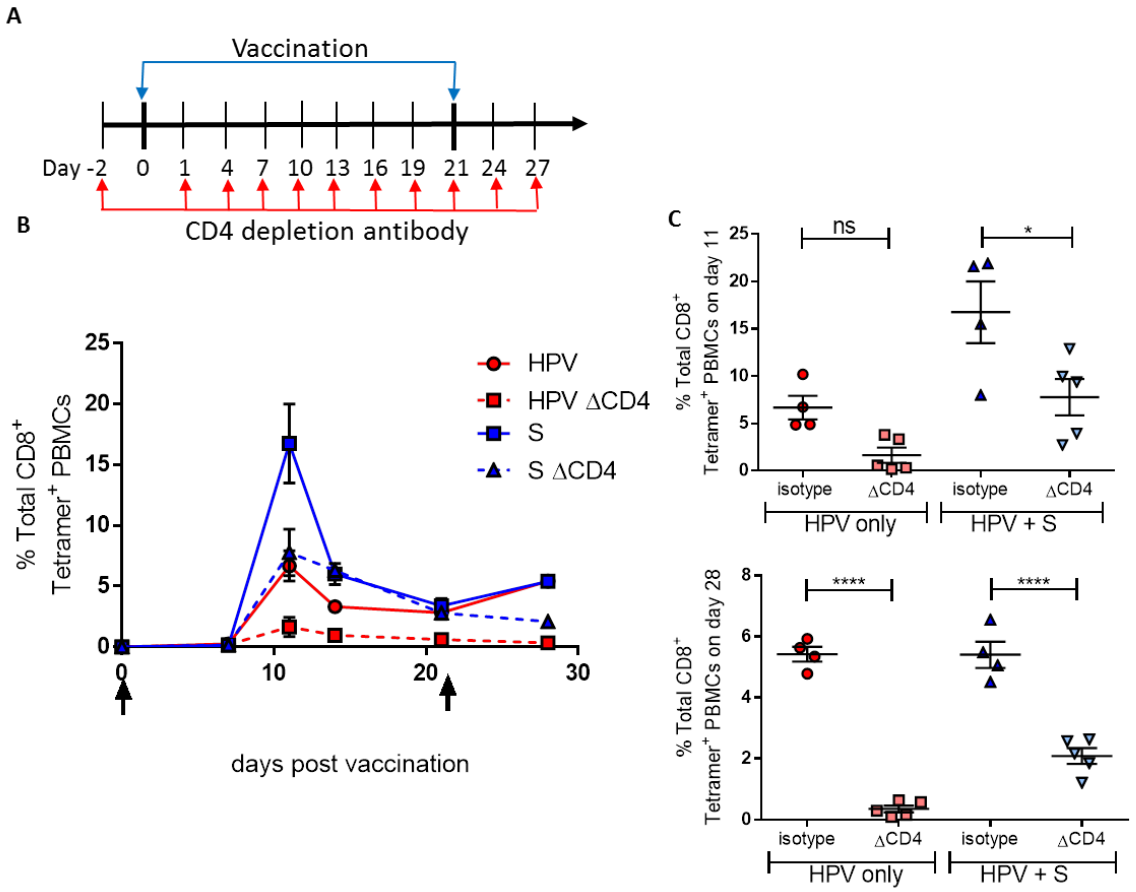

Figure 5.6: Immune responses induced by pS-CD40L were only partially depended on CD4 T cells. (***a***) Diagram of vaccination and depletion. (***b***) CD8 tetramer specific responses in the PBMCs for either HPV only or HPV + pSCD40L with (dotted) or without (solid) CD4 depletion. (***c***) CD8 tetramer^{+} responses on day 11 (top) or day 28 (bottom). Significance determined by modified ANOVA *<0.05, **< 0.01 ***< 0.001 ****<0.0001.

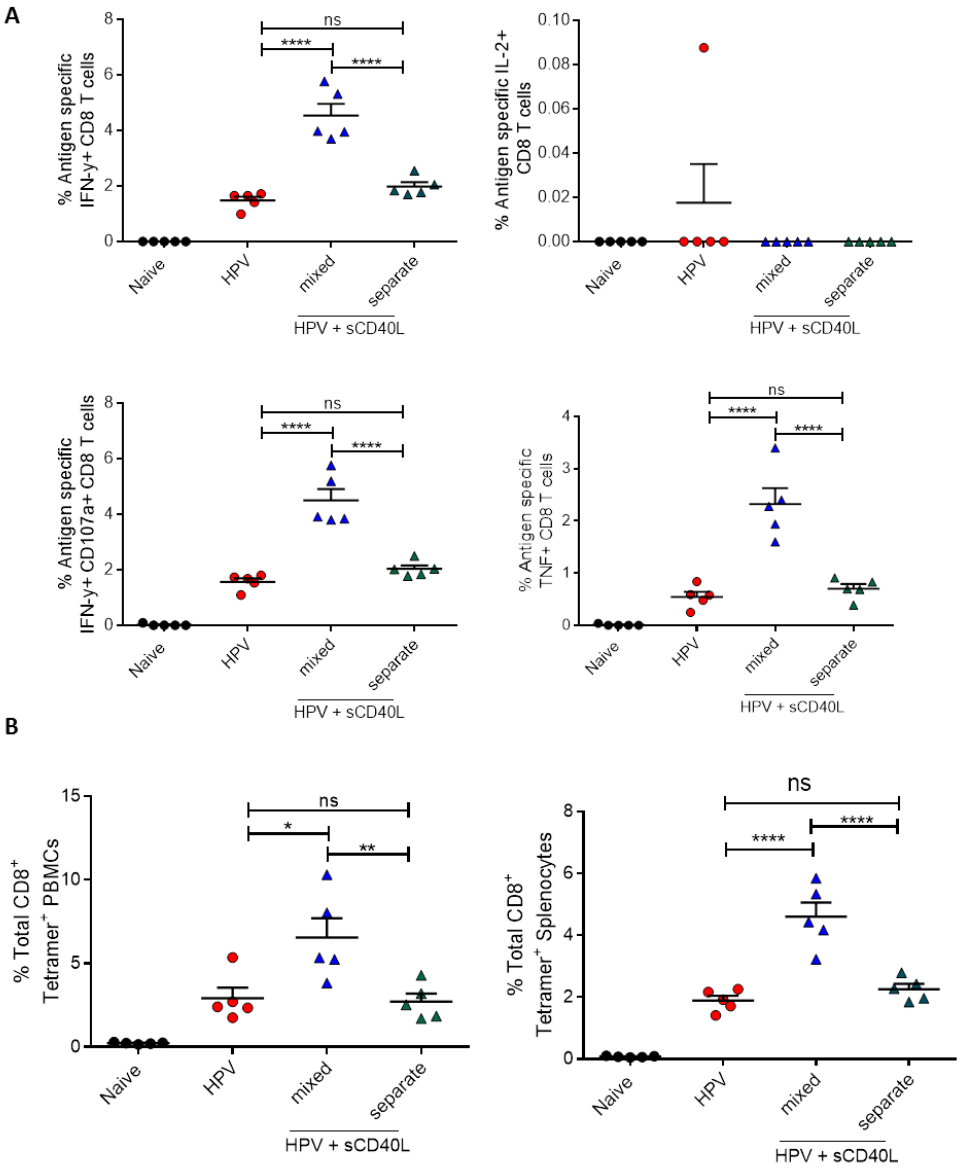

Figure 5.7: pS-CD40L did not have systemic adjuvanting effects. Mice were immunized with either HPV alone, HPV + pS-CD40L formulated together (mixed) or HPV + pS-CD40L delivered to separate contralateral legs (separate). (***a***) Antigen-specific CD8 T cell responses after stimulation with E6 and E7 peptides. (***b***) Tetramer specific CD8 T cells in the PBMCs and splenocytes. Significance determined by modified ANOVA ****< 0.0001.

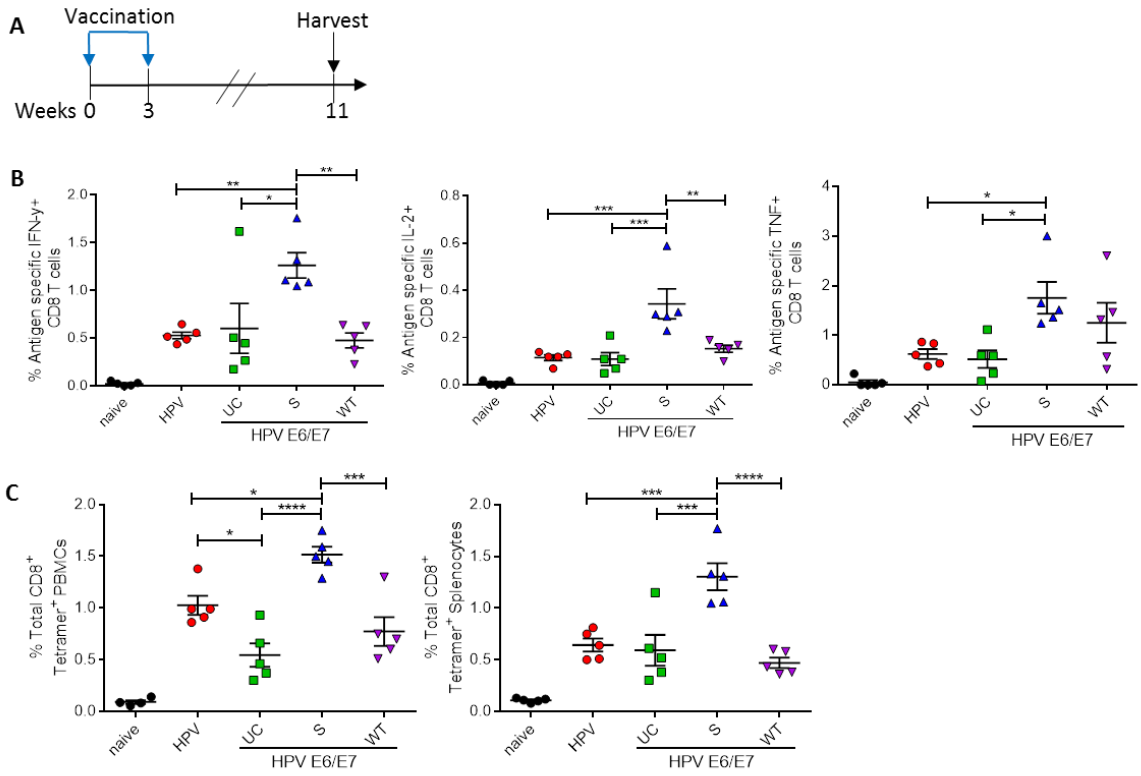

Figure 5.8: Memory responses were maintained when pS-CD40L was added. (***a***) Diagram of vaccination time course. (***b***) Intracellular cytokine staining after stimulation with overlapping peptides to E6 and E7. (***c***) CD8 tetramer^{+} responses in the periphery and spleen. Significance determined by modified ANOVA *<0.05, **< 0.01 ***< 0.001 ****<0.0001.

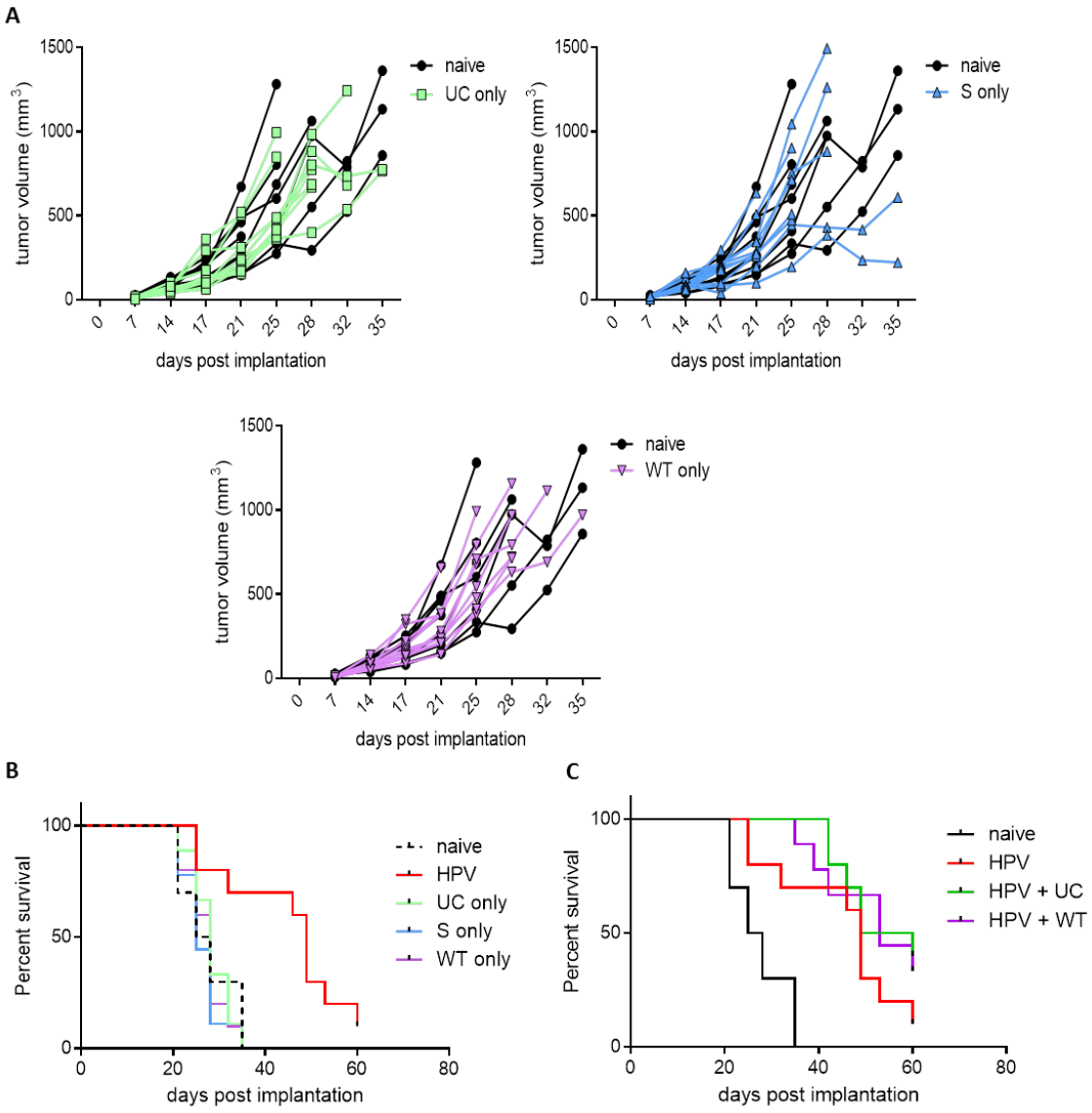

Figure 5.9: CD40L expression alone did not decrease tumor burden. (***a***) Mice were implanted with TC.1 cells. One week later, mice were vaccinated with each of the CD40L constructs followed by three boosts at 1-week intervals. Tumor growth was monitored over time. (***b***) Survival curves of each of the individually delivered CD40L plasmids. (***c***) Survival curves of HPV only, HPV + pUC-CD40L and HPV+ pWT-CD40L.

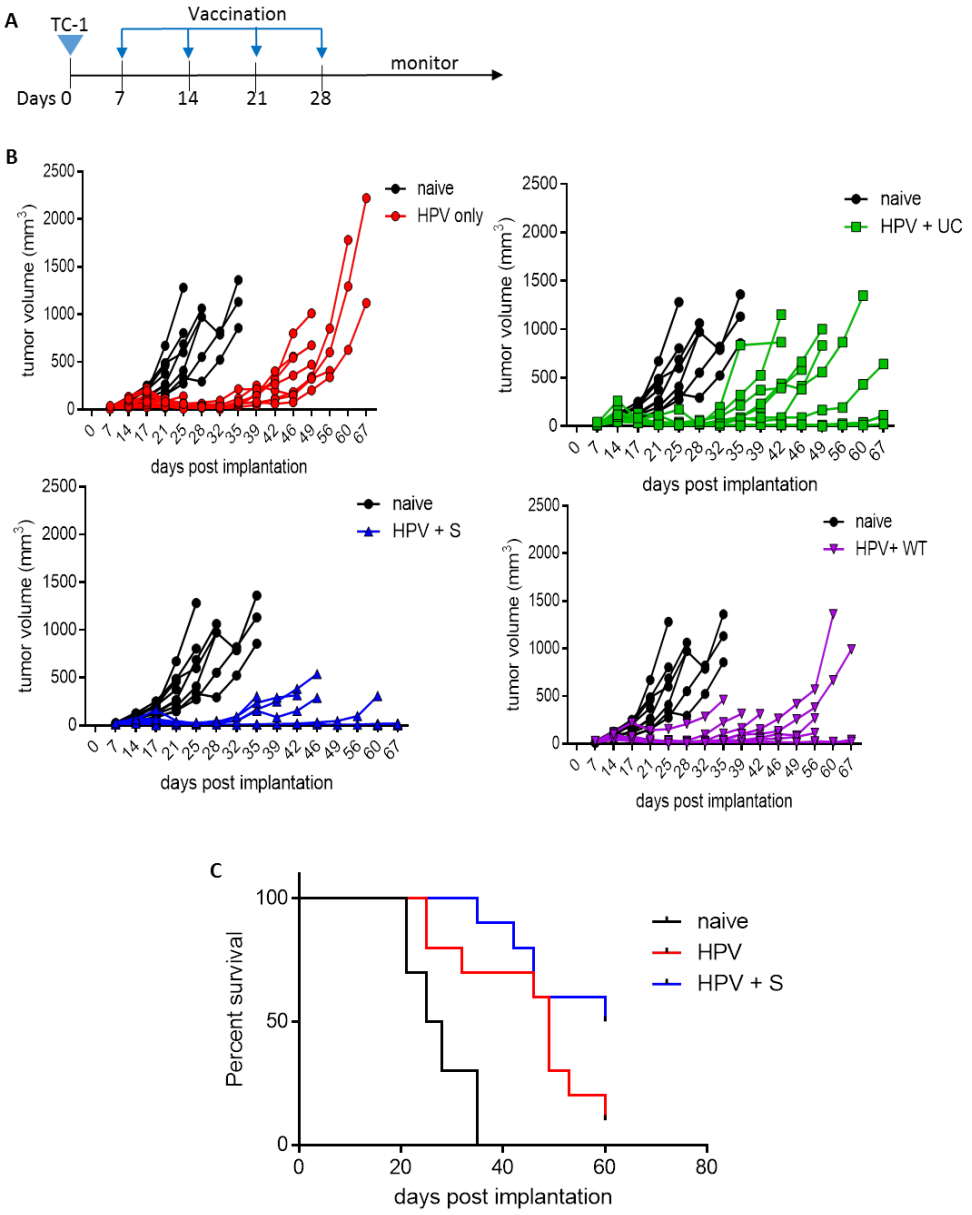

Figure 5.10: HPV + pS-CD40L increased tumor clearance over vaccine alone. (***a***) Diagram of vaccination. (***b***) Tumor growth over time for each mouse. There was a significant difference in tumor growth and control when pSCD40L is included compared to HPV alone ($p<0.0001$), pUC-CD40L ($p<0.0001$) and pWT-CD40L ($p<0.05$) (modified

two-way ANOVA). (***c***) Survival curve of naïve, HPV only and HPV + pSCD40L. A representative of two individual experiments.

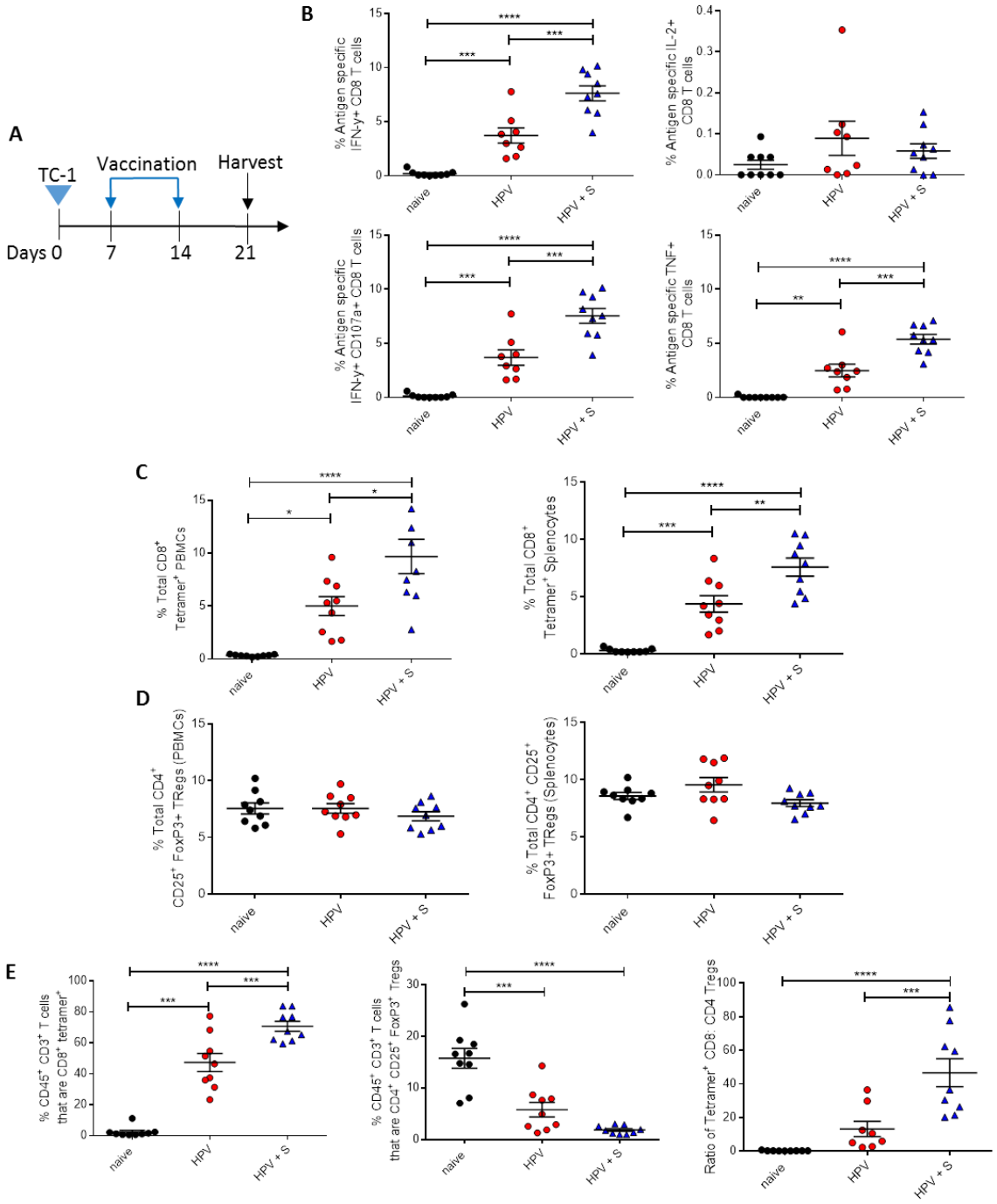

Figure 5.11: Addition of pS-CD40L increased antigen-specific T cells in the periphery and the tumor. (***a***) Diagram of the timeline of implantation, vaccination and harvest. (***b***) Systemic immune responses to E6/E7 peptides. (***c***) Tetramer specific CD8 T

cells (***c***) and CD4 T regulatory cells ($FoxP3^{+}$ $CD25^{+}$) (***d***) in the PBMCs and spleen. (***e***) Tumor infiltrating tetramer-specific CD8 T cells, Tregs, and the ratio of CD8 tetramer-specific T cells to Tregs. Significance determined by modified ANOVA *<0.05, **< 0.01 ***< 0.001 ****<0.0001.

CHAPTER 6: OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Overall conclusions

The overall goal of my thesis work was to improve the potency of DNA vaccines. There are many advantages to the DNA vaccine platform including two which were the focus of this thesis: developing novel immune adjuvants to enhance or tailor vaccine-induced responses and the ability to easily formulate multiple plasmids into a single immunization to increase the breadth of coverage. Here, we have demonstrated that combinations of multiple plasmids expressing consensus HIV-1 Envs enhanced vaccine-induced humoral responses over a single plasmid Env formulation and that these responses were boosted with protein administration. We have built upon these findings and extended the number of plasmids encoding primary acute/early HIV-1 Envs. We now have close to 30 plasmids expressing consensus or primary HIV-1 Envs. These plasmids induced both cellular and humoral responses in small animals and non-human primates. A combination of 14 different primary HIV-1 Env plasmids produced cellular and humoral responses in NHPs. Importantly, these humoral responses displayed functionality as they were able to neutralize a panel of Tier 1 viruses and had strong ADCC activity against gp120/gp140 coated targets. Additionally, we have developed four novel plasmid encoded immune adjuvants. The first set of adjuvants encoded mucosal chemokines for both T and B cells. These chemokine adjuvants had previously shown increased vaccine-induced responses in small animals. The three adjuvants, CCL25 (TECK), CCL27 (CTACK) and CCL28 (MEC) were moved into NHP in combination with a DNA vaccine for SIV Gag, Pol, and Env. Upon challenge with

SIVe660, the addition of CCR10L chemokines (CCL27 and CCL28) significantly enhanced protection over naïve with only a single animal demonstrating progressive infection. Due to the small size of the study, correlates analysis was difficult but did demonstrate that NHPs with abortive or that remained uninfected had stronger induction of vaginal antibodies compared to those with progressive infection. We have also developed a plasmid-encoded immune adjuvant encoding various forms of CD40 ligand. The soluble form of CD40L increased both cellular and humoral responses when combined with our HPV 16 E6/E7 DNA vaccine. These enhanced responses led to increased protection in a therapeutic challenge model with increased infiltration of antigen-specific CD8 T cells into the tumor. Overall, this study has shown that improved formulation and inclusion of plasmid encoded immune adjuvants can increase the potency of DNA vaccine-induced responses.

6.2 Future directions for DNA vaccines against HIV

The combination of plasmids encoding primary and consensus Envelopes.

Another step for improvement of immune responses against DNA vaccines for HIV was to combine the approach for enhanced T cell responses driven by consensus HIV -1 Env immunogens with the immunization power of primary Envs to drive strong humoral responses. NHP are currently being vaccinated with three consensus plasmids encoding clade A, B, and C, as well as, seven primary Envs. These plasmids are formulated together and delivered either ID or IM followed by electroporation. Extensive cellular and humoral characterization are being performed to understand further the responses induced by ID or IM immunizations. Additionally, to compare the effects of

CCL28 (MEC) and IL-12, additional groups of NHPs will be immunized with either IL-12 or MEC. This will allow for a direct comparison of the two adjuvants to determine how vaccine induced responses are influenced.

There are additional questions which remain about polyvalent HIV Env DNA vaccines. Specifically, as seen in **Figure 2.1,** when the multiple Env plasmids were included in the immunization with Gag and Pol, cellular responses to these two antigens decreases compared to the single Env immunization. Most likely, an efficacious HIV vaccine would include the T cell targets of Gag and Pol to try and prevent any dissemination of infection which was able to break through the Env humoral responses. This interference of Gag cellular responses has been observed by others as well. Determining if only certain Envs have this effect and how to prevent or overcome this inhibition is critical for successful HIV vaccine development.

Educating the immune system using Envelopes isolated during progressive infection in a subject who developed a broadly neutralization antibodies (bNab)

We are constantly surveying the field to determine if we can encode for a better antigen. We have formed a collaboration with the Duke Centers for HIV/AID Vaccine Immunology and Immunogen Discovery (CHAVI-ID) to investigate if a DNA vaccine encoding Envs isolated during the time course of infection could help the humoral response down the pathway of bNab development. As discussed in the introduction of this thesis, work from the Duke CHAVI-ID retrospectively sequenced a subject's virus and B-cell receptor to determine how the virus informed the production of the bNab (192-194). Ten Envs from this subject were selected from the transmitted founder viruses to

viruses at week 100 of infection. These Envs were carefully selected as key binders to BCRs expressed during the maturation pathway (**Figure 6.1*a***). These inserts were RNA and codon optimized and expressed as full-length gp160's *in vitro* (**Figure 6.1*b***). To determine immunogenicity, CB6F1 mice (crosses between balb/C and C57Bl/6 mice) were immunized three times at 2-week intervals. All plasmids induced strong cellular responses as measured by IFN-γ spot forming units (SFU) after overnight stimulation of splenocytes with consensus clade C peptides (**Figure 6.1*c***). Since we did not have matched peptides from all of the inserts, we selected the closest clade-specific peptides. Additionally, after two immunizations, all but three mice seroconvert to consensus clade C gp120 (**Figure 6.2*a***). By the third immunization, complete seroconversion was obtained, and endpoint binding titers increase over the post 2^{nd} titers (**Figure 6.2*b***). We also observe a range of binding to consensus clade c gp140 and clade c primary gp120s (CAP45, Du422.1, ZM53) (**Figure 6.2*c***). **Table 6.1** summarized cellular and humoral responses across all 10 Envs. Since all inserts were immunogenic, these plasmids were moved into rabbit studies to further determine vaccine-induced humoral responses. Three groups of 5 rabbits were immunized with three different vaccine regimens (**Figure 6.3*a***). All rabbits received the same dose of DNA (500ug) split across the number of plasmids delivered at each time. Group 1 received 250ug of each of the transmitted founder virus Envs (M11 and M5) for five immunizations. Group 2, termed sequential, is the progress of plasmids through time. Thus the rabbits first received the transmitted founder Env followed by the week 20 Env, week 30 Envs and so on. Group 3, termed additive, is similar to the sequential but instead of only receiving the Env for that time point; the

Envs are added to the previous combination. Endpoint binding titers to 92RW020 (clade A), SF162 (clade B) and ZM197 (clade C) were determined after each immunization (**Figure 6.3*b***). There was no significant difference between each of the groups as all animals seroconverted by the third vaccination. Additional studies regarding antibody binding to matched Env gp120/gp140s, avidity, and functionality of these antibody responses are warranted. Once these studies have been performed, the vaccine regimen with the highest functional antibody titers will be selected to be moved into NHPs for a comparison between the combinations of primary/consensus Envs.

6.3 Future directions for novel plasmid encoded immune adjuvants

Based on the details of the impact of CCL28 adjuvant in an initial NHP study, CCL28 (MEC) is moving into a follow-up NHP study to directly compare its adjuvanting effects with IL-12. This will be a novel head to head comparison.

Soluble CD40L

As demonstrated in chapter 5, pS-CD40L increased vaccine induce cellular and humoral responses when combined with our HPV vaccine. We have also performed studies to investigate if CD40L can also adjuvant other DNA vaccines. First, our HIV consensus clade C Env plasmid was combined with all three CD40L isoforms (**Figure 6.4*a***). Mice were immunized two times at a three-week interval and sacrificed one week after final vaccination. Similar to our HPV results, the inclusion of pS-CD40L increased vaccine-induced CD8 T cell responses and binding titers to matched gp120 (**Figure 6.4 *c-d***). However, in this model, we did not observe an increase in CD4 T cell responses (**Figure 6.4*b***). To address memory responses, we also performed the same vaccination

followed by a two-month rest (**Figure 6.5*a***). Again similar to HPV, memory responses were enhanced with the addition of pS-CD40L (**Figure 6.5**). Surprisingly, unlike after final vaccination, CD4 T cell responses in memory were significantly higher for the expression of IFN-γ, IL-2, and TNF-α compared to vaccine alone (**Figure 6.5*c***). Additionally, the inclusion of the uncleavable surface bound form of CD40L (pUC-CD40L) significantly enhanced CD4 but not CD8 or humoral responses. Whether or not an increase in these responses increases protection remains an open question.

Due to the limitations of mouse models for HIV, to test humoral correlate of protection, we have also tested the ability of our CD40L adjuvants to increase responses against one of our Influenza constructs. Mice were immunized in a similar manner to the HIV study with mice receiving 1ug of our consensus H1 HA vaccine (**Figure 6.6*a***). Immune responses assessed after final vaccination demonstrated similar responses as seen in both HIV and HPV (**Figure 6.6**). Both CD4 and CD8 T cell responses were significantly enhanced over vaccine alone when pS-CD40L is included (**Figure 6.6*b-d***). There was a slight increase in antibody binding titers with pS-CD40L, but the highest increases were observed with pWT-CD40L (**Figure 6.6*e***). Memory responses were again maintained with increases in CD4 and CD8 T cell responses with pS-CD40L (**Figure 6.7*b-d***). Interestingly, there was no difference in humoral responses between each of the groups which could be due to maxing out the responses (**Figure 6.7*e***). Additional studies need to be performed to determine if these antibody titers have the same avidity and ability to inhibit hemagglutination. Since a flu challenge is feasible in mice, it is

important to demonstrate that inclusion of pS-CD40L can increase protect against both morbidity and mortality.

There are additional questions which need to be addressed for the use of pS-CD40L. For instance, we have not performed any studies on determining how pS-CD40L affects the activation of antigen presenting cells. Depletion of CD4 T cells (**Figure 5.6**) suggest that pS-CD40L is partially working on both the APCs and the CD4 T cells. Further understanding the mechanistic action of pS-CD40L could allow us to better pair it with antigens or other plasmid-encoded adjuvants. Furthermore, due to the ability of pS-CD40L to induce strong cellular responses, it is important to see if it can increase vaccine-induced responses to self-cancer antigens. These antigens tend to be much less immunogenic due to tolerance and thus we are always exploring new ways to either enhance or broaden the vaccine-induced responses. Additionally, many plasmids immune adjuvants have performed well in mice only to fail in inducing similar responses in NHPs and humans. It is imperative to move pS-CD40L into NHPs to determine if similar immune responses are induced. Another potential avenue of future investigation is physically linking soluble CD40L to a secreted antigen of interest. Similar studies have been performed with recombinant protein or viral vectors and have shown that the fusion of CD40L to the antigen leads to targeting of this antigen to APCs and increase presentation. It would be interesting to see if there is a difference in immune responses when s-CD40L is delivered fused directly to an antigen or if delivered on a separate plasmid.

Table

Insert	T cell response	Endpoint binding titers	Binding to primary gp120s
30.28	1	3	10
100.B6	2	8	4
M5	3	10	3
78.15	4	2	1
30.21	5	1	2
20.14	6	9	5
53.16	7	5	6
78.33	8	7	8
53.31	9	6	7
M11	10	4	9

Table 6.1: Immune responses in mice vaccinated with 10 CHAVI-ID Env plasmids.

Cellular and humoral responses ranked between each plasmid. 1 is the highest with 10 being the lowest.

Figures

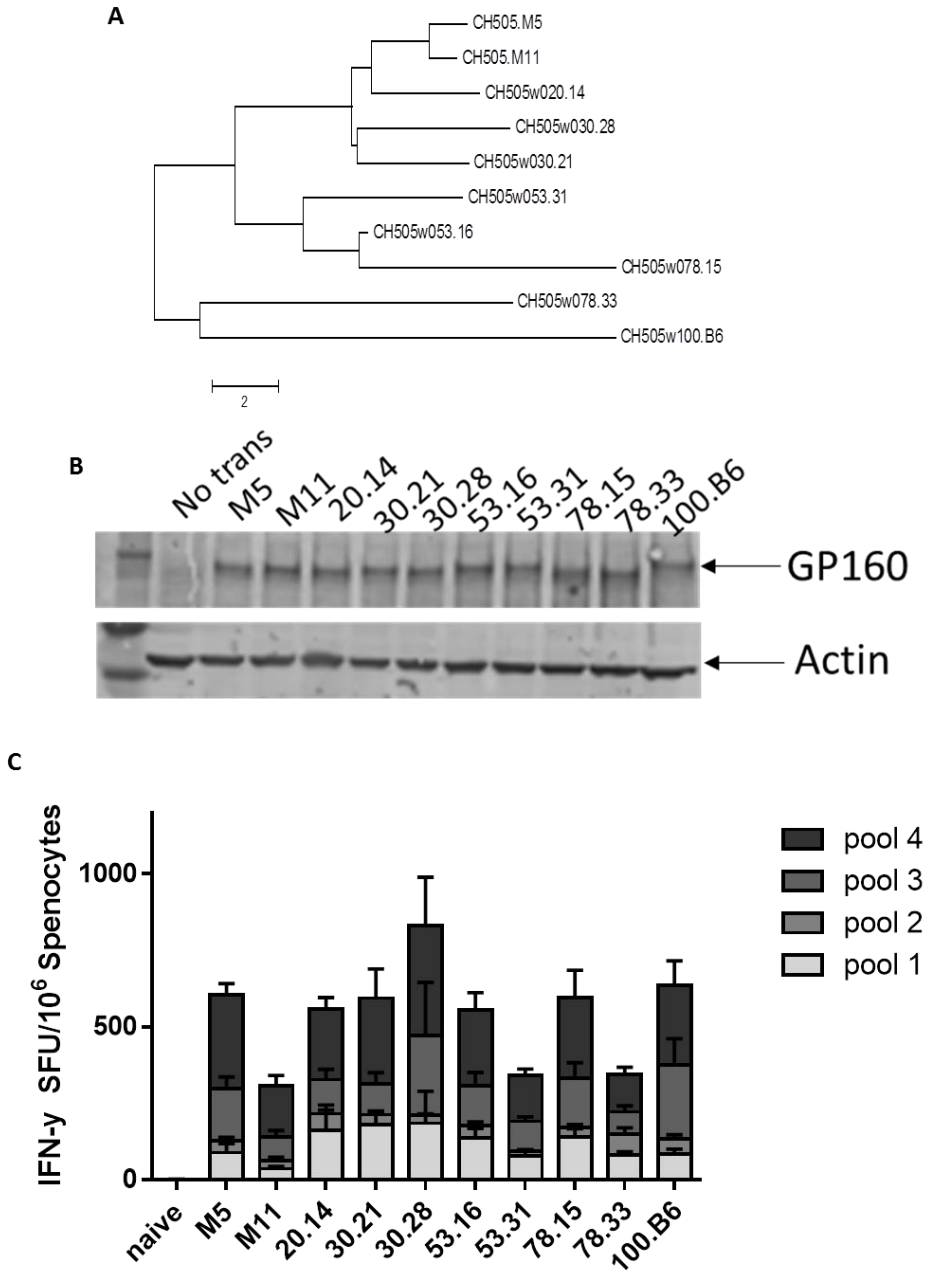

Figure 6.1: ***In vitro and in vivo*** **expression of 10 CHAVI-ID Env plasmids.** (***a***) Phylogenetic tree diagram demonstrating the relationship between the 10 Envs. The sequences were aligned using ClustalX, and a neighbor-joining tree was created. (***b***) *In*

vitro expression of each plasmid in transfected 293T cell lysate. (***c***) CB6F1 mice were immunized three times at two-week intervals and sacrificed one week after final immunization. Cellular responses were detected using IFN-γ ELISpots using splenocytes stimulated with overlapping peptides for consensus clade C gp160.

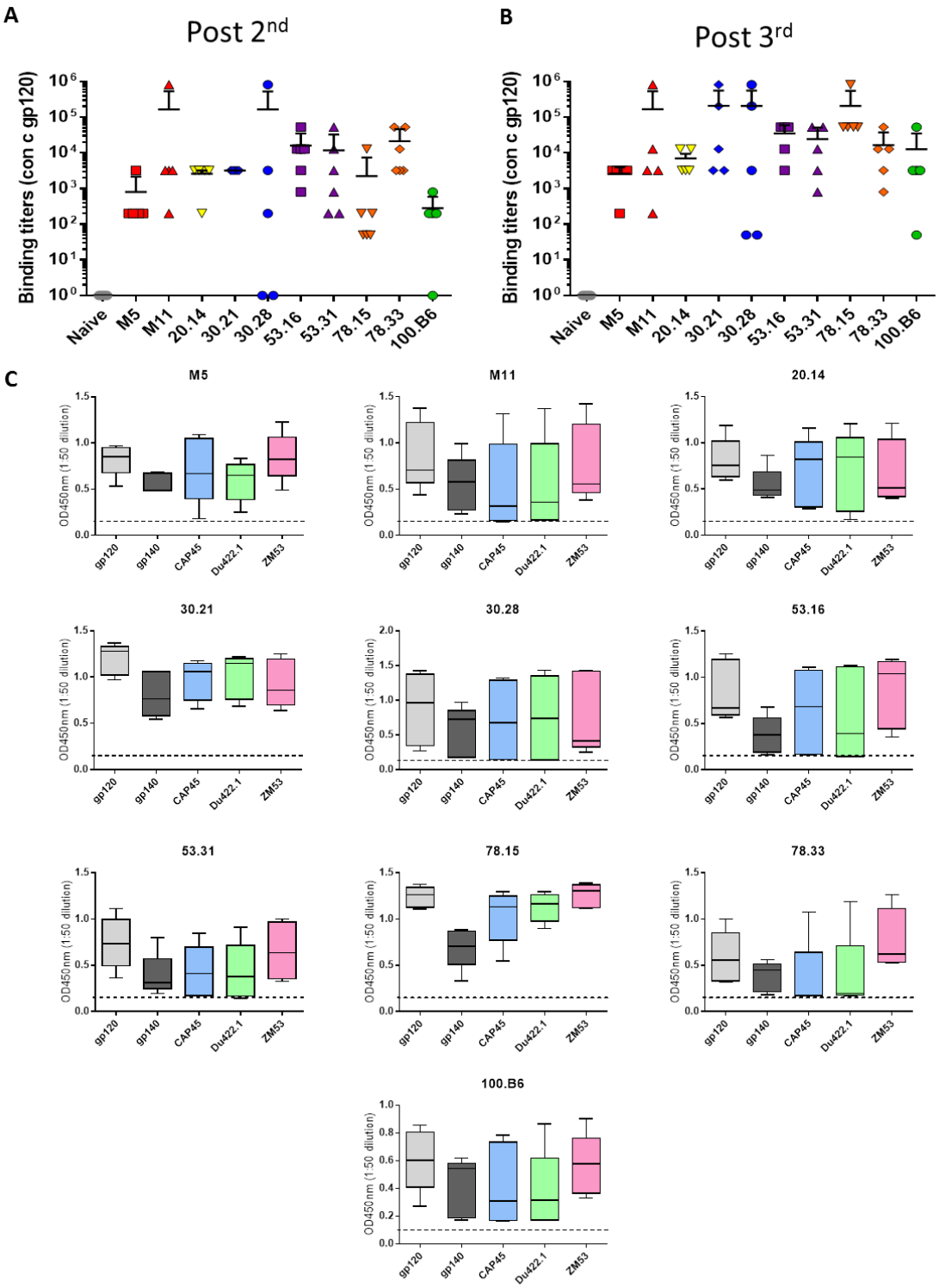
A
Post 2nd
Binding titers (con c gp120)
Naive
M5
M11
20.14
30.21
30.28
53.16
53.31
78.15
78.33
100.B6
B
Post 3rd
C
OD450nm (1:50 dilution)
gp120
gp140
CAP45
Du422.1
ZM53

Figure 6.2 Humoral responses induced in mice vaccinated with a single CHAVI-ID plasmid. Endpoint binding titers against consensus clade C gp120 post 2nd (***a***) or 3rd (***b***). (***c***) Binding to consensus clade C gp120 and gp140 as well as additional clade C primary gp120s (CAP45, Du422.1, and ZM53) after final immunization.

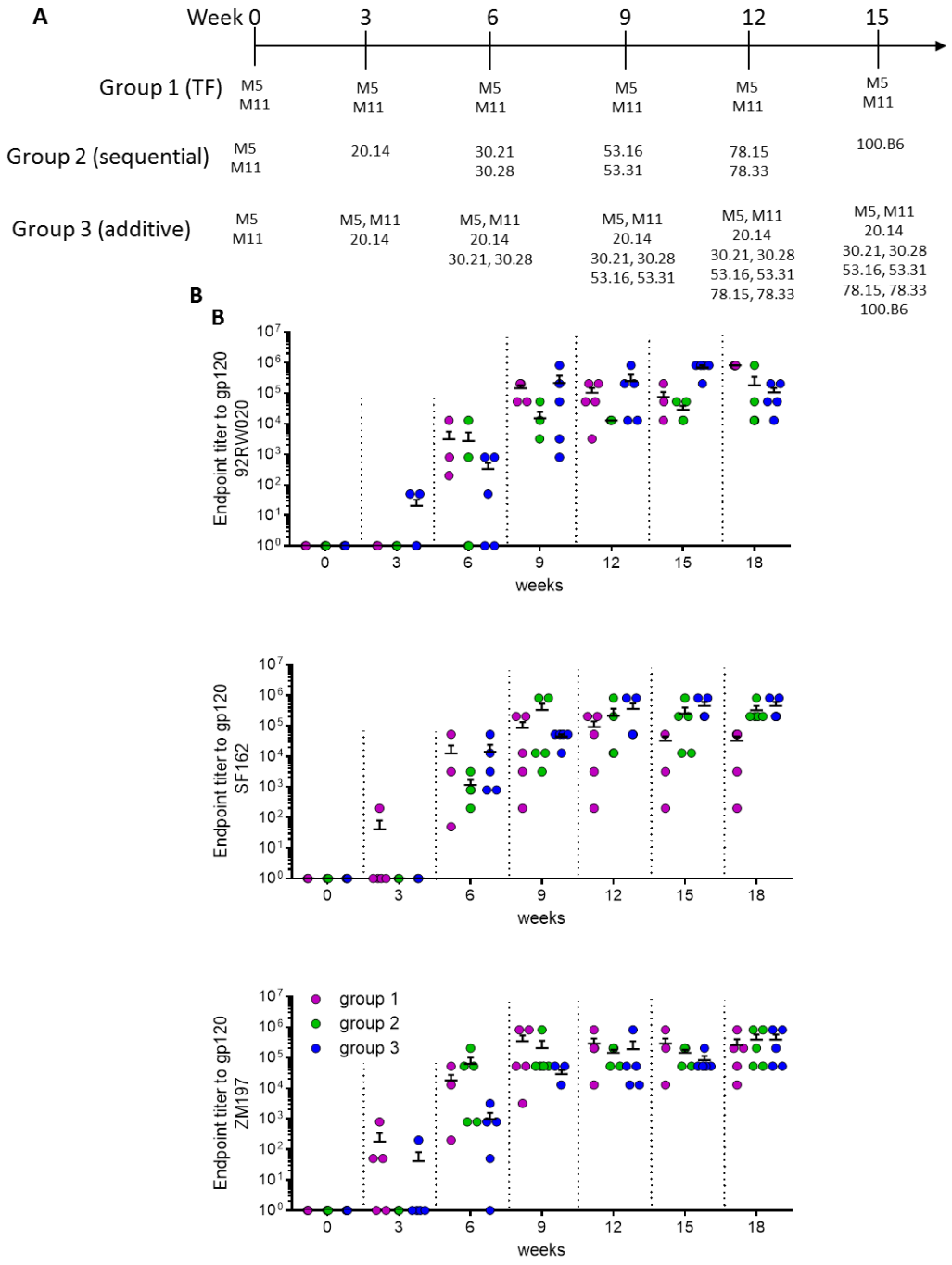

Figure 6.3: Humoral responses induced by different combinations of the 10 CHAVI-ID Envs. (***a***) Rabbits were immunized with three different combinations of DNA plasmids expressing the CHAVI-ID Envs. Each rabbit received the same total dose of

DNA (500ug) and the same number of immunizations. All immunizations were giving in the muscle. (***b***) Endpoint binding titers over time for 92RW020 (clade A), SF162 (clade B), and ZM197 (clade C).

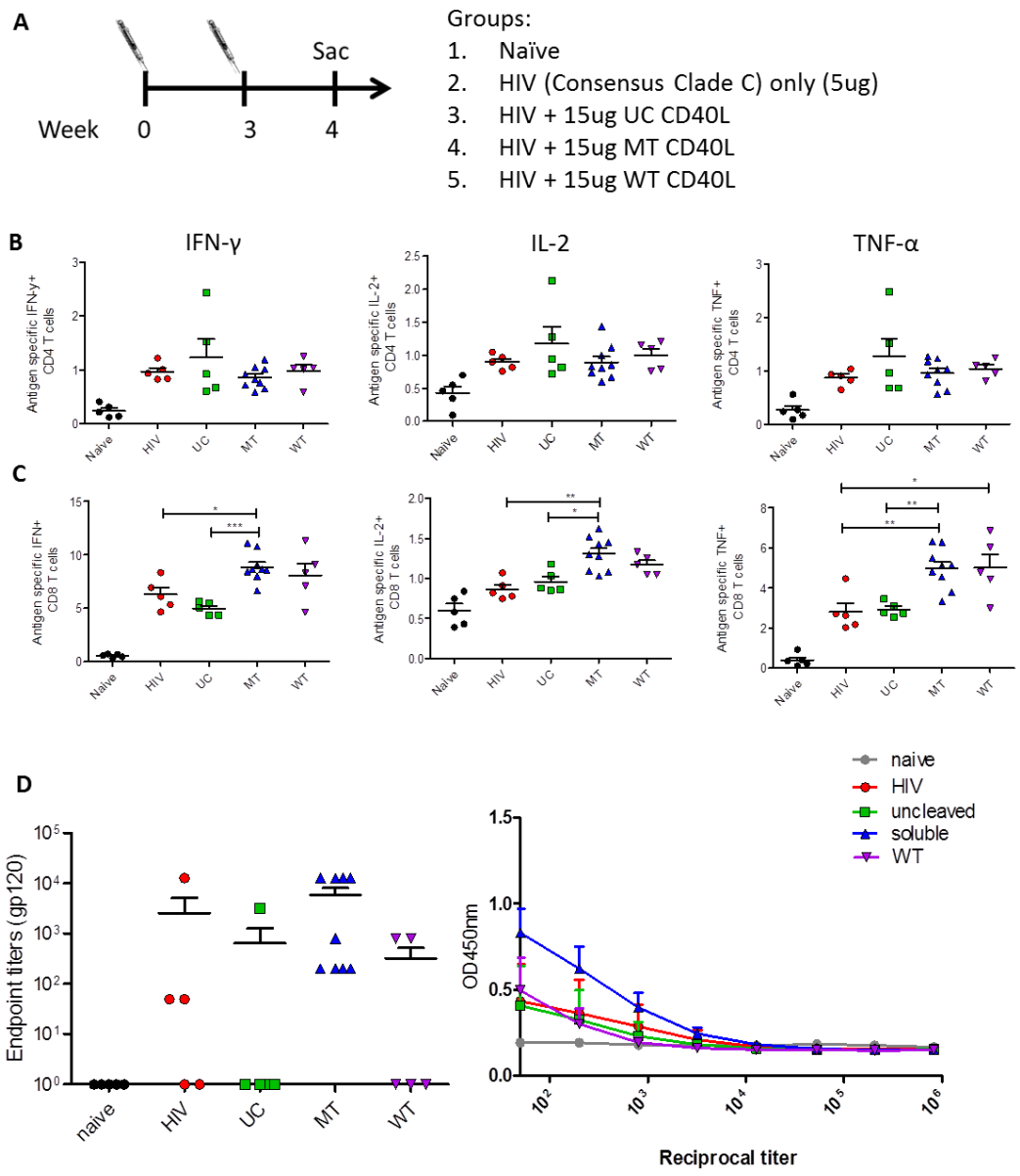

Figure 6.4: Inclusion of pS-CD40L with consensus clade C HIV-1 Env DNA plasmid increased CD8 T cells and humoral responses. (***a***) Mice were vaccinated two times at three-week intervals and sacrificed one week after final vaccination. Intracellular cytokine staining of CD4s (***b***) and CD8s (***c***) after stimulation with overlapping consensus

clade C peptides. (***d***) Endpoint binding titers and dilution curves against consensus clade C gp120 after the final immunization. Significance determined by modified ANOVA *<0.05, **< 0.01 ***< 0.001.

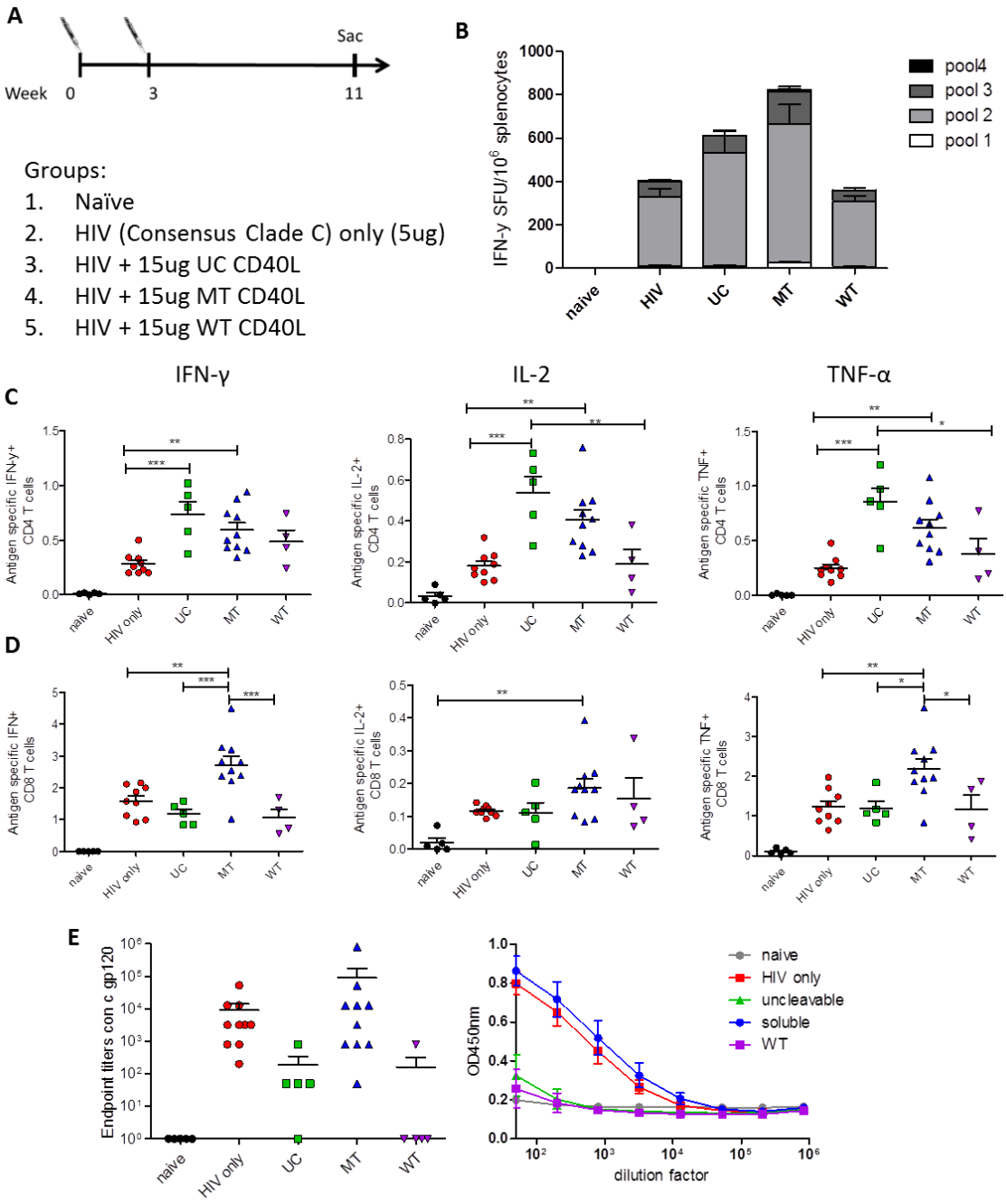

Figure 6.5: Including pS-CD40L with HIV Env DNA vaccine increased memory responses. (***a***) Mice were immunized similar to in Figure 6.4. Mice were sacrificed two months after final vaccination. (***b***) IFN-γ ELISpot responses detected against overlapping consensus clade C peptides. Intracellular cytokine staining for CD4s (***c***) and CD8 (***d***)

after stimulation with homologous peptides. (***e***) Endpoint binding titers and dilution curves to consensus clade C gp120 after final immunization. Significance determined by modified ANOVA *<0.05, **< 0.01 ***< 0.001.

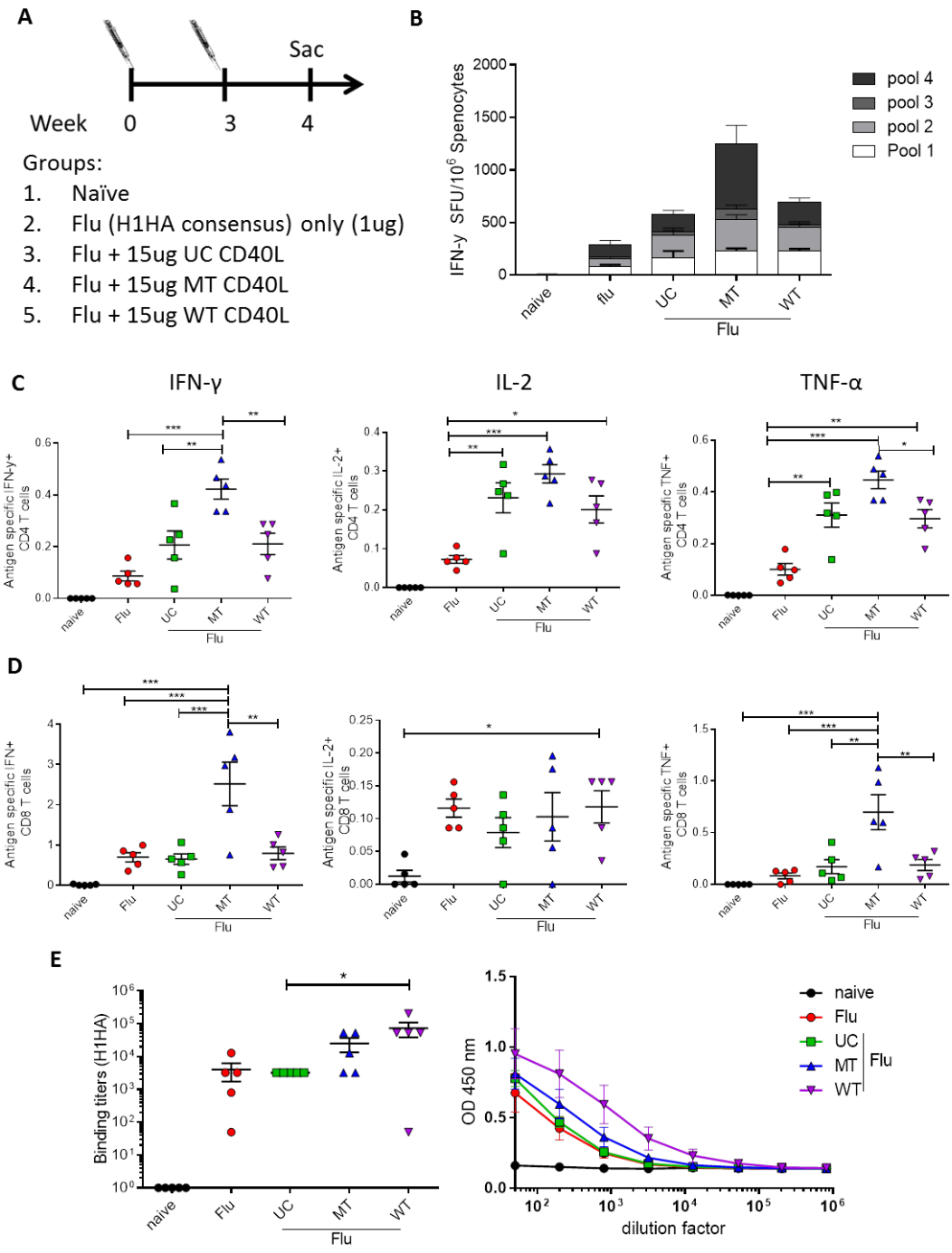

Figure 6.6: Including pS-CD40L with consensus H1 HA (Flu) increased vaccine induced responses. (***a***) Mice were immunized two times at three week intervals and sacrificed one week after final immunization. (***b***) IFN-γ ELISpot responses detected

against overlapping consensus H1 HA peptides. Intracellular cytokine staining for CD4s (***c***) and CD8 (***d***) after stimulation with homologous peptides. (***e***) Endpoint binding titers and dilution curves to H1 (New Caledonia/20/99) after final immunization. Significance determined by modified ANOVA *<0.05, **< 0.01 ***< 0.001.

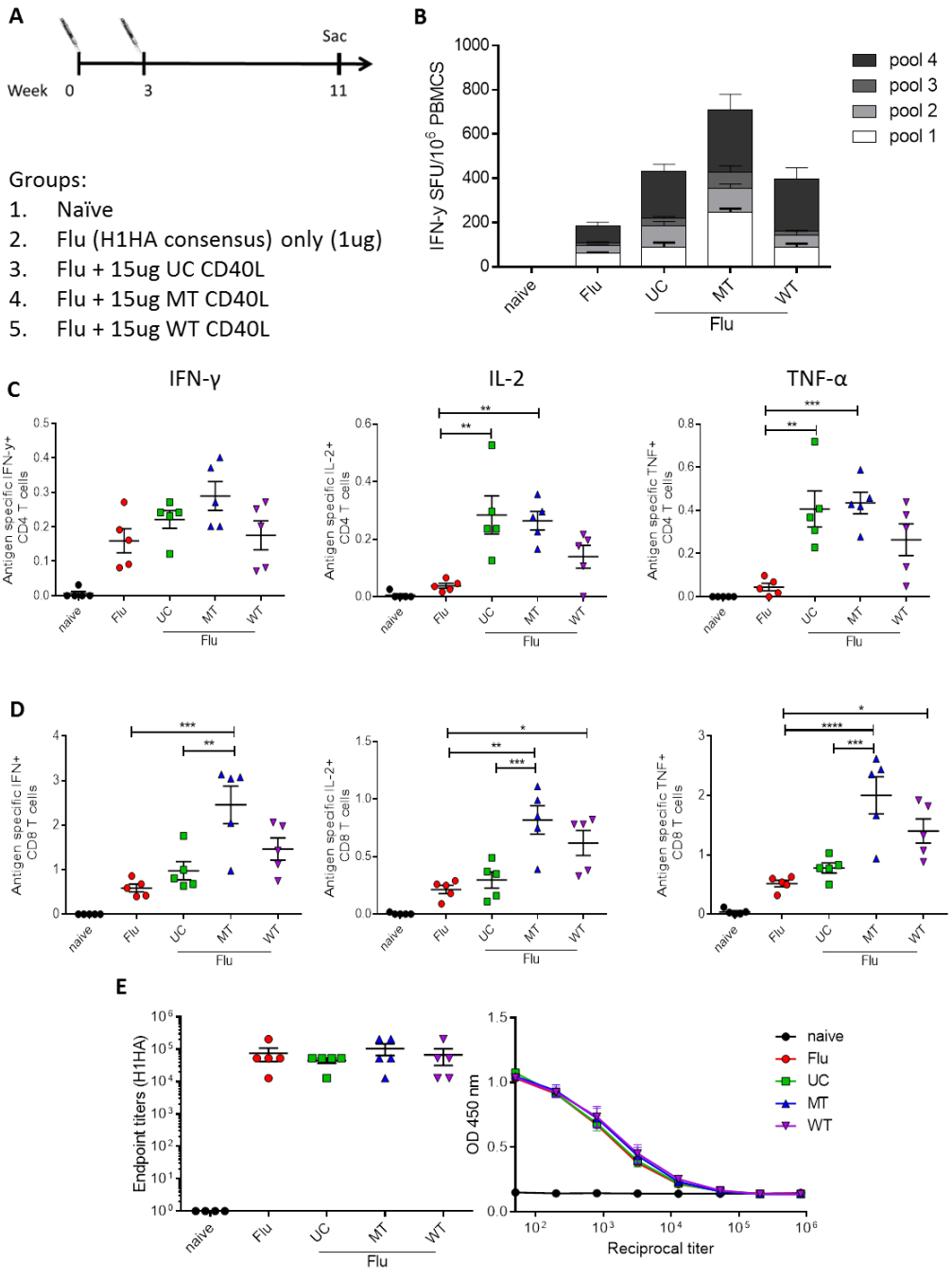

Figure 6.7: Enhanced vaccine-induced responses were maintained into memory with pS-CD40L addition. (***a***) Mice were immunized two times at three-week intervals and sacrificed two months after final immunization. (***b***) IFN-γ ELISpot responses detected

against overlapping consensus H1 HA peptides. Intracellular cytokine staining for CD4s (***c***) and CD8 (***d***) after stimulation with homologous peptides. (***e***) Endpoint binding titers and dilution curves to H1 (New Caledonia/20/99) after the final immunization. Significance determined by modified ANOVA *<0.05, **< 0.01 ***< 0.001.

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