Therapeutic conserved elements (CE) DNA vaccines in simian-human immunodeficiency virus (SHIV) or SIV-infected macaques

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Abstract

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Antiretroviral therapy (ART) is highly effective at reducing viral replication, restoring $CD4^+$ T-cells in the blood, and preventing AIDS in human immunodeficiency virus (HIV) infected people. However, ART cannot deplete the latent reservoir of dormant long-lived infected cells, thus requiring a lifetime of treatment. Lifelong ART has limitations including its high cost, potential side effects, and inability to fully reverse the inflammation and immune exhaustion induced by HIV-infection. These limitations have prompted the field to pursue immunotherapies and therapeutic vaccines the could provide durable viral control in the absence of ART, otherwise known as a “viral remission”. A major barrier for a successful therapeutic HIV vaccine is the vast viral genetic diversity and the ability for the virus to mutate and “escape” effective immune responses with little or no loss of fitness. Encouragingly, broad HIV-specific T-cellular immune responses to conserved and functionally constrained epitopes have been associated with control of HIV in humans, and of simian immunodeficiency virus (SIV) in macaques. Vaccine immunogens have been designed that remove immunodominant variable portions of the viral proteome and consist exclusively of immunologically subdominant, conserved, and functionally constrained amino acid sequences. Towards this end, HIV/SIV conserved elements (CE) DNA vaccines have been developed and have demonstrated immunogenicity in SIV

uninfected macaques. However, it was unknown if these responses could be induced in the immunodominant setting of viral infection and redirect T-cellular immune responses to CE sequences. I demonstrated that CE DNA vaccination induced broader cellular and humoral immunity to CE sequences in SHIV infected ART naïve as well as SIV infected ART treated macaques. Furthermore, I demonstrated that T-cellular “exhaustion”, or a limited functional capacity, prior to vaccination was inversely correlated with vaccine immunogenicity. Additionally, I identified that cellular exhaustion in the blood was correlated with SIV-induced perturbations in gut immune cell subsets and microbial translocation. In all, these findings demonstrate that CE DNA vaccines can overcome the canonical immunodominance hierarchies induced by viral infection, and that approaches to reverse cellular exhaustion (i.e. anti-PD-1 antibodies) and improve gut homeostasis may improve CE DNA vaccine immunogenicity.

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Goals of This Dissertation

It has previously been demonstrated that conserved elements (CE) HIV/SIV DNA vaccines are immunogenic in SIV naïve macaques. However, these CE are immunologically subdominant, making it unclear if these results would translate to SIV-infected macaques in the immunodominant setting of a viral infection. The primary goal of this dissertation is to determine the immunogenicity of a therapeutic HIV/SIV CE DNA vaccine in SHIV/SIV infected macaques. In **Chapter 1,** I review the overall progress towards therapeutic HIV/SIV vaccines with an emphasis on DNA vaccines. In **Chapter 2**, I explore the hypothesis that a therapeutic CE vaccine would induce more potent CE-specific cellular immune responses compared to a full-length (FL) vaccine in SHIV-infected non-progressor macaques. In **Chapter 3**, I characterize the different specificities of the cellular and humoral immune responses elicited by CE and FL immunogens in SIV-infected ART treated macaques. Additionally, I examine how the exhaustion marker programmed death-1(PD-1) may limit vaccine immunogenicity. In **Chapter 4**, I expand upon the exhaustion analyses to more fully assess what factors contribute to immune exhaustion and whether immune exhaustion limits vaccine immunogenicity. Lastly, in **Chapter 5** I synthesize the overall results and explain how they contribute to HIV cure efforts and the development of CE vaccines for other variable pathogens.

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“America rewards you for your people skills.” – Tom Munson

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DEDICATION

This thesis is dedicated to my parents for always encouraging me to follow my dreams, to love learning, and instilling in me the confidence that I could accomplish anything.

Chapter 1: Background & Progress towards a therapeutic HIV vaccine

"Whatever the problem, be part of the solution. Don't just sit around raising questions and pointing out obstacles." – Tina Fey, *Bossypants*

Background & Progress towards a therapeutic HIV vaccine

HIV

During the early 1980's, in the United States, there was dramatic increase in the number of cases of *Pneumocystis carinii* and Kaposi's sarcoma in men who have sex with men (MSM)[1]. Initially, scientists and clinicians were baffled by this phenomenon and originally dubbed the phenomenon gay-related immune deficiency (GRID), later renamed Acquired Immune Deficiency Syndrome (AIDS), although the etiology of the disease remained unknown. Ultimately, a team in Paris believing AIDS may be linked to a viral infection successfully isolated the virus from a lymph node biopsy from an AIDS patient[2]. Further confirmation of this virus as the etiologic cause of AIDS was accomplished by the development of a serological diagnostic test that measured virus-specific humoral responses [3]. The virus was ultimately named the human immunodeficiency virus (HIV) and additional studies demonstrated the virus belonged to the *Retroviridae* family.

HIV is an enveloped virus with a single stranded, positive sense, RNA genome. The protein Gag (HIV = p55, SIV = p57) encodes the internal structural components of the virion including the matrix, capsid, and nucleocapsid after it is cleaved by the viral protease (**Figure 1.1)**. Each virion contains two copies of genome enclosed in the viral capsid protein (HIV = p24, SIV = p27). The viral Envelope (Env), is a heterodimeric glycoprotein composed of the surface antigen (gp120) and the transmembrane proteins (gp41). Env first binds to $CD4^+$ on $CD4^+$ T-cells or macrophages, then binds the co-receptor CCR5[4] (although may use CXCR4[5] among others), thus mediating fusion and entry of the "viral core" which consists of the capsid and the RNA genome. Upon entry, the error-prone reverse transcriptase (RT) enzyme synthesizes complementary DNA (cDNA) from the RNA genome. Next, the viral enzyme Integrase inserts

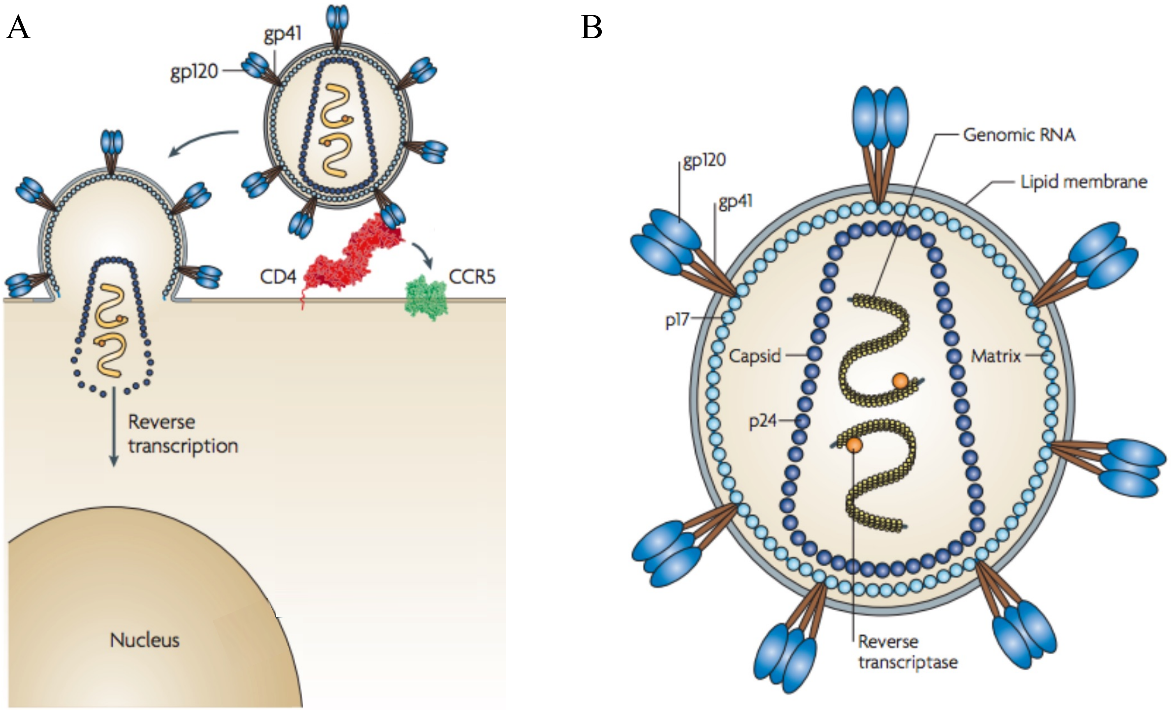


Figure 1.1. Human immunodeficiency virus (HIV) entry and structure. A) HIV binds CD4 as well as the co-receptor CCR5 via the Envelope (Env, gp120 and gp41) protein on T cells and macrophages. Following fusion of the viral and cellular membranes, the capsid enters the cytoplasm, the RNA genome is reverse transcribed to DNA, and the genome travels to the nucleus where it is integrated into the cellular DNA. B) The structure of the HIV virion, Gag (p55) is cleaved by the viral protease to form the matrix protein (p17), capsid (p24) and nucleocapsid (p7) (Adapted from Hedestam *et al.* [6]).

the viral double stranded cDNA into the host genome. The presence of long lived cells harboring viral cDNA, without expression of viral antigen, represents the “latent reservoir”[7].

HIV has several mechanisms that contribute to its enormous genetic diversity and rapid ability to evade host immune responses and therapies. An infected individual produces 10^{10} virions per day, given the mutation rate of RT is 3.4×10^{-5} and the size of the genome is 10^4 base pairs, this results in every possible single mutation and many of the double mutations are generated daily[8]. Viral diversity can also be driven by recombination events that can occur when two different viral strains infect the same cell[9]. Additionally, many viral variants that are resistant to drug or harboring immune “escape mutations” can lay dormant as latent provirus, further complicating a patients’ treatment[10,11]. Rapid viral replication and high mutation rates, as well as lifelong infection, have given rise to an enormous amount of HIV diversity.

There are two types of HIV, HIV-1 which is the cause of the global pandemic, and HIV-2 which is less virulent and predominantly in West Africa and only shares 35-40% Env amino acid identity[12]. Within HIV-1 there are four groups comprising groups M (major), N (non-Major), O (outlier) and P. Groups M, N, and O originated from transmission from chimpanzees (*Pan troglodytes troglodytes* and *Pan troglodytes schweinfurthii*) [13] and P from gorillas (*Gorilla gorilla*)[14]. Group M is divided into subtypes (denoted by letters) and sub-subtypes (denoted by numbers) and currently consists of A1-4, B, C, D, F1-2, G, H, J, and K[8]. Intra-subtype and inter-subtype variability can be as high as 20% or 35% respectively[15]. This enormous genetic diversity represents an enormous challenge for development of vaccine and therapies.

HIV: Epidemiology & Pathogenesis

Since the beginning of the HIV pandemic greater than 70 million people have been infected and approximately 35 million have died according to the World Health Organization (WHO). The (WHO) estimates that in 2016, 36.7 million people were living with HIV, including 16 million women and 3.2 million children. Approximately half of people living with HIV reside in eastern and southern Africa. In 2016, 1 million people died from HIV related morbidities and 1.8

million people were infected. While annual AIDS related deaths decreased by one third from the year 2000 to 2016, HIV/AIDS remains a significant public health and economic burden.

HIV replication results in gradual depletion of CD4$^+$ T-cells[16,17] which results in loss of immune function, leading to opportunistic infections, and ultimately death in most individuals. However, a small number of patients termed “elite controllers” can maintain undetectable viremia in the absence of cART, control that is likely the result multiple immunological and virological mechanisms[18–21]. Different stages of disease progression can be classified according to their Fiebig stages[22]. The Fiebig stages include the eclipse phase as well as stages I-VI. Eclipse corresponds to the period of undetectable viremia (days 7-21) and Stage I is characterized by the presence of viral RNA in the blood, and the beginning Stage VI is defined by the detection of viral proteins, including integrase (p31), in the blood by Western blot and corresponds to the early chronic infection. Later stages of infection may have no symptoms or sporadically may include fatigue or weight loss. As infection progresses, AIDS is defined by a CD4$^+$ T-cell count of less than 200 per uL of blood or the presence of opportunistic infections including: Kaposi sarcoma, *Cytomegalovirus, Mycobacterium tuberculosis, Pneumocystis jirovecii* pneumonia. If untreated, these complications typically worsen and cause death in most individuals.

cART: Effectiveness and Limitations

Coinciding with a better understanding of the molecular biology of HIV was the development of anti-retroviral therapy (ART), which are drug regimens that inhibit viral replication at various stages of the life cycle. The first drug approved in the United States for HIV was Ziduvudine (AZT)[23], a nucleoside reverse transcriptase inhibitor (NRTI). While initially effective at controlling virus, monotherapy with AZT could select for mutants that were highly resistant to AZT[24,25]. ddI (didanosine), another NRTI, was the second FDA approved ART and was effective in AZT-intolerant patients[26]. To limit escape and prolong viral suppression, drugs were being developed to target other steps in the viral replication pathway. Towards this goal various drugs including protease inhibitors (PI's), non-nucleoside reverse transcriptase inhibitors (NNRTI's), integrase inhibitors, and fusion inhibitors have been developed. Currently, combination antiretroviral therapy (cART) refers to drug combinations from at least two different drug classes. The first single tablet once daily cART regiment was Atripla (2 NNRTI's, 1 NRTI) in 2006. Now there are several single tablet once daily regimens including Complera (1 NNRTI, 2 NRTI's: 2011), Stribild (1 Integrase Inhibitor, 1 cytochrome P450 inhibitor, 2 NRTI's: 2012), and Triumeq (1 Integrase Inhibitor, 2 NRTI's). Combining multiple potent HIV inhibitors in single pill provides a simpler experience for patients, limits immune escape, and is highly effective at limiting viral replication and preventing AIDS.

cART potently reduces viral replication and extends life for HIV infected people, but it cannot fully restore normal immune function, enhance HIV specific immune responses, or significantly reduce the reservoir of long lived quiescent cells[7,27–41]. The stability of the latent reservoir during cART means that the patient must remain on therapy for the duration of their life as cART cessation most often leads to viral recrudescence. Furthermore, the high cost of cART limits its availability in low-income countries [42]. However, the issue with access is not limited to resource poor setting, even in the United States only 30% of HIV positive people were virally suppressed on cART, largely driven by only 66% of those diagnosed being engaged in HIV medical care, according to a VitalSigns report published by the Center for Disease Control and Prevention[43]. Given the drawbacks of lifelong cART, there is an intense interest in HIV therapies, including latency reversal agents (LRA's), gene therapies, and vaccines that could induce viral remission in the absence of cART.

SIV/Rhesus macaque model

Developing an effective vaccine for HIV is a major goal for scientists and physicians. Animal models for HIV offer the opportunity for more invasive longitudinal sampling and greater control over parameters such as timing, route, and strain of challenge virus. An ideal animal model should recapitulate aspects of HIV in humans but develop disease at a faster rate for experimental logistics.

Natural hosts of simian immunodeficiency virus (SIV), such as African green monkeys and sooty mangabeys do not develop disease. However, inoculation of Asian macaques with African primate lentiviruses recapitulates many relevant characteristics of HIV infection in humans such as robust viral replication and $CD4^+$ depletion. Common non-human primate models for AIDS include rhesus macaques (*Macaca mulatta*), pig-tailed macaques (*Macaca nemestrina*), and cynomolgus macaques (*Macaca fascicularis*). The most common primate model for AIDS is the Indian origin rhesus macaque.

Various immune-genetic factors play a role in viral control in the SIV/rhesus model. Like humans, rhesus macaques with certain MHC-I haplotypes are better able to control virus due to the different repertoire of viral peptides that can engage with MHC. Primarily this corresponds to *Mamu-A*01*, *B*08*, and *B*17*, which are overrepresented in animals that can robustly control viremia[44–46]. Another important factor for viral control is TRIM5α, a member of the tripartite motif (TRIM) protein family. TRIM5α restricts lentiviral replication at a post-entry step and restricts viral uncoating prior to reverse transcription. When designing macaque experiments, great care should be taken when evaluating animals for their MHC-I and TRIM5α genetics to either avoid controllers or ensure they are evenly divided amongst treatment groups.

Simian immunodeficiency virus (SIV) and chimeric simian-human immunodeficiency viruses (SHIV) have been developed for use in macaques. Typically, SIV replicates to higher titers and is more pathogenic relative to SHIV viruses in macaques. Two commonly used SIV strains are SIVmac239 and SIVmac251. SIVmac239 is the first molecular clone that was consistently pathogenic and is highly resistant to neutralizing antibodies. SIVmac251, is a clone that is closely related to SIVmac239. Typically, SHIV's either carry HIV *env* gene to better evaluate antibody-based vaccines, or HIV enzymes such as *reverse transcriptase* so the virus is susceptible to NNRTI's. SHIV89.6P is a more pathogenic version of the SHIV89.6 virus, and utilizes HIV envelope and is a commonly used SHIV model. SIV∆B670 is a highly pathogenic uncloned primary isolate that replicates to extremely high titers, is neurotropic, and can cause AIDS within 10 months of untreated infection. Researchers should carefully evaluate the characteristics of the available SIV/SHIV strains when determining which is the most appropriate to test their hypothesis.

Particle-mediated epidermal delivery (PMED) DNA Vaccines

DNA vaccines refer to vaccines comprised of plasmid DNA encoding antigens of interest. DNA vaccines have several theoretical advantages over modalities, including ease of manufacture, rapid design, can be administered multiple times without anti-vector immunity, and elicit T-cell and antibody responses.

Particle mediated epidermal delivery (PMED), also known as gene gun, uses a burst of helium to deliver DNA coated gold particles into the epidermis. Since the DNA can be coated on separate gold particles and delivered to separate cells, thereby potentially limiting immune competition between antigens. Encouragingly, PMED is painless and has demonstrated protective levels of immune responses in small and large animal models as well as humans. In particular, PMED vaccination induced protective levels of anti-Hepatitis B antibodies in patients who did not respond to conventional vaccination[47]. Importantly, PMED stimulates potent mucosal responses making it an attractive platform for mucosally acquired pathogens or ones that preferentially replicate in the mucosa, including HIV and influenza. In support of this concept, our laboratory has demonstrated therapeutic SIV vaccine efficacy in the macaque model[48]. Specifically, we found the correlate of protection was a broad T-cell response in the gut mucosa, underscoring both the robust ability of PMED to elicit mucosal responses and their importance in controlling lentiviral replication. The most robust control of viral replication, and most potent SIV-specific T-cell responses, was observed in the group that received antigen encoding DNA co-formulated with DNA encoding the heat labile *E. coli* enterotoxin (LT) a potent mucosal adjuvant. Taken together, these results indicate that PMED + LT is a potent vaccine platform generally, and is specifically well suited for HIV therapeutic vaccine development.

HIV-specific T-cellular Immunity

CD8$^+$ T-cells recognize MHC-I restricted viral 8-11mer peptides. Upon recognition, CD8$^+$ T-cells can kill virally infected cells through the release of the cytotoxins perforin and granzyme, as well as cytokines such as IFN-γ, TNFα, and IL-2. In 1986, CD8$^+$ T-cells were shown to control HIV *in vitro*[49]. *In vivo* CD8$^+$ T-cell arise acutely during infection and are temporally associated with the initial decline in viral load after peak viremia, however, these studies were correlative in nature[50–52]. The first causal role for CD8$^+$ T-cells in control of lentiviral replication came from the SIV/macaque model in the late 90's by administering monoclonal anti-CD8 antibodies to monkeys infected with SHIV/SIV and observing a rise in viral load[53–55]. While the exact mechanisms of CD8$^+$ T-cell control of HIV are unknown there is a growing appreciation for **1)** targeting more conserved epitopes or antigens[56,57] **2)** a broad epitope specificity[48,58,59] **3)** polyfunctional responses[60] and **4)** potent cytotoxicity[61].

CD4$^+$ T-cells recognize MHC-II restricted viral 12mer or greater peptides. HIV-specific CD4's are preferentially infected[62], they also have direct antiviral properties such as cytotoxic effector function[63], thus making their role in HIV infection unclear. The first evidence that CD4$^+$ T-cells can also have direct virally specific cytotoxic activity *in vivo* was demonstrated in the lymphocytic choriomeningitis virus (LCMV)/mouse model, a common model for studying chronic viral infections[64]. As early as 1997, HIV-specific CD4$^+$ T-cells were inversely correlated with viral control, suggesting they may have anti-viral properties or that limited viral replication better preserves them[65]. Furthermore, HIV-specific cytolytic CD4$^+$ T-cells were detected during acute HIV infection and inversely correlated with viral load[63]. While HIV-specific CD4$^+$ T-cells may fuel the infection, it may be possible to harness their antiviral capacity for improved vaccine efficacy.

A crucial barrier for an efficacious therapeutic vaccine for HIV is the ability of the virus to evade effective T-cellular immune responses due to **1)** T-cell dysfunction via upregulation of exhaustion markers and **2)** epitope mutations or “immune escape”.

Exhaustion Markers

As HIV infection progresses, HIV specific CD4$^+$ and CD8$^+$ T-cells are impaired in their ability to persist, proliferate, and produce cytokines[66,67]. This is in part due to lentiviral induced increases in T-cell exhaustion markers including: programmed death-1 (PD-1) T-cell immunoglobulin and T-cell Ig and ITIM domain (TIGIT), which may limit the ability of a therapeutic vaccine to increase HIV-specific immune responses[66,68].

The surface marker PD-1 was first identified on mouse T-cells as a marker of programmed cell death[69]. Later, the PD-1/PD-L1 interaction was determined to be an important inhibitory pathway contributing to T-cell exhaustion in the LCMV/mouse model[70]. Similarly, HIV replication increases PD-1 expression on HIV-specific CD4$^+$ and CD8$^+$ T-cells in progressors but not long-term non-progressors (LTNPs) [66,71–74]. However, exhaustion is not limited to HIV-specific T-cells. HIV infection causes gut damage resulting in microbial translocation, which leads to amplified PD-1 expression on monocytes that is correlated with their production of IL-10 that binds the IL-10R on CD4$^+$ T-cells, thus leading to global impairment of CD4$^+$ T-cells[75]. Similar to HIV infection, SIV infected rhesus macaques display increased levels of PD-1 expression on SIV-specific T-cells and SIV specific immunity has been increased by *in vivo* blockade of PD-1/PD-L1 pathway[76–79].

α-PD-1/ α-PD-L1 antibodies are potent immunotherapies that can block PD-1 inhibition of cellular immune responses and boost immunity. In support of this concept, α-PD-1 antibodies have shown enormous promise in treating human cancers, albeit with 41% of patients experiencing adverse events[80]. In the SIV/macaque model, a therapeutic infusion α-PD-1 antibodies improved cellular and humoral immunity, decreased viremia, and prolonged survival[77]. In combination with a vaccine, PD-1 blockade in rhesus macaques improved responsiveness to prophylactic recombinant adenovirus vaccine[81]. However, PD-1 blockade in chronically SIV infected rhesus macaques transiently increased viremia before returning to pre-infusion levels[81]. The lack of antiviral efficacy observed by Finnefrock *et al* may have been due to α-PD-1 treatment increasing immune responses directed at immunodominant epitopes that the virus had already mutated.

The T-cell Ig and ITIM domain (TIGIT) can inhibit $CD4^+$ and $CD8^+$ T-cell function by outcompeting CD226 for binding with CD155 on dendritic cells (DC's), skewing them to produce more IL-10 and less IL-12[82–86]. In addition to indirect T-cell inhibition via DC's, TIGIT can directly inhibit T-cell function[83]. TIGIT also works synergistically with PD1 to impair antigen specific $CD8^+$ T-cell responses[87]. Notably, it has been demonstrated that TIGIT is upregulated during SIV infection and correlates with viral load, CD4 decline, and disease progression in the SIV macaque model[68]. Importantly, when TIGIT ligand is present, TIGIT and PD1 work in concert to impair $CD8^+$ T-cell in the context of tumor antigen specific $CD8^+$ T-cells in melanoma patients. However, the role of $PD\text{-}1^+$ $TIGIT^+$ $CD8^+$ and $CD4^+$ T-cells in the context of SIV/HIV infection is less understood.

While several lines of evidence suggest viremia limits both therapeutic vaccination immunogenicity and increases exhaustion marker expression, a direct link between exhaustion markers (PD-1, TIGIT) expression and responsiveness to therapeutic vaccination has not been demonstrated in HIV/SIV. Several observations support that viremia limits responsiveness to therapeutic vaccines, including the LCMV/mouse model, demonstrating that high viral loads were inversely correlated with therapeutic vaccine responses[88]. Our lab demonstrated in the SIV/macaque model that ART non-responders also failed to respond to therapeutic vaccination[48]. Furthermore, a therapeutic vaccine clinical trial with recombinant Env antigen found an inverse correlation between plasma viremia and induction of cellular immune responses[89]. Understanding how T-cell exhaustion limits responsiveness to vaccination could lead to more potent immunotherapies, potentially by combining vaccination with α-PD-1 antibodies to augment anti-HIV specific immunity.

CTL Escape

HIV specific CTL are observed early during infection, even upon the development of AIDS[90]. A key question that began to emerge was, how can HIV persist in the presence of high levels of anti-HIV CTL? One possibility is that HIV could evade T-cell responses by "CTL escape"[91].

Cytotoxic T-lymphocyte (CTL) escape refers to viral immune evasion of CTL responses by epitope mutation. CTL escape was first demonstrated by Pircher *et al* in 1990 using the LCMV mouse model with a transgenic mouse system expressing a single T-cell receptor gene to focus an immense amount of immune pressure on a single epitope[92]. This immune pressure, rapidly selecting for viral variants that could not be controlled by T-cell responses, indicates a novel mechanism that allowed for chronicity in LCMV infection. The authors further speculated these results may elucidate a mechanism that allows for the persistence of HIV in humans.

Perhaps CTL escape could explain how HIV persists in the presence of high frequencies of HIV-specific CTL's? In 1991 the McMichael lab published data from a cohort of HIV positive cART naïve patients and demonstrated that viral genomic variation precluded recognition by autologous T-cells[93], in agreement with the observations made by Pircher *et al*. In a more mechanistic study, Koenig *et al* expanded autologous Nef-specific T-cells from a single HIV positive donor and after adoptive transfer unexpectedly saw a rise in viral load and the loss of the Nef epitope[94]. CTL escape mutations have been documented in acute and chronic infection for both HIV and SIV [95–99]. These findings strongly suggest that CTL escape is an important mechanism for viral persistence and pathogenesis and that CTL's specific to mutable epitopes may be harmful.

This observation suggests that vaccines that aim to limit viral escape may be necessary for T-cell based vaccine strategies. In support of this theory, vaccine failure in the SIV/macaque model was linked with the emergence of escape from a single CTL epitope[100]. Additionally, Deng *et al* demonstrated that the latent HIV-1 reservoir is highly enriched (>98%) in CTL escape mutations for common epitopes[10]. The authors conclude that a broad CTL repertoire is required to clear the latently infected cells due to the preponderance of escape mutations. Importantly, Borducchi *et al* tested a therapeutic vaccine consisting of recombinant Ad26 prime / MVA boost regimen in macaques and found that cellular immune responses before stopping drug were strongly negatively correlated with viral set point post-ART[58]. These data support the concept that if the CTL response was sufficiently broad, the escape pathways would be so complex, requiring several compensatory mutations among multiple amino acids, that escape would likely never occur.

Our laboratory utilized an SIV DNA vaccine co-formulated with the powerful genetic adjuvant the heat labile *E. coli* enterotoxin to investigate its potential as a therapeutic vaccine in the SIV/macaque model[48,101]. A key finding from our study was that a broad CTL in the gut was correlated with durable control of viremia in the absence of ART. This is likely due to the ability of the broad CTL response to recognize multiple viral epitopes thus requiring numerous escape pathways too complicated to occur. This study supports the concept of CTL breadth as an important immunological predictor of viral control, particularly in the gut.

We contend the number of escape pathways are too complex to be practically accommodated by a conventional vaccine. While mosaic immunogens, that compress antigenic diversity by including multiple versions of epitopes, have in some cases improved the breadth of variants recognized by the CTL responses[102,103], we propose the most direct path to broadly reactive CTL is to develop immunogens excluding variable regions and instead focus on highly conserved regions of the proteome[104].

Conserved Immunogens

HIV/SIV infected CD4 T-cells display a variety of variable and conserved MHC restricted viral peptides (**Figure 1.2A**). A problem with most therapeutic vaccine immunogens is the reliance on full-length (FL) or near FL immunogens which tend to elicit narrow responses against immunodominant epitopes that can readily mutate with little or no fitness cost, and render CD8 T-cells ineffective at recognizing and killing virally infected cells (**Figure 1.2B**)[105,106]. Immune responses to variable epitopes, or more variable proteins, may be ineffectual or even deleterious in controlling HIV infection[57,107]. One solution to this problem is to focus immune responses on conserved regions of the proteome that cannot mutate without severe fitness costs (**Figure 1.2C**)[104].

However, conserved regions of the proteome are immunologically subdominant in the setting of natural infection or FL immunization[106]. The importance of subdominant immune responses in the control of HIV infection underscores the goal of developing immunogens that elicit non-canonical immunodominance hierarchies[108]. Therefore, efforts to induce broad immune responses to conserved subdominant parts of the virus have led to the development of immunogens such as HIVConsv [109–115], Vacc-4x[116–125], and Conserved elements (CE)[104,126–129]. CE vaccines are designed by excluding immunodominant and variable regions of the proteome and exist exclusively of subdominant conserved regions connected by linker sequences optimized for immunoproteasome cleavage (**Figure 1.2D**). While these vaccines have shown strong immunogenicity in uninfected macaques[128,129] and humans, it was unknown whether such

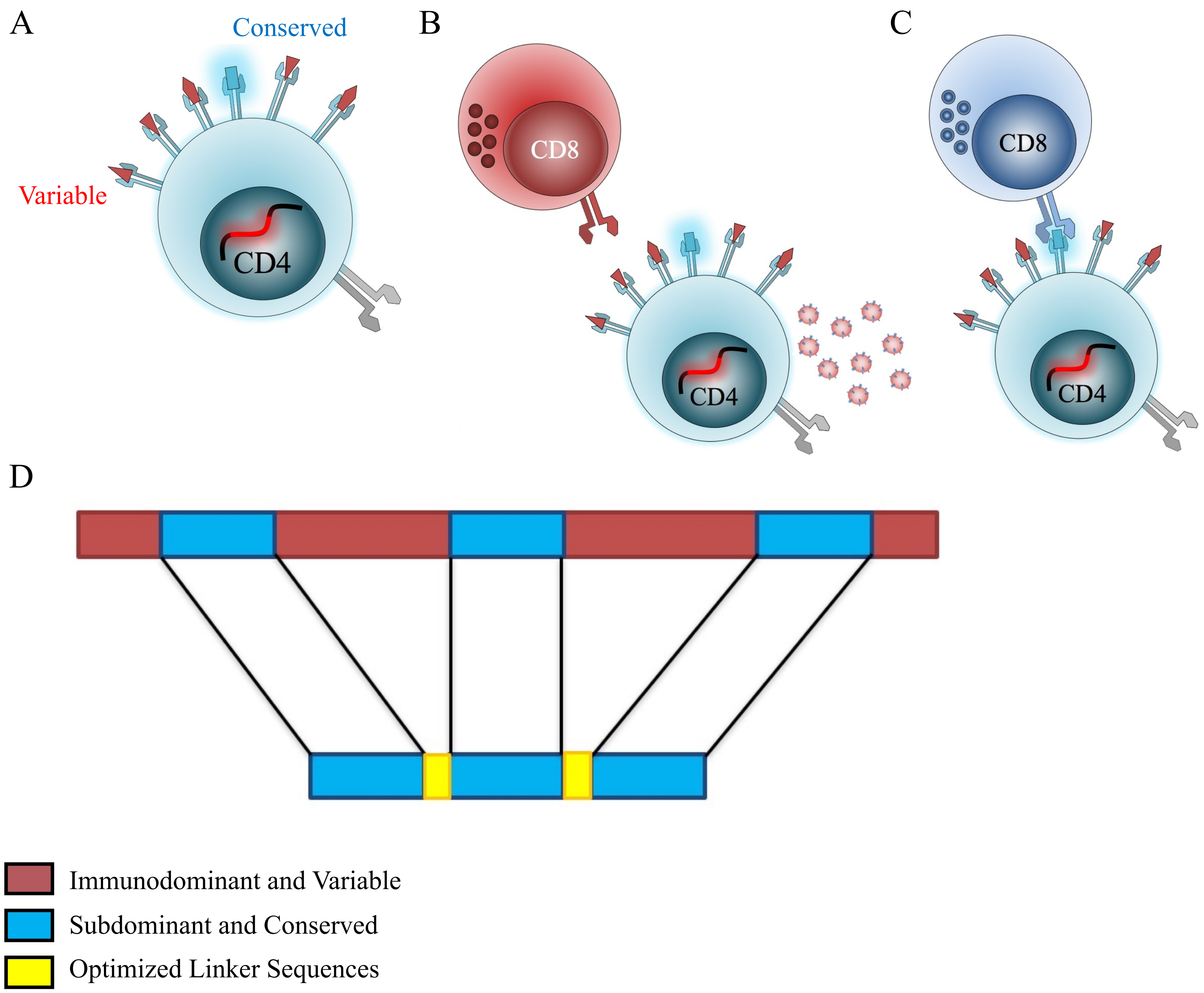


Figure 1.2. Viral diversity and conserved immunogens. A) Shown is a depiction of a productively infected CD4$^+$ T cell displaying MHC restricted viral peptides from variable (red) and conserved (blue) epitopes. **B)** A CD8$^+$ T-cell specific to a variable epitope is unable to recognize an infected cell due to an escape mutation. **C)** A conserved element (CE) specific CD8 T-cell better recognizes infected cells and limits escape mutations. **D)** A CE immunogen excludes immunodominant and variable amino acid sequences and connects CE via optimized linker sequences.

responses could be boosted therapeutically in subjects that have developed immunodominant responses. In support of this, the Felber laboratory demonstrated in uninfected rhesus macaques that FL vaccination prior to CE vaccination resulted in a diminished ability to respond to CE sequences[128].

HIV and SIV CE , and similar conserved vaccines, have shown promise for prophylaxis and therapy and have demonstrated anti-viral efficacy *in vitro* and *in vivo*[126–133]*.* Specifically, the Mullins and Felber laboratories have demonstrated that prophylactic Gag derived **CE DNA** vaccines are immunogenic in mice and macaques[126–129,134]. However, a peptide based vaccine consisting of highly conserved portions of Gag capsid (p24), was designed to elicit T-cellular immunity and recently completed a phase 2 clinical trial[135]. Notably, there was an approximately 0.4 log decrease in viral set point in the **Vacc-4x** group after ART withdrawal among ELISpot responders relative to placebo treated subjects. Additionally, the Hanke group has developed the virally vectored **HIVConsv** vaccine expressing conserved portions of both Gag and Pol[133]. Importantly, in HIV negative subjects, HIVConsv induced $CD8^+$ T-cells that could inhibit HIV replication in autologous $CD4^+$ cells *in vitro*, therefore suggesting therapeutic vaccination may have an antiviral effect *in vivo*[133]. Taken together, these findings suggest that therapeutic DNA vaccines should seek to elicit responses to conserved portions of HIV.

Therapeutic Vaccines

Therapeutic vaccines offer a potentially inexpensive and scalable way to increase HIV specific immunity, with the potential to control viral replication and prevent disease progression in the absence of cART[136]. The goals of therapeutic vaccination are aimed at improving HIV specific T-cell and antibody responses that could delay rebound, or induce functional or sterilizing cures following an ATI (**Figure 1.3**). T-cell based vaccines have focused on increasing the breadth of epitopes targeted, homing responses to lymph node and mucosal tissues, and improving polyfunctional responses[10,48,137]. Antibody approaches focus on improving broadly neutralizing antibodies or Fc mediated effector functions. The feasibility of therapeutic vaccines is supported by rare groups of individuals called elite controllers who are able to control plasma viremia to undetectable levels through robust immune responses[60,61,65,138,139].

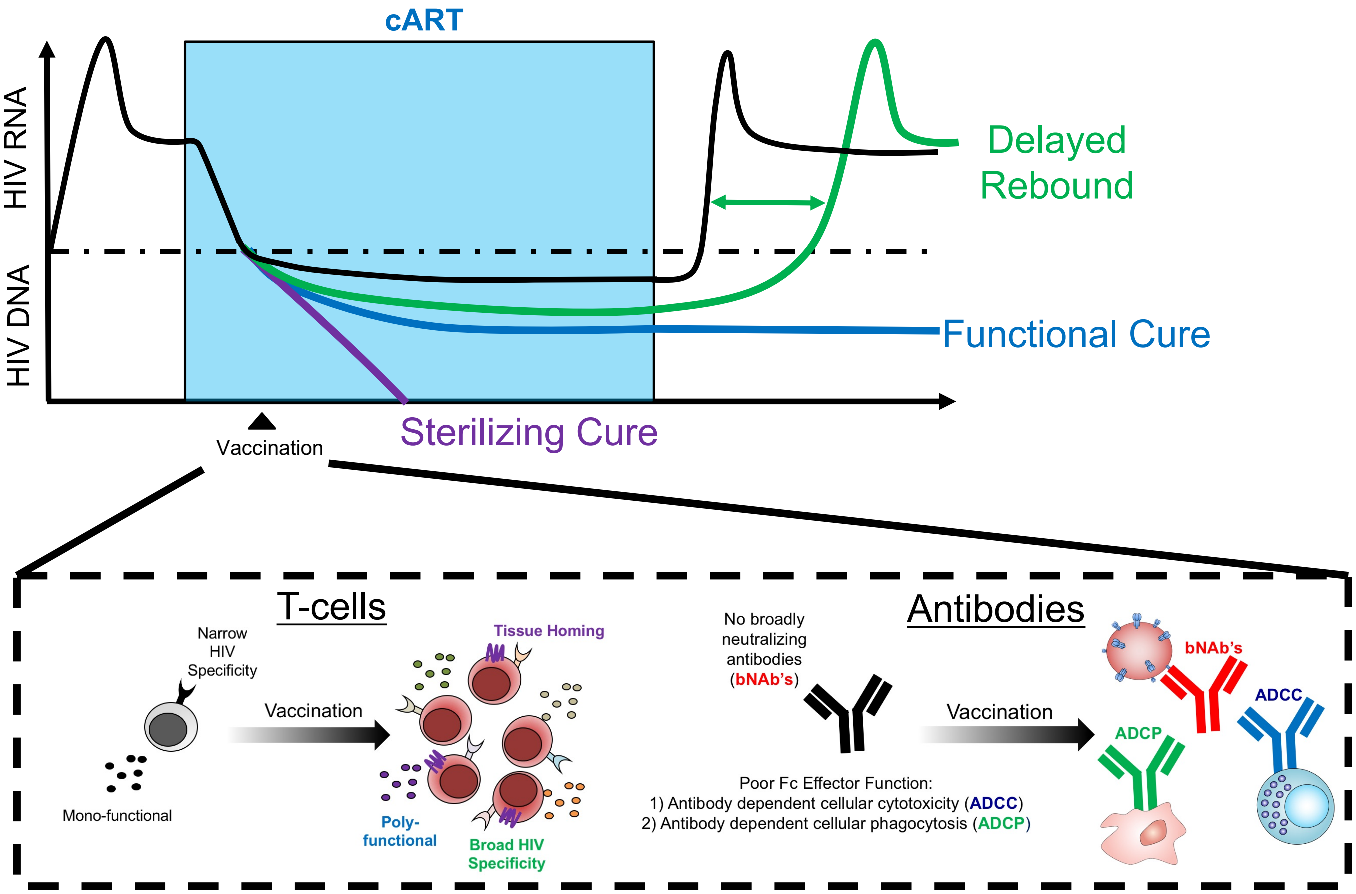


Figure 1.3. HIV Therapeutic Vaccine Concept: A therapeutic vaccine could improve anti-HIV T-cell and humoral responses that would allow for cessation of combined antiretroviral therapy (cART) and delay rebound, or induce functional or a sterilizing cure.

VACCINATION STRATEGIES:

Various therapeutic vaccine approaches have been tested including subunit, inactivated or pseudotyped viral particles, autologous dendritic cells pulsed with viral antigens, and DNA or viral vectors containing various SIV/HIV genes (**Figure 1.4**).

Subunit:

Tat (trans-activator of transcription): The HIV protein Tat (transactivator of transcription) plays an important role in immune activation[140], shielding Envelope (Env) from neutralizing antibodies[141], and induces viral co-receptor expression[142]. Importantly, immune responses to Tat are associated with the asymptomatic phase of infection and non-progression to AIDS[181,182].

Phase I clinical trials of biologically active recombinant Tat were safe and well tolerated[145]. In Longo *et al.*, Tat was delivered either subcutaneously with alum (Arm A) or intradermally without adjuvant (Arm B), as well a placebo groups that received alum and saline subcutaneously or saline alone intradermally[145,146]. While Arm A generally had greater antibody responses (IgM, IgG, and IgA), Arm B had more potently induced cellular immunity as measured by IFN-γ and IL-4 ELISpot, and by T-cell proliferation assays. In a separate study, Goldstein *et al.* evaluated an anti-Tat B-cell epitope vaccine, Thymon universal Tat immunogen-16 (TUTI-16)[147]. TUTI-16 was safe and able to induce anti-Tat antibodies (Abs), thereby achieving the primary endpoints. Unfortunately, no delay in viral rebound was observed between vaccinated and placebo subjects.

Recently, a Phase II randomized multicenter trial of the biologically active intradermally injected Tat vaccine was completed by Ensoli *et al.*[148,149]. Subjects in the trial were HIV positive, virologically suppressed (<50 copies per/mL) by cART,

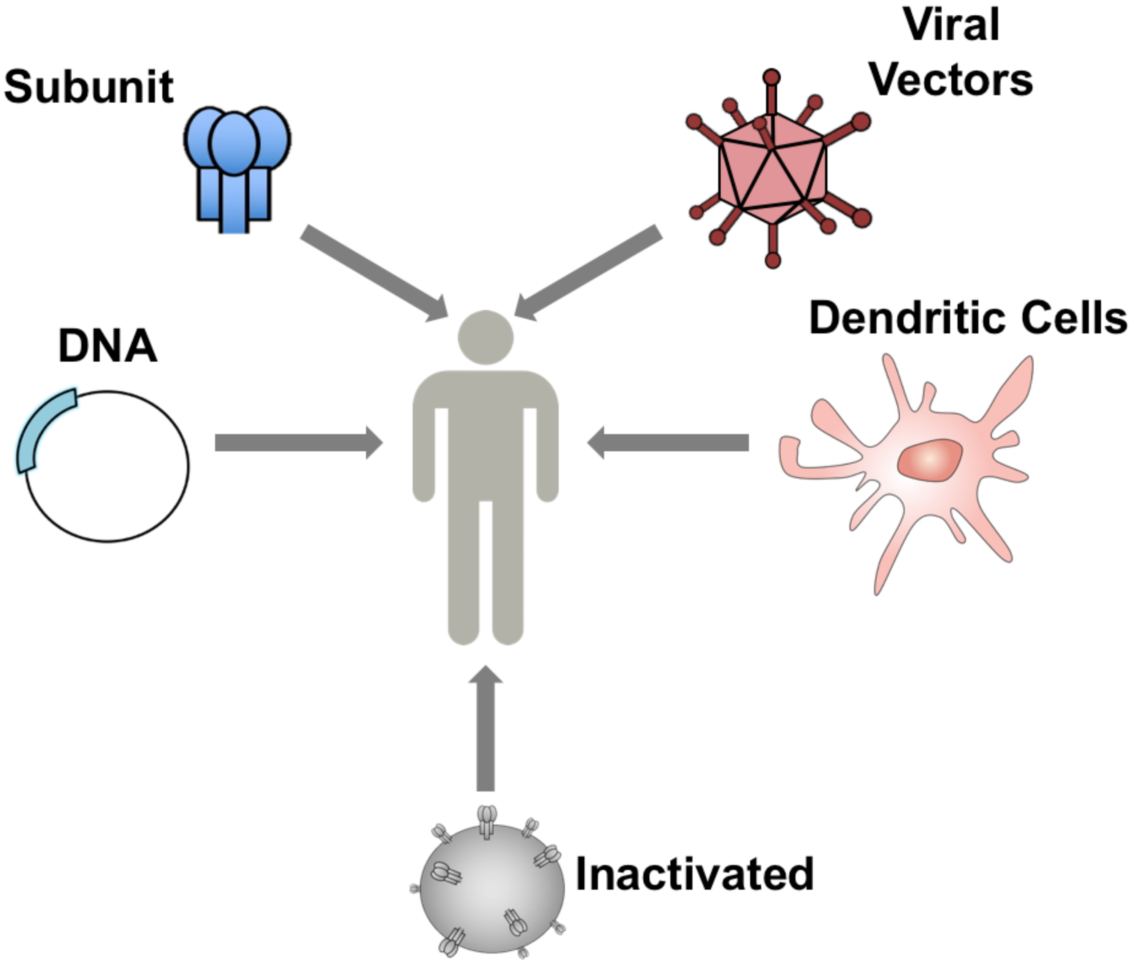


Figure 1.4. HIV Therapeutic Vaccination Strategies: A variety of vaccine modalities have been tested for HIV therapeutic vaccines including subunit, recombinant viral vectors and DNA that express viral antigens, dendritic cells pulsed with viral peptides or inactivated virions, and inactivated viruses.

and anti-Tat antibody (Ab) negative. This trial was not placebo controlled, but included an external reference group (OBS) of individuals that were Tat Ab negative that were virologically suppressed by cART. When compared to OBS, Tat vaccinated individuals had an increase of approximately 100 CD4+ T-cells per microliter of blood. There were also significant increases in B-cells and NK cells in the blood of Tat vaccinated persons compared to the OBS. Importantly, blood HIV-1 DNA was reduced in Tat vaccinees when compared to OBS patients. Furthermore, HIV-1 DNA decay in the blood of vaccinated patients was associated with the presence of anti-Tat IgM and IgG Abs. Similarly, a separate study which delivered Tat intradermally in a phase I/II randomized controlled trial also found reduced HIV RNA and DNA in vaccinees[150].

While anti-Tat antibody based vaccines remain a controversial approach, given their mixed results from macaque studies and the suggestion that CTL responses to Tat would be more efficacious, these studies underscore the potential use of Tat therapeutic vaccinations as potential for cART intensification or simplification[15199].

Vacc-4x: The Vacc-4x vaccine consists of four synthetic peptides corresponding to conserved amino acid sequences of the HIV capsid protein (p24)[119]. The vaccine was found to be safe, but only elicited modest humoral and cellular immunity in an open, single center, Phase I safety study[119]. A follow up Phase II clinical trial with a cohort of 40 HIV-1 infected asymptomatic patients found the vaccine to be safe. Vacc-4x was injected intradermally along with granulocyte macrophage colony stimulating factor (GM-CSF), with the goal of activating Langerhans' dendritic cells. The vaccine was immunogenic, 90% of patients developed a Vacc-4x specific delayed type hypersensitivity (DTH) response skin test and 80% developed Vacc-4x specific CD4$^+$ and CD8 T-cell proliferative responses by a carboxyfluorescein diacetate succimidylester (CSFE) assay[123]. These patients then underwent a structured treatment interruption and the patients with the greatest Vacc-4x responses tended to have lower viral loads relative to Vacc-4x low-responders, however, this trial was not placebo controlled[117].

Furthermore, immune responses elicited by Vacc-4x were associated with delayed resumption of antiretroviral therapy[121]. However, attempts to further enhance these immune responses by administering boosting vaccinations only increased immune responses in some patients, while in others it dampened vaccine specific T-cell responses[125]. Recently, a Phase II placebo controlled, randomized, double blind trial sought to determine the safety and efficacy of Vacc-4x[135]. While the vaccine was immunogenic, it only afforded an approximate 0.4 log reduction in median viral set point compared to placebos during a structured treatment interruption. Interestingly, among ELISpot responders, the Vacc-4x group had a significantly lower viral set point relative to placebo subjects, whereas there was no difference in viral set point between placebo and Vacc-4x vaccinated ELISpot negative subjects. These results suggest that T-cellular immunity induced by Vacc-4x is more potent than responses developed during natural infection.

OPAL: In another approach, autologous blood was incubated with overlapping SIV peptides, an immunotherapy termed OPAL (Overlapping Peptide-pulsed Autologous ceLls)[152]. Juvenile cynomolgous macaques were infected with $SIV_{mac}251$, put on cART, and received peptide vaccinations corresponding to Gag or full proteome peptides (All) or remained unvacinated. The vaccinations were performed by isolating autologous peripheral blood mononuclear cells, briefly incubating them with corresponding peptides, and then re-infusing intravenously into the autologous animal. Unsurprisingly, the Gag vaccinated group had the greatest magnitude of Gag-specific $CD4^+$ and $CD8^+$ T-cells. In contrast, the All vaccinated macaques exhibited the most robust cellular immunity to Env. Upon cART cessation both groups of macaques maintained ~1 log reduction in viral load for 1 year when compared to unvaccinated controls. The authors concluded that Gag alone was effective for T-cell based immunotherapies. Unfortunately, when this approach was taken into a phase I clinical trial it failed to elicit immune responses and resulted in potentially life threatening cardiac events[153,154]. The mechanism behind this pathology is currently unknown.

An alternative approach is to fuse viral proteins to each other or to immunogenic proteins. One group developed an HIV-1 gp120/Nef Tat vaccine adjuvanted with $AS02_A$[155]. $AS02_A$ is an oil-in-water emulsion that contains the immunostimulants monophosphoryl lipid A (MPL) and QS-21 which is a triterpene glycoside derived from the tree *Quillaja saponaria*[157,158]. Vaccinated subjects had significantly greater gp120 specific CD4+ T cell proliferation and did not have any serious adverse events (SAEs) when compared to control subjects.

The first therapeutic vaccine trial in Uganda was a detoxified anthrax polypeptide fused to the HIV capsid (p24)[156]. The vaccine was safe and vaccinees had a modest but statistically significant increase of CD4+ T-cell count in the blood (p=0.02) at 12 months, and no such increase was observed in historical controls (p=0.41). A separate approach fused the peptide AT20, derived from the HIV matrix protein (p17), to the keyhole limpet hemocyanin (AT20-KLH)[159]. KLH is a glycoprotein derived from the mollusk *Megathura crenulata* that has a large complex structure with complex carbohydrate moieties on its side chains and is used as a carrier protein to increase immune responses[160]. p17 has a variety of immunomodulatory and chemokine-like functions and it was hypothesized that antibodies directed to p17 could reduce these pathologies[161162]. Importantly, antibodies induced by natural infection are typically rare and of lower titer[163]. AT20-KLH induced robust and long lasting anti-p17 Ab's in HIV infected cART treated patients that were able to neutralize p17 receptor interactions and the biological activity of p17. An analytical treatment interruption (ATI) was not performed in this study, so it is unknown whether these responses have antiviral activity. Given the inflammatory activity of extracellular p17, it may be of interest to determine whether vaccination in this cohort led to a decrease in cellular activation or inflammatory cytokines[162].

INACTIVATED

Inactivated vaccines consist of pathogens that have been chemically or physically rendered uninfectious[164–167]. These approaches are considered controversial for HIV due to safety concerns. However, the efficacy of this approach was supported by a *nef* deleted SIV vaccine (SIVmac239∆nef) that in some cases protected rhesus macaques from an intravenous pathogenic SIV challenge[168]. Unfortunately, SIVmac239∆nef was able to mutate and restore normal *nef* function *in vivo* and cause CD4+ T-cell depletion and progression to AIDS [169]. Additionally, SIVmac239∆nef can cause AIDS in neonates without recovery of *nef* function[170]. Future work on inactivated and attenuated approaches has focused on maintaining efficacy while improving safety.

Remune® / HIV-1 Immunogen: An excellent overview of the Reumune® is covered by Graziani and Angel[171]. Initially proposed and designed by Dr. Jonas Salk[172], Remune® is a gp160 or gp120 depleted, inactivated HIV-1 formulated with incomplete Freund's adjuvant[173,174]. Over nearly thirty years dozens of trials have been performed with little clinical success[175–193,176].

Clinical trials by Kahn[180], Trauger[193], and Churdboonchart[184] *et al* reported positive clinical findings, but have been eclipsed by controversies. Kahn *et al* disagreed with Immune Response Corporation, the study sponsor, on the best methodology for analyzing the clinical data, and addressed the controversies in JAMA[194]. Whereas Kahn *et al* did not find a reduction in viral load[180], Trauger[195] *et al* found decreased viral load in the Remune treated group. Trauger *et al* were warned by the FDA for removing two patients from the final data set[196]. Finally, Glidden *et al* reported that Churdboonchart *et al* allegedly did not report the primary $CD4^+$ T-cell analyses, and instead used area under the curve (AUC)[197].

A study by Moss *et al.* performed an ATI on individuals that received Remune® (n =13) and unvaccinated persons (n =12)[198]. Immunized individuals had improved HIV specific $CD4^+$ T-cell immune responses and β-chemokine production. Interestingly, these immune responses correlated with reduced viral replication in the second ATI, but not in the first.

In a separate single arm study, Huang *et al.* examined a cART intensification strategy that included GM-CSF, diadenosine, hydroxyurea and Remune® in ten chronically HIV infected adults[199]. During an ATI, vaccinees had elevated HIV specific responses as well as decreased viral replication, but a correlation between these two observations was not found.

AT-2 Inactivation: In a study by Vagenas *et al.,* aldrithiol-2 (AT-2) inactivated SIVmac239 was delivered with TLR binding adjuvants to the tonsils of ART treated SIV$^+$ rhesus macaques[200]. AT-2 inactivation eliminates viral infectivity while maintaining proper structure and antigen processing[201]. Vaccination induced potent neutralizing antibody titers and afforded a robust decrease in plasma viremia during an ATI. During the ATI, animals were re-vaccinated without cART, however this did not result in a reduction in viral load. This study supports the concept of mucosally delivered therapeutic vaccines for HIV.

HIVAX: HIVAX is a replication defective HIV vaccine containing multiple mutations in env, pol, nef, vif, and vpu and can only initiate one round of infection[202]. The virus is pseudotyped from the vesicular stomatitis virus G protein (VSV-G) which expands the cellular tropism, including Langerhans cells[203]. In a randomized, controlled trial vaccines received a subcutaneous injection of HIVAX (n =7) or placebo (n = 5)[204]. HIVAX, in addition to being safe, induced more potent CD4+ and CD8 cellular immunity as determined by ELISpot and intracellular cytokine staining (ICS) assays. Additionally, the HIVAX vaccinated subjects had significantly lower viral loads during an ATI compared to pre-treatment levels.

DENDRITIC CELL VACCINES

Dendritic cells (DCs) are potent antigen presenting cells that are critical for initiating robust adaptive immune responses. However, HIV infection causes DC dysfunction that is only partially restored by cART. One way to boost SIV/HIV specific responses is to obtain autologous DCs and expose the cells to: 1) inactivated autologous HIV virions[205–209] 2) HIV RNA electroporated into DCs[210–212] 3) pulsing with HIV peptides[213–218] and 4) viral vectors delivered to DCs *ex vivo*[219,220].

Thus far, the most successful HIV therapeutic vaccine in humans was comprised of DCs pulsed with inactivated autologous virus[209]. Because each vaccine is developed with a patient's autologous virus the genetic difference between the dendritic cell vaccine and the virus is minimized compared to , therefore potentially eliciting more effective immune responses tailored to each patient. Indeed, a vaccine consisting of aldrithiol-2 (AT-2) inactivated SIV loaded onto dendritic cells afforded an impressive 3 log decrease in viral load compared to macaques receiving DC alone[205]. A placebo controlled study Garcia *et al.* demonstrated that 55% of patients vaccinated with DCs pulsed with autologous heat inactivated HIV had a 1 log decrease in viral load during an ATI, whereas viral load was not signifiancly decreased in the placebo subjects. However, vaccination did not prevent CD4$^+$ T-cell decline in the blood, with values decreasing to pre-cART values among all treatment groups. While the efficacy was modest and the immune correlates were not entirely clear, this vaccine represents the most efficacious therapeutic HIV vaccine in humans.

Efficacy in other dendrtic cell vaccine clinical trials has been mixed with some demonstrating a virological benefit to vaccination[205–208,221], others with no virological benefit [213,214,216,220,222]. Recently a phase IIB, randomized, double-blind placebo controlled study assessed the therapeutic efficacy of AGS-004, a vaccine that uses autologous translation competent RNA encoding for Gag, Rev, Vpr and Nef and electroporates them into DCs[223]. Additionally, DCs were electroporated with CD40L RNA with the goal to circumvent the need for CD4$^+$ T-cell help. AGS-004 induced poly-functional effector memory CD8's versus placebo, these immune responses did not translate to a virologic benefit. Importantly, it was demonstrated that AGS-004, while effective at inducing HIV-specific CD8$^+$ T-cells, did not potently induce HIV-specific CD4$^+$ T-cells. One explanation for this is that RNA delivered to the cytosol does not primarily engage the MHC-II presentation pathway, which is necessary for antigen specific CD4 stimulation. While the immunological basis for the lack of viral control is unknown, using autologous antigen and the stimulation of a CD8$^+$ T-cell response may not be sufficient for viral control during an ATI.

VIRAL VECTORS

Viral vectors expressing SIV/HIV antigens have been shown to be safe, immunogenic, and have provided efficacy in prophylactic vaccine studies[224,225]. Furthermore, early therapeutic vaccine studies in non-human primates demonstrated immunogenicity and antiviral efficacy[226–228].

ALVAC (Canarypox-virus):

The prophylactic RV144 phase III clinical trial utilized the non-replicating canarypox vector ALVAC (vCP1521) followed by a gp120 AIDSVAX® B/E protein boost that provided ~30% protection against HIV infection[229]. Unfortunately, several therapeutic trials utilizing ALVAC-HIV vaccines did not statistically significantly decrease viral load during ATI or reduce the size of the viral reservoir[185,230–235].

Some trials using ALVAC-HIV vectors did detect modest anti-viral efficacy. A randomized, double blind, placebo controlled study of ALVAC (vCP1452) expressing *env* and *gag* genes, as well as CTL epitopes from *nef* and *pol* was administered with or without Remune[175]. Vaccinations, regardless of Remune® co-administration, tended to delay viral rebound after ATI, however it did not lower the viral load set point compared to the placebo arm. The addition of Remune did not provide a clinical benefit compared to the ALVAC alone arm. A separate trial by Lévy *et al.* tested a therapeutic regimen consisting of ALVAC-HIV (vCP1433) and Lipo-6T vaccination followed by three cycles of subcutaneous IL-2 injection and was compared with control patients only receiving cART[236,237]. Following an ATI, 24% of vaccinated patients had lowered viral set point versus only 5% of control patients (P = 0.027). Furthermore, a logistical linear regression analysis demonstrated the correlate of viral control was a stable and broad HIV-specific $CD4^+$ T-cell repertoire.

One trial employing an ALVAC vaccine demonstrated increased viral replication versus placebo during an ATI[238]. In a double blind, placebo controlled, phase II study by Papagno *et al* found that the ALVAC-HIV vaccine (vCP1452) preferentially stimulated HIV-specific CD4$^+$, but not CD8$^+$, T-cells[238]. The increased viral load may be caused by the vaccine induced expansion of HIV-specific CD4$^+$ T-cells that may fuel viral replication[62,238,239].

MVA:

The Modified Vaccinia Ankara (MVA) was developed by serial passaging in chicken embryo fibroblasts, resulting in genome reduction and an inability to replicate in virtually all mammalian cells while still being immunogenic[225,240–244]. A single arm study of an MVA-*nef* vaccine was safe and immunogenic, but after cART interruption viral rebound occured in all patients[245]. Interestingly, the MVA-*nef* vaccine did increase MVA and Nef-specific T-cell immune responses, indicating that anti-vector immunity did not completely inhibit vaccine immunogenicity.

A limitation of therapeutic vaccines for HIV is they require infected cells to produce viral proteins to render them susceptible to immune clearance, however there is a reservoir of long lived latently infected cells that do not produce viral proteins. Combining a vaccine with a latency reversal agent (LRA) could overcome this problem and more effectively decrease the size of the reservoir. Mothe *et al.* investigated a MVA-based (MVA-B) vaccine expressing gp120 and a Gag-Pol-Nef polyprotein with and without disulfiram in a Phase I clinical trial[246]. The vaccination did not induce robust antibody responses but did induce modest, but significant, T-cell responses, particularly to Gag compared with placebo recepients. After an ATI, there was a transient but significant reduction in plasma viral load in the MVA-B vaccinated versus placebo group. However, differences in viral load between the placebo and the MVA-B + disulfiram groups did not reach statistical significance. These results may be due to the modest immunogenicity of the MVA-B vaccine or the limited *in vivo* efficacy of disulfiram.

Hancock *et al* reported the immunogenicity and efficacy of an MVA vaccine expressing conserved portions of the proteome, termed MVA.HIVconsv, in HIV-infected cART treated patients[247]. While ELISpot responses were similar between vacinees and placebo groups, there was a modest increase in the viral inhibitory activity of $CD8^+$ T-cell in the vaccinees. However, vaccination did not result in a decrease in the size of the viral reservoir.

Adenovirus:

Adenoviral vectors (Ad) have several attributes that make them attractive for vaccine design: safety, manufacturability, infectivity of dividing/non-dividing cells, and induction of robust immune responses[248,249]. Furthermore, Ad vectors have the benefit of being replication competent in mucosal surfaces and may induce mucosal immune responses, including the gut, which harbor large numbers of virally infected cells[250,251].

Schooley *et al.* evaluated a replication deficient recombinant Ad5-*gag* vaccine in a placebo controlled, randomized, double blind study and was considered safe and modestly immunogenic, but only provided a modest transient decrease in viremia following an ATI[252]. Interestingly, the immunological correlate of viral control was Gag-specific $IFN\gamma^+/CD4^+$ T-cells, however it is unknown whether these cells acted directly on virally infected cells or provided support for $CD8^+$ T-cells and/or other immune cells. A separate randomized, and placebo controlled, clinical trial by Li *et al.* used a rAd5-HIV *gag* vaccine and also found decreased viral load in vaccinated recipient's with neutral HLA alleles, however this viral control was not correlated with Gag-specific $IFN\gamma^+$ $CD4^+$ or $CD8^+$ T-cells[253].

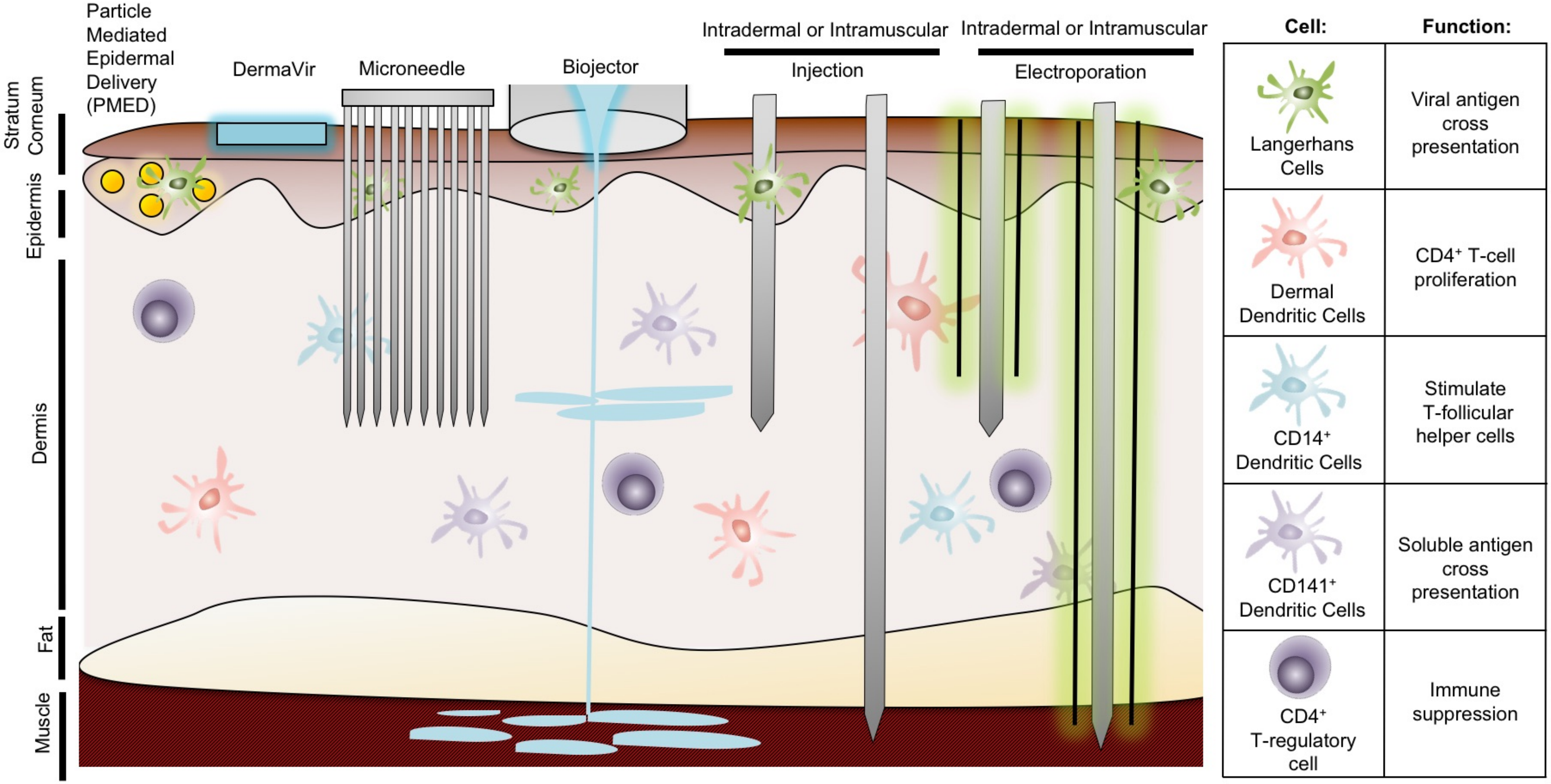
A study in macaques tested a novel Ad26/MVA vaccine co-delivered with a TLR7 agonist (GS-986) as a LRA. Macaques were treated with cART seven days after SIV infection and received a sham injection or received the following treatments: Ad26/MVA, GS-986, or Ad26/MVA + GS-986. GS-986 alone did not lower viral set point during ATI compared to the placebo animals. In contrast, vaccination lowered viral load by 0.66 logs, Ad26/MVA vaccination plus GS-986 administration resulted in a 1.74 log reduction and complete viral suppression in 33% (3/9) of animals. Broader vaccine induced SIV-specific T-cell responses correlated with reduced viral replication. This study, along with the MVA/disulfiram study by Mothe *et al.*, demonstrate the potential for combining therapeutic vaccines with other cure approaches.

DNA: DNA has several advantages for a therapeutic HIV vaccine including the ability to elicit $CD8^+$ T-cell and antibody responses, do not elicit anti-vector immunity, and are quick and simple to manufacture.

INJECTION WITH NEEDLE AND SYRINGE

The first therapeutic DNA immunization of an AIDS virus infected subject was performed by Boyer *et al* in 1997 and consisted of two chronically HIV-1_{IIIB} infected (>4 years) chimpanzees (*Pan trogolodytes*). One subject received DNA encoding the Env glycoprotein of HIV-1_{MN} and the regulatory gene *rev*, while the other subject received a non-coding plasmid as a placebo [254]. Both chimpanzees received 3 doses of 100 micrograms of DNA inoculated into the quadricep muscle. The vaccine induced antibody responses to several HIV antigens, including the gp120 V3 MN peptide as well as several regions of gp41. Furthermore, the vaccinated chimpanzee experienced decreased viral replication, whereas viremia was unchanged in the placebo. Although this study only employed a single subject in each cohort, it represents the pivotal beginning of therapeutic HIV DNA vaccines.

Shortly thereafter, in 1998 MacGregor *et al* performed the first human trial of an HIV DNA vaccine consisting of the structural gene *env* , as well as the regulatory gene *rev*, in asymptomatic HIV-infected subjects [255]. Similar to Boyer *et al*, human subjects received three DNA vaccine doses, with a range of DNA concentrations from 30-300 μg. The three doses at all concentrations of DNA were safe and well tolerated, with no local injection site reactions, and no perturbations in liver chemistries. Additionally, MacGregor *et al* did not detect the presence of anti-DNA antibodies, which was an initial concern with the use of DNA vaccines. As for immunogenicity, while the numbers of individuals enrolled in each dose cohort was small, they did observe the most potent CTL activity in the intermediate dose group (100 μg of DNA) and not in the 30 or 300 μg of DNA cohorts. The safety and immunogenicity of the first human trial of a therapeutic HIV DNA vaccine thus warranted further investigation.



Cell:	Function:
Langerhans Cells	Viral antigen cross presentation
Dermal Dendritic Cells	CD4⁺ T-cell proliferation
CD14⁺ Dendritic Cells	Stimulate T-follicular helper cells
CD141⁺ Dendritic Cells	Soluble antigen cross presentation
CD4⁺ T-regulatory cell	Immune suppression

Figure 1.5. DNA Delivery Technologies & Skin Immune Cell Subsets. Owing to the limited immunogenicity of intra-muscular injection of DNA there is significant interest in alternative delivery methods. Towards this end a variety of methods including particle mediated epidermal delivery (PMED), DermaVir, Biojector, intradermal injections, and electroporation aim to deliver DNA to more immonocompetent anatomical sites.

Consistent with these findings, several studies from the years 1998-2005 using intramuscular delivery, induced modest or undetectable HIV-specific T-cell responses, and did not decrease viral load [255–259]. This paradigm changed in 2005 when MacGregor *et al* showed a reduction of viral "blips" compared in vaccinated subjects in a double blind placebo controlled Phase I clinical trial[260]. In this study, MacGregor *et al* vaccinated asymptomatic HIV positive subjects on cART with DNA plasmids expressing *gag/pol* and *env/rev* genes. In addition to being safe and well tolerated, this vaccine was moderately immunogenic, with slightly over half of the vaccinated subjects (8/13) developing detectable ELISpot responses to MHC class-I restricted epitopes. Encouragingly, only one of 13 vaccinated subjects experienced viral "blips" (defined as temporarily detectable viremia) during follow-up, as compared to three of five placebo vaccinees. This Phase I placebo-controlled study, provided crucial evidence of the therapeutic potential of DNA vaccines for HIV, and more broadly underscored the growing evidence that vaccines capable of augmenting HIV-specific CD8$^+$ T-cells could hinder viral replication.

Overall, the modest success of un-adjuvanted intramuscular injections of therapeutic HIV DNA vaccines prompted the field to pursue the use of novel adjuvants. Adjuvants are a widely-used strategy to improve vaccine immunogenicity. Of particular interest, are cytokines that enhance CTL function, such as IL-12 which is a potent stimulator of NK cells[261] and CTL maturation[262], as well as IL-15 which supports the persistence of virus-specific memory CD8$^+$ T-cells[263]. Promisingly, genetic cytokine based adjuvants such as IL-12[264] and IL-15[265] showed enhanced DNA vaccine immunogenicity in mouse models.

Building on this work, in 2008 Halwani *et al* published the immunogenicity of a SIV DNA vaccine, with molecular adjuvants IL-12 and/or IL-15, in acutely SIV infected macaques on cART (PMPA & FTC)[266]. Interestingly, they demonstrated that an IL-12 genetic adjuvant primarily stimulated a SIV-specific CD8^{+} effector memory T-cell (T_{EM}) response, that was further boosted by a SIV-DNA IL-15 adjuvanted vaccine[266].

In a separate study, von Gegerfelt *et al* demonstrated that DNA vaccines ± IL-12 and IL15 adjuvants, administered to chronically SIV infected, cART treated macaques, could enhance immunity and reduce viremia after an ATI[267]. However, von Gegerfelt *et al* did not demonstrate an enhanced ability to control viremia with inclusion of IL-12 or IL-15 adjuvants in this study, likely as a result of the small number of animals receiving cytokine DNA [267].

In addition to adjuvants, other efforts have focused on antigen design to elicit broader responses to inhibit a wider variety of viral variants. Towards this end, Vardas *et al* 2012 tested a multi-HIV antigen consisting of DNA encoding HIV subtype B derived Rev, Nef, Tat, parts of Gag, and a string of helper T-cell (Th) and CTL epitopes, in chronically HIV infected, cART naïve, and clade C infected subjects, in a Phase II placebo-controlled trial in South Africa[268]. Although subtype C infected subjects received a clade B derived vaccine, they did demonstrate enhanced HIV-specific CD4^{+} and CD8^{+} T-cell responses, and a slight viral load decrease in subjects receiving intramuscular DNA vaccination. These results suggest the vaccine may have elicited responses to conserved epitopes thus stimulating cross-protective immunity. Interestingly, while Vardas *et al* administered vaccinations both intramuscularly (IM) and intradermally (ID), they only observed a >0.5 log decrease in plasma viral RNA in 40% of vaccinated subjects in the IM cohort with HLA B*57, B*8101, and B*5801 haplotypes, yet not in the ID vaccinated subjects, although the mechanisms for this disparate outcome are not yet known.

In another Phase II study (PEDVAC), Palma *et al* 2013 tested a multigene, multiclade HIV DNA vaccine in the first study of a therapeutic HIV DNA vaccine in vertically infected children[269]. The PEDVAC vaccine was shown to be safe and developed modest increases in HIV-specific immune responses, primarily in Gag-specific CD8^{+} T-cells releasing perforin; however, the anti-viral potency of these responses was not assessed in this study.

Taken together, injection of DNA vaccines expressing multi-antigens have demonstrated moderate immunogenicity[269], and in one trial, a modest viral load decrease[268]. These results encourage the further development of immunogens designed to elicit immune responses capable of inhibiting a broad repertoire of viral variants.

Overall, compelling improvements have been made for the syringe injection of DNA since the initial studies in the 1990's and mid 2000's. However, a substantial focus of the field is on improving DNA vaccine delivery to the immunocompetent skin and/or physical methods for increasing the uptake of DNA by antigen presenting cells.

BIOJECTOR

The Biojector is a needle free device that uses high pressure to force a liquid directly into the skin or muscle [270,271]. Encouragingly, the Biojector has demonstrated safety and efficacy for the delivery of enfuvirtide, the only ART medication that must be injected [272,273], yet this delivery method has not been approved for enfuvirtide use in the United States. However, several DNA vaccines have shown safety and immunogenicity, including for prophylactic HIV vaccines in humans[274], delivered intramuscularly and intradermally by the Biojector animal models[275,276].

The vast genetic diversity of HIV-1 poses a challenge for choosing the appropriate antigen, to address this problem in 2006 Graham *et al* performed the first phase I dose-escalation study of a multi-clade DNA vaccine, delivered into the muscle using a Biojector 2000^{TM} [277]. Graham *et al* utilized a mixture of four plasmids expressing subtype B *gag/pol/nef* fusion gene, as well as subtypes A, B, and C *env*, delivered in 1 ml injections via Biojector at concentrations ranging from 2-8 milligrams of DNA, for a total of 3 doses. There were no severe adverse events and reports of pain were similar between vaccine and placebo groups. Interestingly, Env-specific $CD4^+$ and $CD8^+$ T-cells were significantly increased after the third vaccination, but not to other HIV antigens, potentially due to immune interference between the vaccine plasmids. This study represented an important step to design more complex immunogens, with the goal of generating cross-reactive HIV-specific T-cell responses.

Many therapeutic vaccines were tested in humans either not receiving cART, or who initiated cART during chronic HIV infection, thus potentially limiting their ability to respond to therapeutic immunization. One hypothesis to improve therapeutic DNA vaccine immunogenicity, was to enroll subjects who initiated cART early during infection, who theoretically had a better preserved immune system and would be more likely to respond to vaccination. To address this question, Rosenberg *et al* 2010 conducted a phase I/II study on people who initiated ART early/acute during HIV infection[278], using a four plasmid mixture as previously utilized by Graham et al[277]. This plasmid mixture was delivered by Biojector at a dose of 4 mg of DNA, for a total of four vaccinations. Cellular immune responses were poorly induced by vaccination as assessed by ELISpot and a lymphoproliferation assay. As expected from the anemic immune responses, there were no differences in viral load between vaccinees and placebos during the ATI.

Graham *et al* and Rosenberg *et al* are the only two studies evaluating the use of the Biojector for therapeutic HIV DNA vaccines to our knowledge[277,278]. It should be noted, that both trials utilized un-adjuvanted DNA vaccines, which typically only induce weak immune responses. Additional studies utilizing potent adjuvants would need to be pursued to fully evaluate the Biojector as a potential platform for therapeutic DNA vaccine delivery.

DERMAVIR

DermaVir was designed to improve DNA vaccine immunogenicity by directly delivering DNA to the immunocompentent skin[279]. For DermaVir, DNA encoding most of the viral structural and regulator genes are formulated on a patch, and then applied to exfoliated skin, to allow uptake of DNA by epidermal Langerhans cells. After the uptake of plasmid DNA, Langerhans' cells mature to dendritic cells, traffic to the draining lymph nodes, and stimulate naïve T-cells into CTL.

DermaVir$_{SHIV}$ was initially tested by Lisziewicz *et al* in 2005, on chronically SIV$_{mac251}$ infected rhesus macaques with AIDS, and provided an enhanced virological and clinical benefit[280]. In this study, 0.1 mg of DNA were applied to four skin sites over axillary and inguinal skin sites. A preliminary study in swine demonstrated only temporary site reactogenicity and no obvious toxicity after two DermaVir$_{SHIV}$ vaccinations. Additionally, Lisziewicz *et al* demonstrated that immunocompromised hosts with AIDS can still respond to therapeutic immunization, and that those responses have antiviral potency[280].

After showing promise in macaques, Lisziewicz *et al* conducted the GIHU004 phase I single-arm dose-escalation study of DermaVir in HIV infected people on cART, published in 2012[281]. People were vaccinated with doses of Dermavir containing either 0.1, 0.4, or 0.8 mg of DNA administered in a single dose to the upper back and upper thigh. Consistent with swine and macaque studies[280], no serious adverse events were reported at any dose, and only mild temporary skin irritation was observed in one subject. While subjects remained on cART, no viral blipping was observed, suggesting vaccination did not alter latency. Interestingly, the medium dose cohort (0.4 mg of DNA) had the most robust cellular immunity compared to placebo, low (0.1 mg of DNA) and high dose (0.8 mg of DNA) cohorts. However, these responses HIV-specific T-cell responses waned after a 48-week follow up. Although, these responses lacked robust persistence, immunogenicity of un-adjuvanted DermaVir vaccine in HIV infected subjects is an exciting advance for this technology.

Generally speaking, T-cell based vaccines primarily aim to elicit HIV-specific CD8$^+$ T-cell responses and limit activation of HIV-specific CD4$^+$ T-cells because they can drive infection. However, few studies have included immunomodulatory agents that could dampen vaccine induced CD4$^+$ T-cell activation. To address this, Gudmundsdotter *et al* 2011, co-administered a DermaPrep delivered DNA vaccine alone or in combination with hydroxyurea (HU), with the intent of limiting CD4$^+$ T-cell activation, in HIV infected subjects on cART[282]. Notably, these DNA vaccinations could form virus-like particles that may enhance immunogenicity. The HIV-DNA, but not the HIV-DNA + hydroxyurea, group had significantly greater HIV specific ELISpot responses over the placebo recipients. The enhanced HIV-DNA vaccine immunogenicity did not result in reduced viral replication during multiple ATI's in either vaccine group. While HU did not have the desired effect, this study is unique for combining an immunomodulatory agent with therapeutic HIV-DNA vaccination and represents an underexplored area in the field.

In the most recent DermaVir trial, Rodriguez *et al* 2013 conducted a placebo controlled dose-escalation study in HIV infected men and women on cART [283]. Similar to Lisziewicz *et al* 2012, the doses tested were 0.1, 0.4, and 0.8 mg of DNA with the placebo group receiving glucose. Consistent with the previous studies of DermaVir, no subjects experienced severe adverse reactions or developed anti-DNA antibodies. Also, consistent with Lisziewicz *et al* 2012, this study found the most potent increase in Gag-specific ELISpot responses in the medium dose (0.4 mg of DNA) cohort. The authors speculate the poor immunogenicity of the highest dose could be the result of "high-dose antigen-induced hyporesponsiveness", although additional mechanistic studies will likely be required to confirm this hypothesis. The nature of the longer-term incubation ELISpot utilized in this study detects central memory (T_{CM}) cells, which could have been elicited due to the repeated doses of Dermavir. The potential elicitation of long lived HIV-specific T_{CM}, could represent a significant advancement over the shorter-lived responses reported by Lisziewicz *et al* 2012[281].

In short, there have been substantial improvements in Dermavir from Lisziewicz *et al* 2005[280] to Rodriguez *et al* 2013[283]; however, more substantial innovations beyond dosage and number of vaccinations will likely be required for this approach to become clinically efficacious.

ELECTROPORATION

One hypothesis regarding the modest immunogenicity of DNA vaccines, delivered via injection, is the reliance on inefficient cellular processes for the uptake of DNA. A potential solution to this problem is the use of electric fields, which have been used for decades to increase cellular uptake of DNA *in vitro*. To determine if cellular uptake of DNA was a limiting factor of DNA vaccine immunogenicity, Widera *et al* demonstrated that electric fields enhanced DNA vaccine immunogenicity to various antigens in mice[284]. Shortly thereafter, several groups demonstrated the enhanced immunogenicity of DNA vaccines delivered by electroporation, including for an HIV vaccine[285].

HIV/SIV induces immune dysfunction, in part due to the decreasing in $CD4^+$ T-cells, that may interfere with therapeutic vaccine immunogenicity. To specifically address if *in vivo* electroporation, as well as IL-2 administration, would enhance the immunogenicity and efficacy of therapeutic SIV DNA vaccines, zur Megede *et al* 2008 utilized SIVmac239 infected rhesus macaques treated with cART (PMPA, FTC) [286].

$Gag_{181-189}$-specific $CD8^+$ T-cell responses significantly increased in vaccinated $MamuA01^+$ macaques, and IL-2 administration increased $CD4^+$ T-cell counts compared to controls, yet these responses did not enhance control of viremia. While IL-2 may have increased $CD4^+$ T-cell counts generally, it can potently increase *FOXP3* expression in T-regulatory cells ($CD4^+CD25^+$), which may have blunted vaccine immunogenicity[287].

While zur Megede et al 2008 utilized a cytokine to attempt to enhance vaccine efficacy, IL-2 is a rather blunt instrument, and likely induced a myriad of un-intended effects, in addition to a possible expansion of T-regulatory cells, that inhibited vaccine efficacy. As the understanding of cytokine based adjuvants became more refined, more avenues became available to more precisely enhance the desired cellular responses. For example, Valentin *et al* 2010 conducted a single arm study in SIVmac251 infected macaques on cART (PMPA,FTC, and ddI) with a electroporated DNA vaccine along with the genetic adjuvants IL-15/IL-15 receptor alpha, with the goal of eliciting robust and persistent T-cell responses[288]. The DNA vaccines consisted of 1 mg of DNA injected intramuscularly in the left and right things, followed by an *in vivo* electroporation using the CELLECTRA® device. Interestingly, they found enhanced Gag, Pol, and Nef-specific CD4$^+$ T-cell responses, whereas as CD8$^+$ T-cell responses were primarily Env-specific. These cellular responses were associated with a ~1 log reduction in viremia, suggesting cytokine adjuvants can enhance the immunogenicity and efficacy of therapeutic HIV DNA vaccines. Although this was a single arm study with small numbers of animals (n=3), it provided evidence that genetic cytokine adjuvants in conjunction with DNA vaccines via electroporation, may have a therapeutic benefit to HIV infected people.

In addition to IL-15, IL-12 represents another widely-used cytokine adjuvant for vaccines due to its ability to promote naïve cell differentiation to CTL, and was shown to be immunogenic by Halwani *et al* 2008 for intramuscular injection of DNA vaccines[266]. To determine if IL-12 could similarly benefit electroporation, Jacobson *et al* 2016 evaluated a multiantigen therapeutic HIV DNA vaccine co-administered with increasing doses of a molecular IL-12 adjuvant, in a phase I placebo controlled study of HIV infected cART treated humans[289]. Interestingly, The HIV DNA vaccine co-delivered with a low dose of IL-12 resulted in the most profound increase in HIV-specific CD4$^+$, but not CD8$^+$, T-cells compared to placebo subjects. This CD4 bias is in partial agreement with Valentin et al 2010[288], however the inability to induce CD8 T-cell responses could be due to differences in the immunogen, adjuvant, or species. While the tolerability, safety and immunogenicity of this vaccine are important milestones, the inability to induce HIV-specific CD8$^+$ T-cells with a cytolytic phenotype may limit the therapeutic potential. Further studies will be required to more completely understand the immunological mechanisms favoring a CD4$^+$, over a CD8$^+$, T-cell response described in Jacobson *et al* 2016[289].

To summarize, while the advancements in electroporation are encouraging, improvements such as enhancing cellular uptake of plasmid DNA, incorporation of intradermal electroporation[290], as well as the inclusion of more potent adjuvants may be required to demonstrate improved immunogenicity and anti-viral efficacy in humans.

GENE GUN

Particle mediated epidermal delivery (PMED or gene gun) works by delivering plasmid DNA coated gold particles into the epidermis, resulting in *de novo* synthesis of antigen. In the early 1990's gene gun delivery of DNA vaccines was shown to be immunogenic in mice[291,292]. The precise mechanisms explaining the ability of gene gun to elicit potent cellular and humoral immunity are not entirely clear. Langerhans cells have been implicated in the immunogenicity of gene gun DNA vaccines because of their ability to persist in the epidermis as well as traffic to lymphoid tissue[293,294]; however, other studies have suggested they are not necessary for gene gun immunogenicity[295]. Importantly, our laboratory demonstrated immunogenicity and efficacy of prophylactic influenza vaccines in non-human primates[296,297]. Specially, we found that the gene gun induced potent T-cellular immune responses localized to the lung mucosa, the nidus of influenza infection, and may explain the robust blunting of viral replication following a heterologous influenza challenge[297]. Our promising results are not only restricted to animal models, we were the first to demonstrate gene gun delivered DNA vaccine inducing antibody and T-cell responses in humans[47]. Given the promising immunogenicity and efficacy of gene gun delivered DNA vaccines in animal models and humans, we have pursued the development of novel vaccine therapies for the treatment of HIV.

A major hurdle for the development of an effective HIV vaccine is the predilection for the virus to mutate and escape immune responses. In an effort to expand SIV-specific CD8$^+$ T-cell breadth and limit CTL escape, Fuller *et al* 2006 tested a DNA vaccine consisting of 19 CD8$^+$ T-cell epitopes that were inserted in the hepatitis B core antigen (HBcAg) to improve immunogenicity in SIVB670 infected macaques on ART (PMPA) two weeks after infection[298]. Towards that end, we demonstrated that DNA vaccination increased SIV-specific CTL breadth in macaques that responded to ART, and 17/20 of the vaccinated macaques controlled viremia for 7 months after ART was withdrawn, as compared to 0/4 placebo vaccinated animals.

Impressively, the study demonstrated robust immunogenicity and antiviral efficacy in the absence of genetic adjuvants. This study is the first to demonstrate the therapeutic efficacy of a therapeutic DNA vaccine for a pathogenic AIDS virus delivered by gene gun.

In our previous therapeutic vaccine study Fuller *et al* 2006, demonstrated an un-adjuvanted vaccine demonstrated immunogenicity and efficacy. Notably, the gut mucosa represents an important anatomical reservoir of HIV infected cells, but vaccine strategies designed to elicit mucosal immunity were limited. To improve on our previous results, Fuller *et al* 2012 tested a SIV DNA vaccine co-formulated with the genetic mucosal adjuvant the heat labile *E. coli* enterotoxin (LT) delivered for its ability to enhance SIV-specific immunity, particularly in the gut mucosa, of SIVB670 infected macaques on ART (PMPA, FTC)[48]. Importantly, the correlate of this protection was broad T-cell immune responses in the gut, whereas the breadth between controllers and non-controllers was similar in the blood. Our results indicate that the specificity of the response, as opposed to the magnitude, may be a more important factor for containment of the virus in the mucosa.

Our laboratory has made exciting advancements in gene gun DNA vaccines generally, and therapeutic vaccines specifically. To build on these results, the Fuller laboratory, in collaboration with Orlance, Inc., seek to develop a more clinic friendly version of the gene gun research device, as well as to design immunogens eliciting cross-protective immune responses for variable pathogens.

COMPLEMENTARY APPROACHES

While therapeutic HIV DNA vaccines have largely been shown as safe and often immunogenic, profound reduction of viremia in humans remains an elusive goal. These DNA vaccines have largely been tested in isolation from other cure-based strategies.

Some of these failures may be due, in part, to deficiencies in the immune system of humans and macaques that DNA vaccines alone cannot fully address. In this section, we will review select cure strategies that may be particularly well-suited complement DNA vaccination.

A weakness of adaptive immune responses is they require that HIV infected cells produce viral proteins to render them susceptible to immune clearance. An imposing barrier to an HIV cure is the pool of long lived latently infected cells that do not produce viral proteins thus making them impervious to immune clearance[7,37,299]. The primary goal of latency reversal agents (LRAs) is to induce transcription of proviral DNA in latently infected CD4$^+$ T-cells to make viral proteins. This causes death of the cell by viral cytopathic effects or rendering the cell susceptible to CD8 T-cell or antibody dependent cellular cytotoxicity (ADCC) clearance, sometimes referred to as the "shock and kill" approach. A number of latency reversal agents (LRA's) have been tested in clinical trials[300–307] with some resulting in increased plasma HIV RNA[304–307]. On their own, LRA's are modest at reducing the size of the HIV reservoir, due to HIV induced impairment of CD8 T-cell function and/or the preponderance of CD8 escape mutations in the latent reservoir[10,308]. However, an LRA given in combination with a therapeutic DNA vaccine able to direct the immune responses to non-mutated CD8 epitopes present in the latent reservoir may have a more dramatic reduction in the viral reservoir. This approach has had antiviral efficacy in two studies[246,309].

α-PD-1/ α-PD-L1 antibodies are potent immunotherapies that can block PD-1 inhibition of cellular immune responses and boost immunity. In support of this concept, α-PD-1 antibodies have shown enormous promise in treating human cancers, albeit with 41% of patients experiencing adverse events[80]. In the SIV/macaque model, a therapeutic infusion α-PD-1 antibodies improved cellular and humoral immunity, decreased viremia, and prolonged survival[77]. In combination with a vaccine, PD-1 blockade in rhesus macaques improved responsiveness to prophylactic recombinant adenovirus vaccine[81]. However, PD-1 blockade in chronically SIV infected rhesus macaques transiently increased viremia before returning to pre-infusion levels[81]. The lack of antiviral efficacy observed by Finnefrock *et al* may have been due to α-PD-1 treatment increasing immune responses directed at immunodominant epitopes that the virus had already mutated.

DISCUSSION:

Overall the progress towards a therapeutic DNA vaccine for HIV has been modest (**Figure 3**). To date, the most potent therapeutic HIV vaccine in human relied on incubating dendritic cells *ex vivo* with heat inactivated autologous virus Encouragingly, vaccine candidates tested in the macaque model exhibit potent control or even functional cures[310], however these results have not yet translated to humans. Improvements in therapeutic vaccines will likely focus on: **1)** improvements in immunogen design, **2)** novel and/or combinational adjuvants, and **3)** new delivery methods

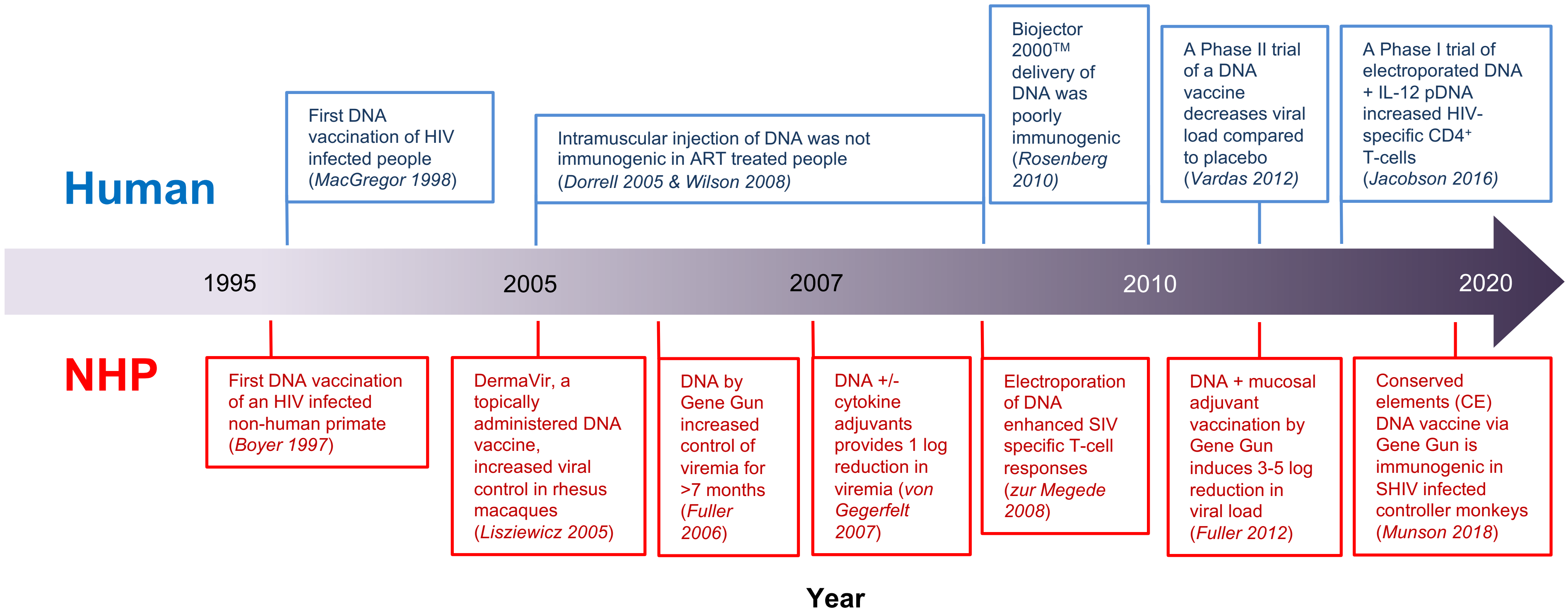


Figure 1.6. Progress towards a therapeutic HIV DNA vaccine. This timeline represents a selection of noteworthy HIV/SIV therapeutic vaccines in humans (*blue*) and non-human primates (NHP, *red*). Each box contains a short summary of the novelty and findings and the year the paper was published.

Novel DNA vaccines may need to induce HIV-specific immune responses to conserved and functionally constrained regions of the viral proteome to provide a sustained block from viral rebound following cessation of cART[104]. Infected cells display immunodominant variable epitopes as well as subdominant conserved MHC-I restricted epitopes (**Figure 1.4A**). CTL escape mutations may render infected cells resistant to immunodominant CTL mediated clearance, allowing for continued viral replication (**Figure 1.4B**). CTL escape mutations have been documented in acute and chronic infection for both HIV and SIV [95–99]. These findings strongly suggest that CTL escape as an important mechanism for viral persistence and pathogenesis. In contract, CE-specific CTL may better limit escape mutations and more potently recognize and kill virally infected cells (**Figure 4C**). Standard immunogens have primarily consisted of full length, or near full length genes, based on consensus sequences. These immunogens tend to induce responses against immunodominant variable regions of the proteome at the expense of targeting conserved regions[106,311] (**Figure 4D**). To improve targeting of HIV/SIV derived CE-specific immune responses, DNA immunogens consisting exclusively of CE encoding sequences have been developed, and have shown immunogenicity in uninfected mice[126] and macaques[128,134,129].

Improved HIV therapeutic vaccines will likely require adjuvants that can induce a greater magnitude and quality of CD8$^+$ T-cells which are capable of trafficking to critical anatomical reservoirs, such as mucosal and lymph node tissue. Relatively few adjuvants have been tested in the context of therapeutic HIV vaccines including alum, IL-12, IL-15, and heat labile *E. coli* enterotoxin (LT)[48,89,145,266,312]. However, a large number of adjuvants tested in therapeutic cancer vaccine studies could serve as a guide for improving HIV vaccines, such as a TLR3 adjuvant that has demonstrated increasing the potency of NK's and CTL's[313–315]. Also the use of immunomodulators, such as anti-PD1/PDL1 antibodies, could reverse HIV associated immune dysfunction and improve therapeutic vaccine immunogenicity[66,71,72,316]. In support of this concept, co-administration of immune checkpoint inhibitors with a vaccine has improved efficacy of cancer vaccines[317–319].

Novel delivery methods may also be needed to increase vaccine immunogenicity. Early approaches with DNA vaccines were hindered by delivering DNA to the muscle, which sparsely contains antigen presenting cells (APC's). New DNA delivery technologies such as the Biojector, Dermavir, and gene gun have been primarily aimed at delivering DNA into the immunocompetent dermis or epidermis (**Figure 3)**. Tat protein vaccines delivered subcutaneously while immunogenic did not decrease viral load[146,147], but Tat vaccines delivered intradermally (ID) were shown to decrease viral load or viral DNA[148,150]. While the mechanism for this improved clinical outcome is unknown, it may be due to delivery into tissues richer in APC's and/or stimulating a more robust innate immune response.

There are significant opportunities for improving HIV therapeutic vaccines as well as combining them with other non-vaccine approaches. The SIV/macaque model plays an important for the better understanding HIV pathogenesis and evaluating cure approaches. This model is a uniquely suited for the HIV cure effort given its thorough characterization and recapitulating several important features of HIV infection including $CD4^+$ T-cell depletion in the gut, making it a particular important model for mucosal vaccines[320,321]. Finally, our success toward a functional cure for HIV will require greater insight into HIV pathogenesis including inflammation, dysfunction, and immune escape.

CHAPTER 2

Therapeutic conserved elements (CE) DNA vaccine induces strong T-cell responses against highly conserved viral sequences during simian-human immunodeficiency virus infection

“The beginning is the most important part of the work.” - Plato

Therapeutic conserved elements (CE) DNA vaccine induces strong T-cell responses against highly conserved viral sequences during simian-human immunodeficiency virus infection

Running Title: Therapeutic conserved elements DNA vaccination in macaques

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Abstract:

HIV-specific T-cell responses play a key role in controlling HIV infection, and therapeutic vaccines for HIV that aim to improve viral control will likely need to improve on the T-cell responses induced by infection. However, in the setting of chronic infection, an effective therapeutic vaccine must overcome the enormous viral genetic diversity and the presence of pre-existing T-cell responses that are biased toward immunodominant T-cell epitopes that can readily mutate to evade host immunity and thus potentially provide inferior protection. To address these issues, we investigated a novel, epidermally administered DNA vaccine expressing SIV capsid ($p27^{Gag}$) homologues of highly conserved elements (CE) of the HIV proteome in macaques experiencing chronic but controlled SHIV infection. We assessed the ability to boost or induce de novo T-cell responses against the conserved but immunologically subdominant CE epitopes. Two groups of animals were immunized with either the CE DNA vaccine or a full-length SIV $p57^{gag}$ DNA vaccine. Prior to vaccination, CE responses were similar in both groups. The full-length $p57^{gag}$ DNA vaccine, which contains the CE, increased overall Gag-specific responses but did not increase CE responses in any animals (0/4). In contrast, the CE DNA vaccine increased CE responses in all (4/4) vaccinated macaques. In SIV infected but unvaccinated macaques, those that developed stronger CE-specific responses during acute infection exhibited lower viral loads. We conclude that CE DNA vaccination can re-direct the immunodominance hierarchy towards CE in the setting of attenuated chronic infection and that induction of these responses by therapeutic vaccination may improve immune control of HIV.

Key Words: HIV, SIV, SHIV, therapeutic vaccination, conserved elements vaccine, DNA vaccines, gag

Abbreviations: ART, antiretroviral therapy; CE, conserved elements; FL, full length; LT, heat labile *E. coli* enterotoxin

Introduction:

Antiretroviral therapy (ART) has a tremendous capacity to suppress HIV replication and prolong the life of HIV-infected persons. However, an effective ART regimen requires a combination of antivirals that are costly, cannot completely reverse immune dysfunction, can lead to the emergence of drug resistant viral variants, and cannot eradicate latently infected cells [30,322,323]. In addition, ART can have adverse side effects that can exacerbate non-compliance. Cessation of therapy typically results in viral rebound, thus requiring a lifetime of treatment [324–328]. Several additional approaches are being developed for administration in combination with ART, including gene therapies, latency reversal agents, and vaccines to boost antiviral immunity. These efforts seek to eradicate the residual population of cells harboring infectious proviruses, or achieve sustained viral remission, characterized by a protracted period of undetectable viremia after stopping ART [266,267,288,300,329–331].

Therapeutic vaccines offer a relatively inexpensive and widely applicable means to potentially contribute to or achieve these goals. A particular focus of therapeutic vaccines is to induce strong HIV-specific CD8$^+$ cytotoxic T-lymphocyte (CTL) responses that can control viremia and eliminate residual cells expressing virus and viral antigens [55,332,333]. In support of this possibility, several therapeutic vaccines have been shown to augment virus-specific CTLs and in some cases, reduce viral loads in the plasma and tissues of rhesus macaques chronically infected with SIV [135,226,231,334]. We previously showed that an SIV therapeutic DNA vaccine delivered by particle mediated epidermal delivery (PMED) into the skin of SIV-infected macaques receiving suboptimal ART provided a substantial, 3-5 log reduction in viral load and induced durable control (> 1 year) of viremia in approximately half of vaccinated animals [48].

The vaccine employed in our previous therapeutic vaccine study consisted of full-length (FL) SIV immunogens, and although it broadened the mucosal CD8$^+$ T-cell response, these responses were mostly directed against immunodominant epitopes rather than subdominant conserved regions [10,106,335]. A potential concern with the use of FL immunogens is that HIV diversity is so enormous and pathways to escape mutations are so varied that immunogens driving induction of immunodominant responses against variable epitopes, even if directed against multiple epitopes, are unlikely to provide a sustained block against the wide range of immune escape pathways [336,337].

To address these concerns, we designed a conserved elements (CE) DNA vaccine [126–129], that exclusively expresses CE sequences as a strategy to direct CD8$^+$ T-cells to regions of the proteome that are highly conserved among viruses, where mutations are more likely to decrease viral fitness, and are associated with viremic control [56]. This approach was also developed based on findings showing that CD8$^+$ T-cells targeting conserved regions, particularly within Gag, were associated with lower viral load, whereas responses to other, more variable regions were associated with high viral loads [56,57,107]; and that CD8$^+$ T-cell responses to conserved epitopes were preferentially detected in elite controllers and long term non-progressors [338–340,333,341,332,342]. However, responses associated with viral control are typically subdominant in natural infections [56,106,108,343], with exceptions including individuals with "protective" HLA alleles, who tend to target epitopes whose escape results in a loss of viral fitness [344]. We hypothesize that a therapeutic vaccine that can direct immune responses to CE will be more effective at inducing durable viral remission by inhibiting a wider variety of viral variants, limiting escape mutations, and more often reducing viral fitness if escape occurs. In support of this concept, we previously demonstrated that prophylactic administration of DNA vaccines expressing CE from HIV and SIV Gag can induce stronger CE-specific responses in mice and uninfected rhesus macaques, compared to vaccinating with a FL HIV Gag immunogen [126,128,129]. Interestingly, in these studies, priming with the CE Gag DNA vaccine (hereafter referred to as the CE vaccine) and then boosting with CE plus FL Gag DNA was much more effective at generating CE-specific responses than priming and boosting with the CE vaccine alone [126,128,129]. However, we also found that priming T-cells with a FL Gag DNA substantially reduced recognition of CE epitopes when followed by a subsequent boost with CE [128], suggesting that it may not be possible to redirect immunodominance by therapeutic vaccination during ongoing infection. To experimentally address this, we conducted a pilot study to determine if immunizing SIV-HIV hybrid (SHIV) virus infected rhesus macaques with a CE DNA vaccine could redirect a pre-existing immunodominant SIV-specific T-cell repertoire toward greater recognition of CE epitopes. The CE DNA vaccine significantly increased T-cell responses against subdominant CE epitopes in macaques with attenuated infection, demonstrating the feasibility of using therapeutic immunization to redirect the virus-primed T-cell repertoire toward greater targeting of CE. Furthermore, in a separate study, rhesus macaques that naturally developed CE responses during acute SIV infection were found to exhibit lower viral set points when compared to those that did not develop detectable CE responses, supporting the concept that increasing CE responses during therapy may improve control of HIV.

Materials and Methods:

Ethics statement and animal care. Sixteen Indian origin, adult rhesus macaques were used for these studies. The Washington National Primate Research Center (WaNPRC) is responsible for the humane care of laboratory animals. The WaNPRC is accredited by the American Association for the Accreditation of Laboratory Animal Care International (AAALAC). The University of Washington’s Institutional Animal Care and Use Committee approved all experiments in these macaques. Animals were singly housed in comfortable, clean, adequately-sized cage. Cages, racks, and accessories were sanitized in mechanical cage washers at least once every two weeks and waste pans were cleaned daily. Temperatures in animal quarters were maintained between 72-82°F. Animals were fed a commercial monkey chow, supplemented daily with fruits and vegetables, and drinking water was available at all times provided by automatic watering devices. Throughout the study, animals were checked twice daily by the veterinary technicians to evaluate their physical and clinical condition. The macaques received environmental enrichment for the duration of the study including grooming contact, perches, toys, foraging experiences and access to additional environment enrichment devices. For the CE vaccination study, eight adult Indian origin rhesus macaques previously infected with SHIV89.6P [345] were reassigned to this project after completion of a separate, previous vaccine experiment consisting of a recombinant adenoviral vaccine prime (expressing either HIV_{IIIB} *tat* [346], SIV_{239} *gag* [347], $\text{HIV}_{89.6\text{P}}$ *gp140* and/or SIV_{239} *nef* [348]) and a Tat, gp140, or Nef protein boost performed under the NIH/NIAID Simian Vaccine Evaluation Unit Program (Dr. Nancy Miller, Program Officer, NIH; Dr. Ruth Ruprecht, Principal Investigator). For the acute SIV infection study, eight adult Indian origin macaques were challenged with SIVΔB670 (kind gift, Dr. Michael Murphey-Corb, University of Pittsburgh) intravenously using a previously established protocol [48]. Cryopreserved SIVΔB670 was thawed and diluted in RPMI, and then 1 mL of RPMI containing 100 TCID_{50} was injected into the saphenous vein of each macaque. Macaques were MHC-I typed for *Mamu* alleles (A*01, A*02, A*08, A*11, B*01, B*08, B*17, and B*29) by PCR as previously described [349–351].

All biopsies were performed under ketamine (10 mg/kg) or Telazol (2.5-10 mg/kg) anesthesia and any continued discomfort or pain was alleviated at the discretion of veterinary staff. Euthanasia prior to necropsy was performed on the SHIV infected animals by administration of Euthasol® (Virbac Corp., Houston, TX) while the animal was under deep anesthesia in accordance with guidelines established by the 2007 American Veterinary Medical Association Guidelines on Euthanasia. None of the animals became severely ill during the course of the study and none required euthanasia prior to their experimental endpoint.

Particle mediated epidermal delivery (PMED) of CE and FL DNA vaccines. We designed an SIV Gag CE DNA vaccine [129] corresponding to sequences homologous to the previously described HIV Gag CE DNA vaccine [126] to test the CE vaccine concept in rhesus macaques infected with SHIV89.6P. The SIV Gag CE DNA vaccine consists of two plasmids, each with a

CMV promoter, a granulocyte macrophage-colony stimulating factor (GM-CSF) signal peptide to promote extracellular secretion, followed by a string of seven sequences encoding conserved elements of the viral proteome ranging from 12-24 amino acids in length separated by linker sequences designed for optimal immune-proteasome cleavage, and finally a bovine growth hormone polyadenylation signal (**Figure 2.1A**) [352,353]. To avoid a strongly hydrophobic amino terminus, which may interfere with intracellular trafficking, the CE1 sequence was placed at the C terminus [126]. To enhance coverage, the two plasmids corresponded to alternate versions of CE differing only by "toggled" amino acids. CE2, CE3, and CE5 contain a single toggle amino acid site. CE4 contains 2 additional toggled amino acid sites since those variants were always associated with the primary toggle site in the Los Alamos HIV database [104]. CE1, CE6, and CE7 do not contain a toggle site due to their high conservation among the available SIV sequences. The final SIV Gag CE DNA vaccine consisted of equal amounts of the toggled plasmids (p27CE1 and p27CE2). The FL DNA vaccine consisted of $p57^{Gag}$ from $SIV_{17E\text{-}Fr}$, which is homologous to the SHIV89.P challenge virus [354,355] and contains each of the CE. Each SIV DNA vaccine (CE and FL) was co-delivered at a 10:1 ratio along with a 3rd plasmid expressing the heat-labile *E. coli* enterotoxin (LT) as a genetic adjuvant, previously shown to significantly increase systemic and mucosal immunogenicity of PMED-delivered DNA vaccines [48,101].

To administer the vaccines by PMED, the CE, FL Gag and LT plasmid DNAs [101,129] were formulated separately onto gold particles as previously described [48]. Using the PowderJect® XR1 gene delivery device (PowderJect Vaccines, Inc.), each macaque received 32 μg of the CE or FL DNA vaccine along with 3.2 μg of the plasmid expressing the LT adjuvant administered over 16 skin sites (2 μg CE or FL DNA + 0.2 μg LT per site) along the abdomen and over the inguinal lymph nodes. Skin sites were clipped of fur and swabbed with alcohol prior to PMED administration. The two toggled SIV Gag DNA vaccine plasmids were previously shown to express similar levels of protein [129] but since they were co-delivered, differences in the immunogenicity between the plasmids were not assessed in this study.

IFN-γ ELISpot assay. This assay was performed on PBMC or on Percoll® (GE Healthcare, Catalog #: 17-0891-01) purified mononuclear cells isolated from solid tissues as described below. Cells were stimulated with SIV Gag peptide pools containing 15-mer peptides overlapping by 11 amino acids and spanning the full length SIV Gag p57 viral protein (provided by the National Institutes of Health AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases). In addition, cells were stimulated with peptide pools spanning only the CE regions, including 15-mer peptides overlapping by 11 amino acids and 10-mer peptides overlapping by 9 amino acids. Peptides were resuspended in 80% DMSO and 20% water and then diluted in cell culture media so that the final concentration of DMSO in the culture media was <1%. Peptide stimulations were performed at a final concentration of 1μg/mL. Concanavalin A (5 μg/mL, Sigma-Aldrich, Catalog #: C2272) was used as a positive control, and DMSO at a concentration equal to peptide stimulations was used as a negative control. Antigen-specific cells secreting IFN-γ were detected using anti-macaque IFN-γ monoclonal antibodies (U-Cytech-BV) as previously described [48]. Spot forming cells (SFC) were enumerated using an Immunospot Analyzer with CTL Immunospot Profession Software (Cellular Technology Ltd.). Peptide-specific SFC were determined by subtracting background SFC measured in negative control wells. Responses were considered positive if they were at least twice that of background values and a minimum of 50 SFC per million cells.

Intracellular cytokine staining. Multiparameter flow cytometry was used to determine T-cell immune responses using peptide stimulated PBMC as previously described [48]. One million PBMC were stained for each condition (DMSO, PMA/Ionomycin, or peptide(s)) for 10-14 hours. PBMC were stained with the following antibodies: Live/Dead Yellow (Invitrogen®, Catalog

#:L34959), CD3-APC (BD Biosciences, clone SP34-2, Catalog #: 551916), CD4-PerCP Cy5.5 (BD BioSciences, clone L200, Catalog #: 551980), CD8 APC-Cy7 (BD Biosciences, clone RPA-T8, Catalog #: 55760), IFNγ-BV650 (BioLegend, clone 4S.B3, Catalog #: 502537), IL-2-PE (BioLegend, clone MQ1-17H12, Catalog #: 500307), TNFα-PECy7 (BD Biosciences, clone Mab11, Catalog #: 557647), CD107a-FITC (BD Biosciences, clone H4A3, Catalog #: 555800). Cells were fixed in 1% paraformaldehyde and acquired using an LSR II flow cytometer (BD Biosciences) and the data analyzed using FlowJo software (Tree Star, Inc.). Samples were considered positive if peptide-specific responses were at least twice that of the negative control plus at least 0.01% after background subtraction.

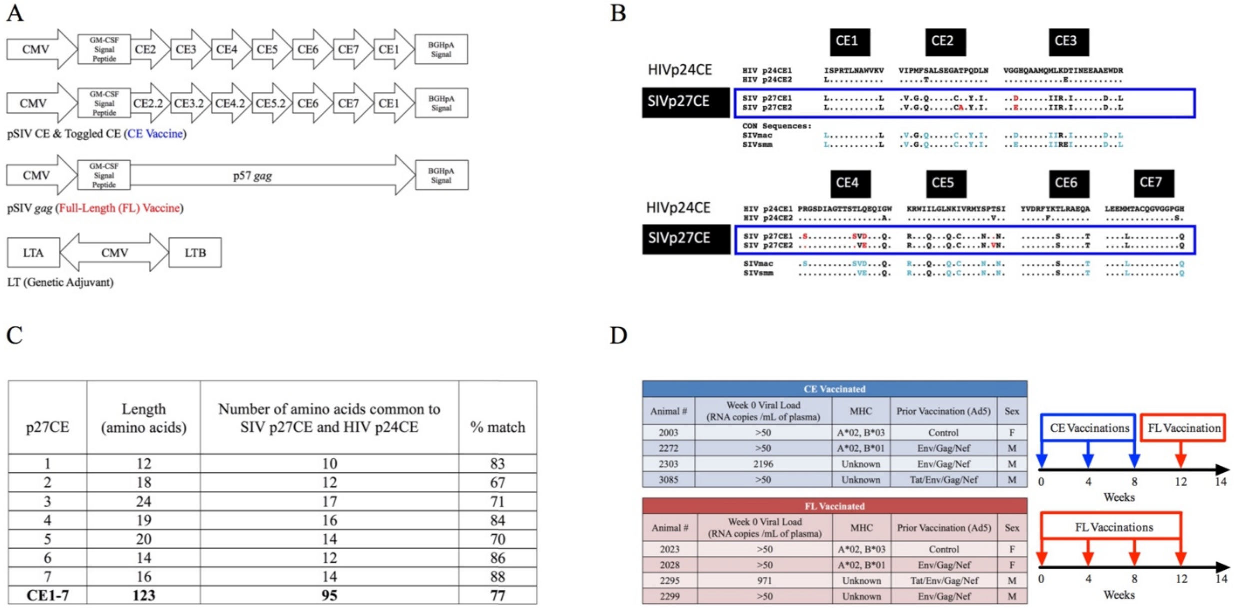
Isolation of gut-associated mononuclear lymphocytes. 15-20 cm of jejunum were removed surgically, then cut length-wise and mucous removed by gentle wiping with a paper towel. Next, fat tissue was excised and the remaining tissue was cut into small (1/3 x1/3 cm) pieces and then enzymatically digested in serum free RPMI-1640 containing L-glutamine, penicillin/streptomycin, gentamycin, DNAse (4 μg/ml; Sigma-Aldrich, Catalog #: DN25) and Liberase® (40 μg/ml; Roche Inc., Catalog #: 5401119001) at 37°C and 5% CO_2 for one hour. Tissue fragments were then crushed onto and passed through 70 μm filters and washed with R10 media. Cells were then counted on a Cellometer® Auto T4 (Nexcelom Bioscience) and cryopreserved. Jejunum and lymph nodes were collected at necropsy (Week 14). Due to limited numbers of cells isolated from the gut, only ELISpot was performed.

Plasma Viral Load Quantification. SHIV89.6P plasma viral loads were determined by nucleic acid sequence-based amplicfication (NASBA) at Advanced BioScience Laboratories Inc. (ABL) as previously described [345]. The Virology Core at the WaNPRC quantified viral RNA in the plasma for the SIVΔB670 infected animals by RT-PCR as previously described [48,345].

Statistical Analyses. Statistical differences between the two groups were calculated using a two-sided Mann-Whitney test in GraphPad Prism (Version 6, GraphPad Software). Correlations between immune responses and viral loads were determined by a Spearman's rank correlation test. $P \leq 0.05$ was considered significant for each test.

Results:

SIV CE therapeutic DNA vaccine and study design. The CE DNA vaccine expresses seven highly conserved peptide sequences derived from $p27^{Gag}$. The FL DNA vaccine expresses $p57^{Gag}$ from $SIV_{17E\text{-}Fr}$. The LT genetic adjuvant plasmid expresses both the A and B subunits of the heat-labile enterotoxin of *E. coli* (LT) (**Figure 2.1A**). The SIV p27CE sequences were selected based on alignments with available SIV sequences and homology to HIV (**Figure 2.1B**). Comparison of SIV p27 Gag CE to the HIV p24 Gag CE sequences revealed a range of 75-100% amino acid similarity per CE (**Figure 2.1C**). As predicted from their strong conservation levels, the SIV p27 Gag CE are contained within both the FL $SIV_{17E\text{-}Fr}$ p57 Gag vaccine and the SHIV89.6P challenge stock that was previously used to infect the macaques in this study [355,356]. To investigate the immunogenicity of the SIV CE DNA vaccines in the setting of attenuated chronic infection, we enrolled a group of eight rhesus macaques that had been previously immunized with either a recombinant adenovirus 5 (Ad5) vaccine expressing HIV_{IIIB} *tat,* SIV_{mac239} *gag*, $HIV_{89.6P}$ *gp140* and/or SIV_{239} *nef* or an empty Ad5 vector control [345]. All animals became infected following intravenous challenge with SHIV89.6P, were infected for nearly five years when enrolled in this study, but had attenuated infection, with viral loads ranging from undetectable to 10^4 copies/ml (**Figure 2.1D**). The eight macaques were stratified equally between the conserved elements (CE, N=4) and full-length (FL, N=4) DNA vaccination groups based on viral load, MHC, prior Ad5 vaccination, and sex (**Figure 2.1D**). The CE group received three priming doses of the CE DNA vaccine at weeks 0, 4, and 8 and one booster dose with the FL



p27CE	Length (amino acids)	Number of amino acids common to SIV p27CE and HIV p24CE	% match
1	12	10	83
2	18	12	67
3	24	17	71
4	19	16	84
5	20	14	70
6	14	12	86
7	16	14	88
CE1-7	**123**	**95**	**77**

CE Vaccinated

Animal #	Week 0 Viral Load (RNA copies /mL of plasma)	MHC	Prior Vaccination (Ad5)	Sex
2003	>50	A*02, B*03	Control	F
2272	>50	A*02, B*01	Env/Gag/Nef	M
2303	2196	Unknown	Env/Gag/Nef	M
3085	>50	Unknown	Tat/Env/Gag/Nef	M

FL Vaccinated

Animal #	Week 0 Viral Load (RNA copies /mL of plasma)	MHC	Prior Vaccination (Ad5)	Sex
2023	>50	A*02, B*03	Control	F
2028	>50	A*02, B*01	Env/Gag/Nef	F
2295	971	Unknown	Tat/Env/Gag/Nef	M
2299	>50	Unknown	Env/Gag/Nef	M

Figure 2.1. DNA vaccines and vaccine study design.

(A) *DNA vaccines.* The CE DNA vaccine consists of two plasmids encoding seven different conserved sequences connected by optimized linker sequences. CE2, CE3, CE4, and CE5 differ by toggled amino acids analogous to the HIV p24CE vaccine previously described (see Methods). The FL DNA vaccine expresses the p57 *gag* coding sequence from $p57^{Gag}$ from $SIV_{17E\text{-}Fr}$. A genetic adjuvant plasmid expressing the heat-labile *E. coli* enterotoxin (LT) was co-delivered with both DNA vaccines (CE and FL) at each dose. (B) Comparison of $p27^{Gag}$ CE in HIV and SIV strains, adapted from Hu et al[32]. SIV $p27^{Gag}$ sequences from SIVmac (macaque origin) and SIVsmm (sooty mangabey), were compared to reported HIV Gag p24 CE sequences[29]. The SIV p27CE1 and p27CE2 toggled amino acids are indicated in red. Blue amino acids indicate SIV sequences that are dissimilar from HIV sequences. (C) The number of CE amino acids (AA) that are common in both SIV and the HIV-1 M group (see panel B), including toggle AA, for each CE is shown along with percent match between the HIV-1 p24CE and the SIV homologues. (D) *Study Design.* Macaques were stratified into either the CE (conserved elements) or FL (full length) DNA vaccine groups based on plasma viral load, MHC class I genotype, prior vaccination, and sex. The CE + LT DNA vaccinated animals (blue) received three doses of the CE + LT DNA vaccine administered into the epidermis of the skin by PMED at weeks 0, 4, and 8 followed by a single boost of the FL + LT DNA vaccine at week 12. The FL DNA vaccinated animals (red) received four doses of the FL SIV Gag DNA vaccine at weeks 0, 4, 8 and 12.

DNA vaccine at week 12 (**Figure 2.1D**), a regimen that was previously shown to induce strong CE responses in uninfected macaques [128,129]. The FL vaccinated group (N=4) received four doses at the same time points. The total amount of DNA per dose was equal in both groups and each dose was co-administered with the LT genetic adjuvant by PMED.

CE DNA vaccination increases CE-specific IFN-γ T-cells in both the blood and mucosal tissues. To determine the impact of the vaccines on T-cell responses in blood, the frequency of IFN-γ responses was determined by ELISpot at week 0 and week 14, the latter corresponding to two weeks following the last vaccination, using peptide pools representing either the full-length p57^{Gag} or CE sequences alone. The magnitude of FL Gag-specific and CE-specific IFN-γ T-cell responses prior to vaccination (week 0) were similar between both groups (**Figures 2.2A & B**). Vaccinating with either the CE or FL DNA vaccine increased overall Gag-specific T–cell responses to similar levels (p = 0.49, **Figure 2.2A**). In contrast, immunization with the CE vaccine followed by one dose of the FL vaccine significantly increased the magnitude of CE-specific responses in all four vaccinated animals in the CE group, whereas immunization with the FL vaccine resulted in no increase or a decline in CE responses. Overall, significantly higher CE responses were observed in the CE vaccine group (p = 0.03, **Figure 2.2B**). Following vaccination, T-cell responses were detected against 5-7 CE in all four animals in the CE vaccine group versus only one animal in the FL group against 2 of the CE sequences (**Figure 2.2C**). Two CE vaccinated animals (2003 and 2303) developed T-cell responses against all 7 CE and all four animals responded to both CE3 and CE5. Overall, the CE vaccine induced greater magnitude, (**Figure 2.2B**, p = 0.03) and broader specificity (**Figure 2.2D**, p = 0.03) against otherwise subdominant CE-specific T-cell epitopes when compared to the FL only group. Notably, the magnitude of the overall Gag-specific T-cell response prior to vaccination (week 0) did not correlate with CE-breadth post-vaccination (week14), indicating that the levels of pre-

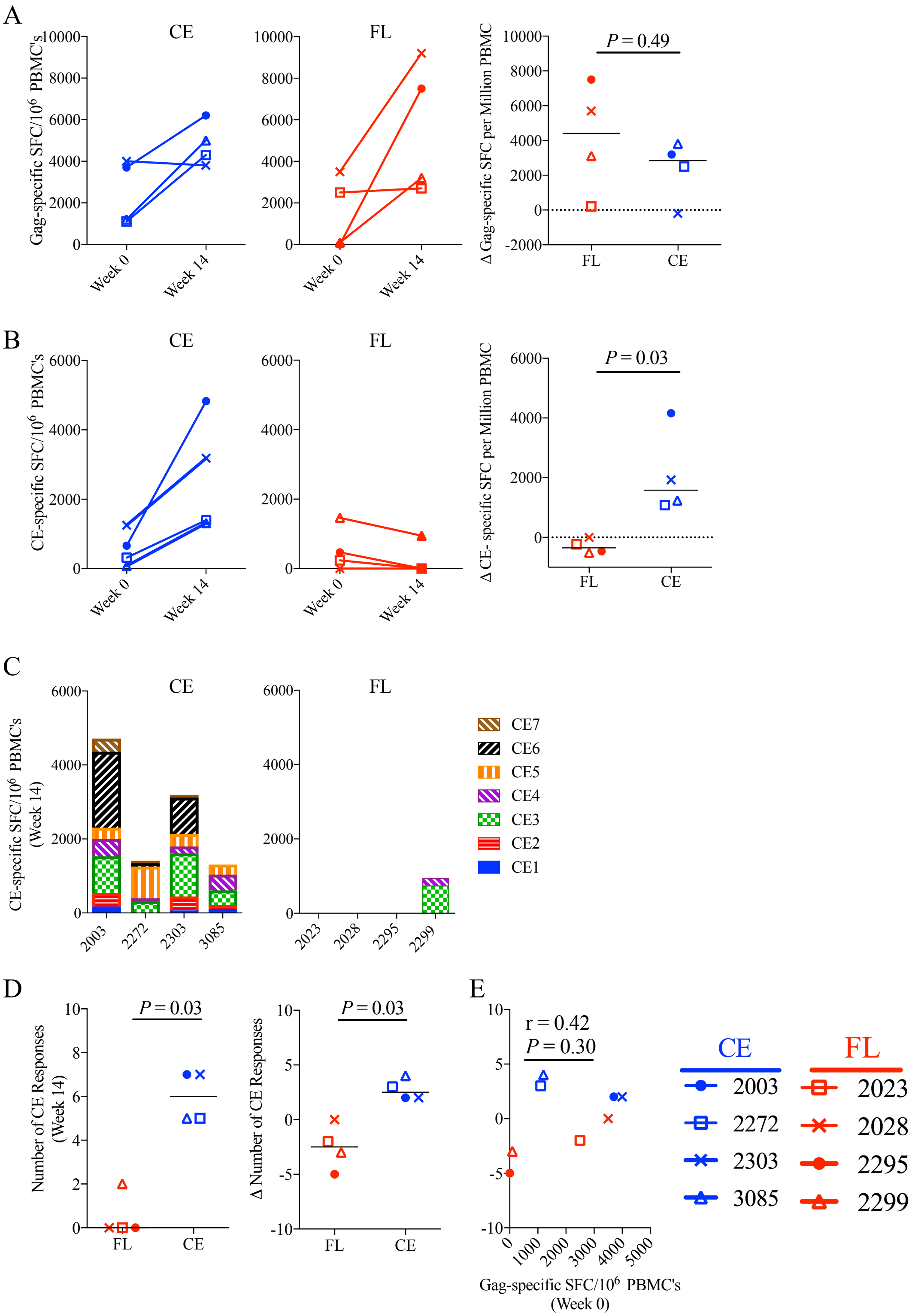


Figure 2.2: CE-specific IFN-γ T-cell responses in the blood.

T-cell responses in Spot Forming Cells (SFC) per on million PBMC were measured before and after vaccination by IFN-γ ELISpot following stimulation with SIV CE or p57 Gag peptide pools.

Shown are the mean spot forming cells (SFC) per 10^6 PBMC corresponding to (A) Gag-specific and (B) CE-specific SFC measured at baseline (week 0) and 2 weeks after the final DNA vaccine dose (week 14) in the CE group (left panels) and FL group (middle panels). (C) The specificities of the CE-specific responses in each animal in the CE group (left panel) and the FL group (right panel) at week 14 were measured against each separate CE peptide pool. (D) Shown are the breadth of CE-specific responses (number of positive CE peptide pools) at week 14 (left panel) and the change of the CE breadth at week 14 versus week 0 (right panel). (E) Analysis of the correlation between the magnitude of Gag-specific SFC prior to vaccination (Week 0) versus the increase in CE breadth post-vaccination (Week 14) was determined by Spearman Rank correlation test. Median values are indicated. *P*-values were determined by non-parametric Mann-Whitney T tests. *P* values ≤ 0.05 were considered significant.

existing Gag responses prior to vaccination did not influence the ability of the vaccine to broaden the CE-specific immune responses (p = 0.30, **Figure 2.2E).** In agreement with prior vaccine studies in uninfected macaques [128], we also did not observe a relationship between a particular MHC class I haplotype and the ability to develop CE responses (**Supplemental Table 1**) [128].

Lymph nodes and intestinal tissues are known viral reservoirs [357], and our previous results showed that the relative ability of a DNA vaccine to induce responses in these tissues correlated with a significant reduction in viral replication in these compartments [48,137]. We therefore investigated the effects of the vaccines on immune responses in mucosal and lymphoid tissues. The CE and FL vaccinated animals had similar levels of ELISpot responses to the whole SIV p57 Gag in the axillary and mesenteric lymph nodes (LN) (**Figure 2.3A**). There was no significant difference between the FL and CE groups in the magnitude of the CE response in the axillary LN, but a strong trend (p=0.06) for higher magnitude responses in the mesenteric LN and jejunum. In addition, all four animals in the CE group exhibited detectable responses to the CE portions of Gag in each tissue whereas only 1 or 2 animals in the FL group developed a detectable CE response in these tissues (**Figure 2.3B**). The CE group may have also had broader CE-specific ELISpot responses in the axillary (p=0.09) and mesenteric LN (p=0.17) with 4/4 animals developing responses against 1-7 CE, whereas only 2/4 animals in the FL vaccine group had CE responses, and only against 1-2 CE sequences (**Figure 2.3C)**. CE breadth was not examined in the jejunum due to sample limitations. In the FL group, the strongest and most frequently targeted CE response in the blood (**Figure 2.2C**) or lymph nodes (**Figures 2.3D)** was biased towards CE3, whereas in the CE group, T cell responses were directed primarily to CE5 and CE6 (**Figures 2.2C & 2.3D**), a result that is in agreement with previous CE DNA vaccine studies in uninfected rhesus macaques [129]. Taken together, these data demonstrate that therapeutic immunization with the CE vaccine was able to significantly increase the magnitude

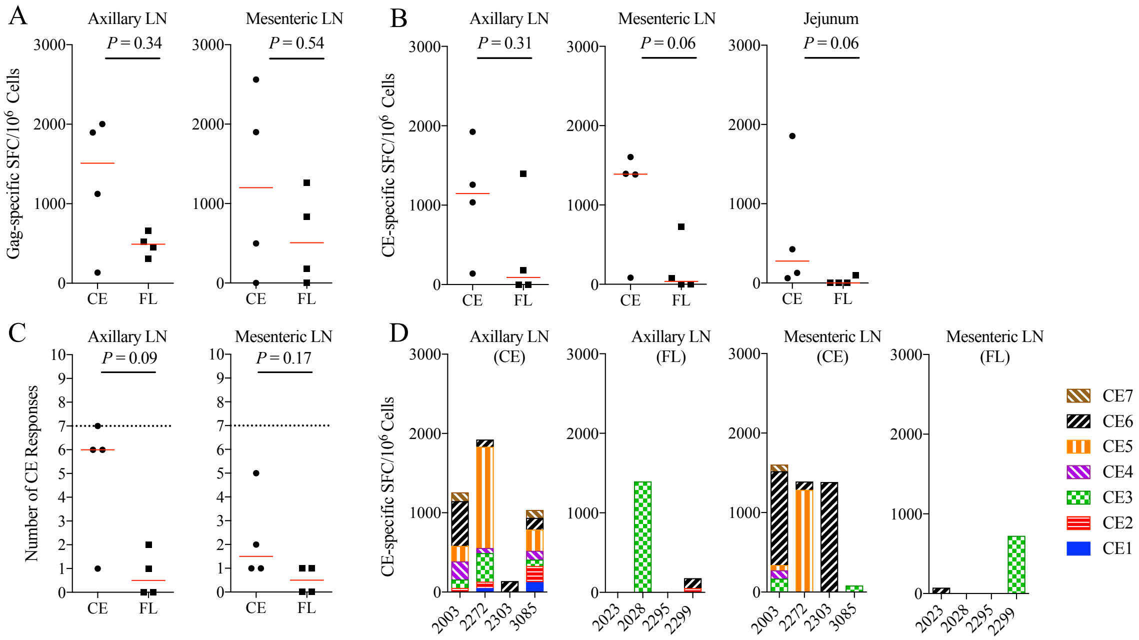


Figure 2.3: CE-specific IFN-γ T-cell responses in tissues.

(A) The total magnitude of Gag-specific IFN-γ T-cell responses as Spot Forming Cells (SFC) were determined by IFN-γ ELISpot at Week 14 following stimulation with pools of Gag peptides at Week 14 in the axillary (left panel) and mesenteric (right panel) lymph nodes (LN). (B) CE-specific T-cell responses are shown as cumulative number of SFC against each CE peptide pool and was determined by summing the responses against each individual CE (1-7) peptide pool in the axillary (left panel) and mesenteric (middle panel) lymph nodes and the jejunum (right panel). (C) The breadth of CE-specific IFN-γ T-cell responses was determined by measuring spot forming cells (SFC) against each individual CE peptide pool in the axillary (left panel) and mesenteric (right panel) lymph nodes. The dotted line at seven indicates the maximum number of CE that can be recognized. (D) The specificity of the CE responses was mapped in the axillary (left two panels) and mesenteric (right two panels) lymph nodes by ELISpot at week 14. Shown are responses against each peptide pool in animals immunized with either the CE or FL DNA vaccines. Median values are indicated in panels A-C with red horizontal lines and *p*-values were determined by non-parametric Mann-Whitney T test. *P* values ≤ 0.05 were considered significant.

and breadth of CE-specific IFN-γ secreting cells in the blood as well as in lymphoid tissues in SHIV-infected macaques with attenuated infection.

CE vaccination induces multifunctional, cytolytic CE-specific $CD8^+$ T-cell responses. CD107a is a marker of degranulation, indicating cytolytic effector function, that has been previously shown to correlate with better control of viremia in SIV/SHIV-infected macaques and HIV infected humans [358]. Since both cytolytic and multifunctional $CD8^+$ T-cell responses have been correlated with better viral control in HIV infected individuals [60,61], the ability of the CE vaccine to induce these responses may be important for enhanced viral clearance and control. Hence, CE-specific effector responses in the blood were characterized by intracellular cytokine staining (ICS) for IFN-γ, TNFα, IL-2, and for CD107a, at week 14. Similar to the IFN-γ ELISpot results, CE vaccinated animals had a greater fraction of CE-specific CD8 T-cells expressing IFN-γ detected by ICS (**Figure 2.4A**). There was also a strong trend for induction of higher magnitude CE-specific $CD8^+$ T-cells expressing CD107a, CD107a+TNFα, or CD107a+IL-2 cells when compared to the FL vaccine (**Figures 2.4B-D)**. Overall, the CE vaccine induced a higher proportion of CE-specific CD8+ T-cells expressing CD107a when compared to the FL DNA vaccine ($p = 0.03$, **Figures 2.4E**). Cytolytic and multifunctional responses to the non-CE portions of Gag were not assessed in these animals so we were unable to determine if the FL or CE vaccinated animals had similar responses to epitopes outside of CE.

To determine if therapeutic DNA vaccination with either the CE or FL vaccines influenced viral load, plasma viral RNA levels were measured by RT-PCR. We found no significant difference in mean viral loads (VL) between the two groups at any timepoint, although only one animal per vaccination group exhibited significant viremia over the course of the study (**Figure 2.5**). Even though VL in the CE vaccinated animal decreased while VL in the FL vaccinated animal increased, we cannot conclude that these results were due to either vaccine.

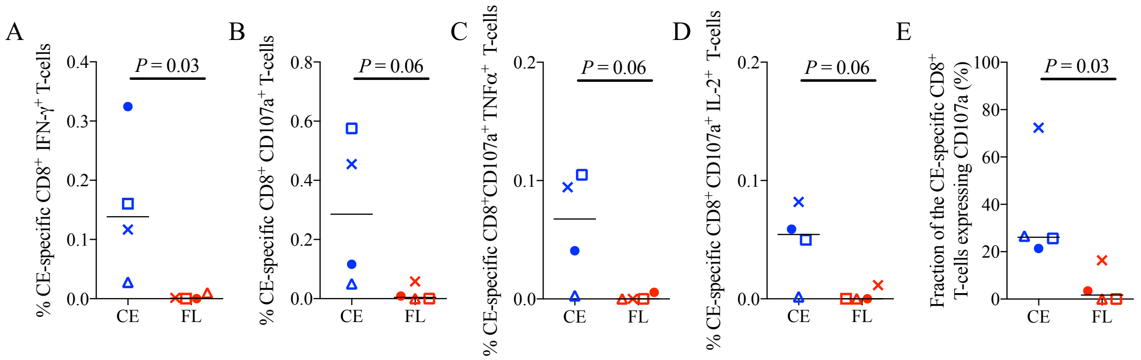


Figure 2.4: Multifunctional and cytolytic CE-specific CD8+ T-cell responses.

The frequency of CE-specific CD8+ T-cells expressing the cytokines IFN-γ, IL-2, and/or TNF-α and/or CD107a, a marker of cytolytic effector function, was determined two weeks after the final DNA vaccine dose (week 14) using cryopreserved PBMC stimulated overnight with CE peptide pools. (A-D**)** Shown are the frequencies of CE-specific CD8$^+$ T-cells expressing the indicated cytokine or cytolytic functions. (E**)** The relative proportion of CE-specific CD8+ T-cells that are expressing the cytolytic degranulation marker, CD107a. The difference in mean response between the two groups was analyzed by a non-parametric Mann-Whitney T test. Median values are indicated with black horizontal lines and a *p*-value ≤ 0.05 was considered significant.

CE-specific T-cell responses are associated with lower viral set point in SIV-infected rhesus macaques. The SIV CE DNA vaccine was derived by homology to HIV CE [56,129]. In acutely HIV infected ART naïve people, lower viral set point has been shown to correlate with the presence of stronger CE-specific responses, a finding that supports the hypothesis that increasing CE-specific responses during infection may provide a benefit in improving viral control [56]. However, it is unknown if CE-specific responses will have a similar correlation in the context of SIV or SHIV infections in macaques. Having demonstrated that it is possible to broaden or redirect the pre-existing immunodominant SIV Gag-specific T-cell repertoire toward increased targeting of SIV CE-specific epitopes in SHIV infected macaques, we sought to determine if, similar to humans, SIV-specific CE responses similarly correlated with lower viral set-point following acute infection in a second cohort of rhesus macaques. In this cohort, rhesus macaques were intravenously infected with SIVΔB670, a primary isolate that results in high viral loads during acute infection, reaches set point viremia by 6 weeks post-infection, and causes AIDS in the majority of animals within 11 months after infection[48,359]. After infection, CE-specific T-cell responses, measured by both ELISpot (**Figure 2.6A**) and ICS (**Figure 2.6B**) were detected in 4 of the 8 macaques. Interestingly, CE-responsive animals exhibited significantly lower set point viremia than the 4 macaques that had no detectable CE responses ($p = 0.03$, **Figures 2.6C & 2.6D**). Furthermore, the breadth of CE-specific T-cell responses measured by ELISpot inversely correlated with set point viral load in these animals, as defined by the averaging the viral loads from weeks 4, 5, and 6 ($p = 0.007$, **Figure 2.6E**), whereas the breadth of the response against the full length p57 Gag did not significantly correlate ($p = 0.11$, **Figure 2.6F**). Similar to previous studies in macaques and humans [56,128], we observed no relationship between MHC class I haplotype and the development of CE responses in these animals (**Supplemental Table 2.1**). Furthermore, in agreement with our previous studies involving SIVΔB670 [48], we also found no association between MHC or TRIM5 genetics and viral load (**Supplemental Table 2.1**).

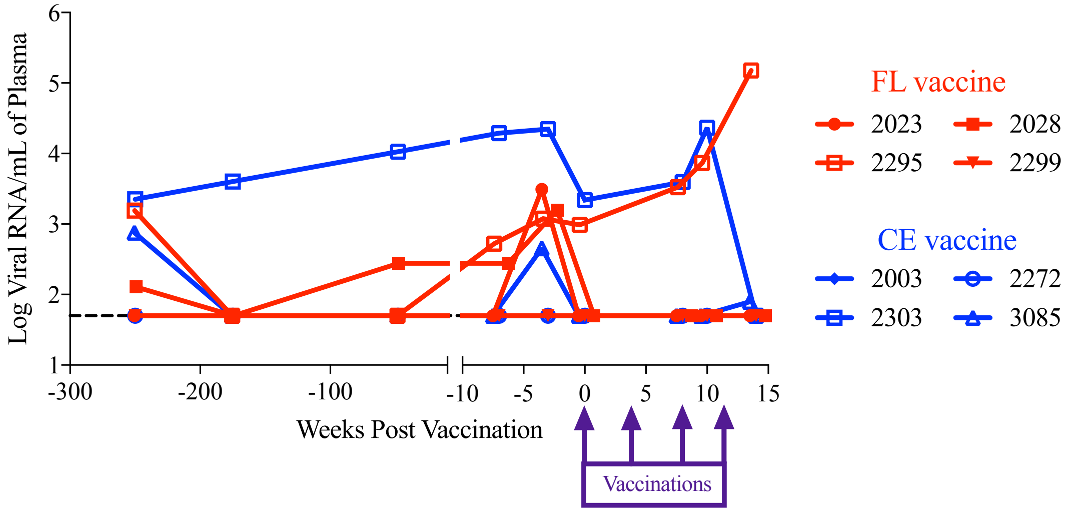


Figure 2.5: Plasma viral RNA load in SHIV infected macaques.

Plasma viral RNA levels before and after vaccination were determined by a nucleic acid sequence-based amplification technique (NASBA) with a limit of detection of 50 copies per 1 mL of plasma.

Discussion:

The results reported here demonstrate that therapeutic immunization with an SIV CE DNA vaccine can broaden T-cell responses primed by SHIV infection toward increased targeting of CE sequences. Our results also provide evidence that CE-specific T-cell responses induced by DNA vaccination have stronger cytolytic effector function when compared to T-cell responses induced by a standard FL DNA vaccine and as such, may be more effective in controlling viral replication. Consistent with this possibility, we found that unvaccinated rhesus macaques that developed stronger CE-specific responses during the acute stage of a highly pathogenic SIV infection also developed lower viral loads. These results are consistent with data from HIV-infected humans where it has been shown that CE responses that appear early in infection correlate with lower viral set point [56] supporting the concept that CE-specific CD8$^+$ T-cell responses may limit SIV replication and suggests that therapeutic immunization with CE vaccines may be able to improve treatment of HIV infection by increasing subdominant CE responses primed by the virus.

Previous studies have suggested that T-cell responses against subdominant Gag epitopes are preferentially lost during chronic HIV and SIV infection, perhaps due to T-cell exhaustion [360–362]. Therefore, vaccination with full-length antigens may mimic, or worsen, the effects of natural infection and fail to induce or even decrease CE-specific T-cell responses, as found here. Our data indicates that immunizing with a CE DNA vaccine can circumvent this problem, likely by excluding immunodominant decoy elements that divert T-cells responses, thereby boosting or inducing *de novo* T-cells with greater TCR affinity to the subdominant conserved regions of the viral proteome. The superior ability of the CE vaccine to induce broader CE responses and greater functional responses against subdominant epitopes when compared to a FL vaccine is in agreement with our prophylactic DNA vaccine studies in uninfected macaques [128,129]. In contrast, Stephenson *et al* reported that prophylactic

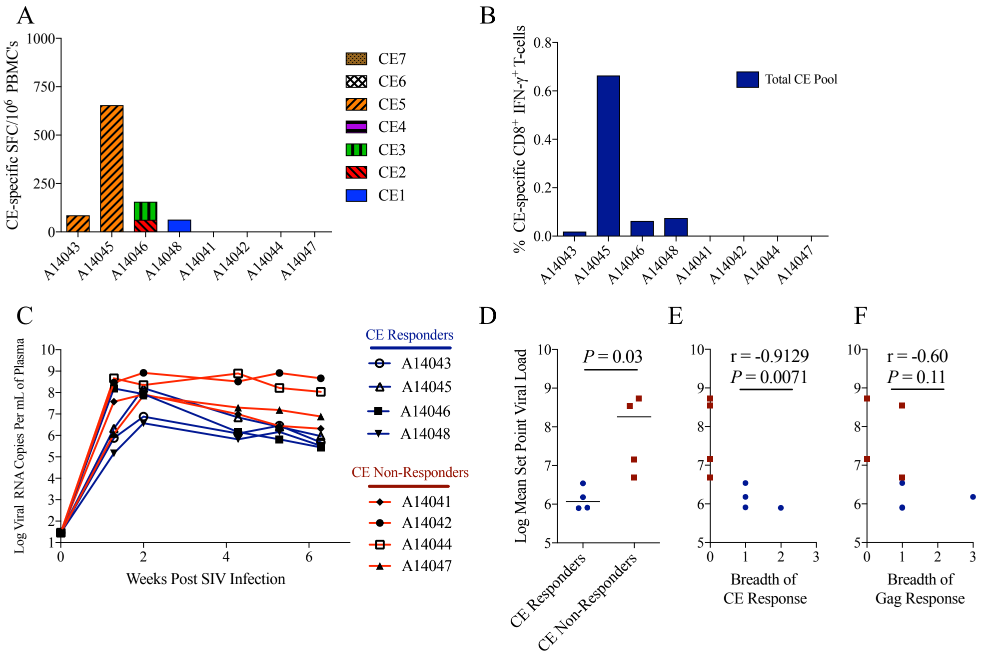


Figure 2.6. Higher CE-specific T-cells responses correlate with lower viral set point during acute SIV infection.

CE-specific T-cell responses in rhesus macaques acutely infected with SIVΔB670 were measured six weeks following intravenous infection using (A) IFN-γ ELISpot against individual CE peptides and (B) IFN-γ ICS against a pool of all seven CE peptides. CE responders were defined as having a detectable CE response measured by ELISpot and/or ICS. (C) Viral loads were measured in the plasma by quantitative RT-PCR. (D) Mean viral set point was determined by averaging the viral loads measured at weeks 4, 5, and 6 and compared between CE responders (animals with detectable CE responses) and CE non-responders (animals with undetectable CE responses) by non-parametric Mann-Whitney T test. Horizontal lines indicate median values. Correlation between the breadth of (E) CE and (F) Gag-specific T-cell responses and mean set point plasma viral load was determined by a Spearman Rank correlation. A *p* value ≤ 0.05 was considered significant.

immunization with full-length mosaic immunogens via recombinant adenovirus vectors in rhesus macaques (Ad35 and Ad26) was superior at inducing responses against conserved regions when compared to Adenoviruses expressing a conserved-region-only HIV immunogen [363]. This could be due to the different composition and configuration of the conserved immunogens, differences between consensus and mosaic immunogens and the single FL immunogen used here, and/or the use of adenoviral vectors versus DNA vaccine delivery, since Ad vectors contain immunodominant epitopes that may compete with subdominant conserved HIV epitopes [364]. Previously, we demonstrated that in uninfected rhesus macaques, CE DNA priming followed by FL DNA boosting was more effective at increasing the magnitude of CE responses, compared to priming with CE without a heterologous boost or priming with a FL DNA vaccine and then boosting with a CE DNA vaccine. The latter result suggested a limited ability to improve CE responses in animals that were previously primed with immunodominant responses to variable regions of the viral proteome [128]. Unexpectedly then, we found that therapeutic immunization increased CE-specific T-cell epitopes in SHIV-infected macaques primed with full-length antigens by the viral challenge, albeit in animals with attenuated infection. In this setting, therapeutic DNA vaccination may have augmented subdominant CE-specific cellular responses that were primed by the virus. This suggests that therapeutic vaccination, in a setting were viral replication is suppressed by antiretroviral drugs may be able to overcome the "original antigenic sin" associated with SIV/HIV infection that typically favors induction of immunodominant responses to more variable sequences.

The enhanced cytotoxic function of the CE-specific responses we observed following CE vaccination is consistent with previous studies in uninfected rhesus macaques immunized with an HIV Gag CE DNA vaccine [128,129]. However, due to the small numbers of animals in this study and low initial viremia in both groups, we cannot determine if CE therapeutic vaccination provided a benefit in the control of viremia.

Animal #	TRIM5	MHC	SIV	Vaccine	Log Viral Load (at end of the study)	CE Responses in PBMC
2299	Not Typed	-	SHIV89.6P	FL	Undetectable	CE3 & 4
2003	Not Typed	A*02, B*03	SHIV89.6P	CE	Undetectable	CE1-7
2272	Not Typed	A*02, B*01	SHIV89.6P	CE	Undetectable	CE3-7
2303	Not Typed	-	SHIV89.6P	CE	Undetectable	CE1-7
3085	Not Typed	-	SHIV89.6P	CE	1.90	CE1-5
A14043	TFP/CYPA	A*01, A*08	SIVΔB670	None	5.68	CE5
A14045	TFP/TFP	-	SIVΔB670	None	5.97	CE5
A14046	TFP/Q	B*01	SIVΔB670	None	5.44	CE2 & 3
A14048	TFP/CYPA	A*01, A*08	SIVΔB670	None	5.52	CE1
2023	Not Typed	A*02, B*03	SHIV89.6P	FL	Undetectable	None
2028	Not Typed	A*02, B*01	SHIV89.6P	FL	Undetectable	None
2295	Not Typed	-	SHIV89.6P	FL	5.18	None
A14041	TFP/TFP	A*01	SIVΔB670	None	6.32	None
A14042	Q/Q	-	SIVΔB670	None	8.67	None
A14044	TFP/Q	B*01	SIVΔB670	None	8.03	None
A14047	TFP/TFP	A*08	SIVΔB670	None	6.89	None

Supplemental Table 2.1. MHC and TRIM5 genetics.

Shown for each animal are TRIM5 haplotype, MHC type (A*01, A*02, A*08, A*11, B*01, B*08, B*17, and B*29), SIV or SHIV infecting strain, vaccination regimen, and the viral load at the end of study (2 weeks post 4th vaccination in SHIV89.6P infected animals and 6 weeks post-infection in SIVΔB670 infected animals), as well as the specificity of CE-specific cellular immune responses. Animals in blue are CE responders whereas animals indicated in red are non-responders.

CE vaccinated animals also exhibited a trend toward greater CE-specific T-cell responses in the gut mucosa and lymphoid tissues. Since the gut and lymphoid tissues contain virally infected cells [10,48,137,357,365,366] that can contribute to viral rebound after cessation of ART [367], inducing CE-specific responses in these tissues may be important to decrease the size of the reservoir and provide more durable control of viremia in the absence of drug.

In the SHIV study, we found no relationship between the magnitude of Gag-specific responses measured prior to therapeutic vaccination and the development of *de novo* CE-specific responses induced post-vaccination. This indicates that the presence of immunodominant HIV or SIV CD8$^+$ T-cell responses against Gag prior to vaccination did not significantly impair the immunogenicity of the CE DNA vaccine. However, we did not measure responses against other viral antigens that were not included in the vaccine, such as Env, Pol, or Nef that are also rich in immunodominant CD8$^+$ T-cell epitopes, so we cannot exclude the possibility that immunodominant responses against other viral antigens could influence immunogenicity of the CE vaccine.

HIV CE sequences were selected based on sequence conservation and fitness constraints as previously described and are generally >98% conserved among all HIV-1 group M isolates evaluated to date. This is the key advantage of using this vaccine strategy over other vaccines in that we can expect a high degree of similarity between the vaccine and any virus that would theoretically be found in humans. The SIV Gag CE DNA vaccine used for these studies was not based on sequences in the challenge virus but rather, they were designed based on homology to the HIV CE sequences as previously described [129]. The 75-100% amino acid similarity between SIV p27CE and HIV p24CE further highlights the high degree of conservation of the CE sequences across not only HIV strains but also SIV strains. The full length (FL) Gag DNA vaccine used in this study was derived from SIV/17-Fr sequence, a macrophage tropic, congenic isolate of SIVmac239[368]. These two strains are about 15% divergent in the Env gene [48] but as is the case for most SIV strains, the Gag sequences for the two strains are 100% identical [356], and each contain CE sequences. The challenge virus SHIV89.6P is derived from $\text{SIV}_{\text{mac239}}$ [355], so it too was at least substantially homologous to the FL vaccine, although we did not sequence virus in the SHIV89.6P-infected macaques in this study, and the sequences may have diverged from the challenge stock prior to therapeutic vaccination.

Taken together, these results show that CE DNA vaccination can significantly increase the magnitude, function and breadth of CE-specific responses even in the presence of a strong immunodominant memory T-cell response induced by chronic SHIV infection in rhesus macaques, although we are unable to draw conclusions about the antiviral efficacy of the CE vaccine in this study. As the overall goal of an effective therapeutic vaccine is to induce viral remission or cure of the infection, future studies will investigate the ability of this vaccine, in combination with antiretroviral drug therapy and potentially reservoir activating agents and immune modulators, to reduce viral replication or block viral rebound after analytic treatment interruption in SIV or SHIV infected macaques. If successful, these studies would support further development of a therapeutic CE vaccine for people living with HIV.

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Chapter 3: Therapeutic conserved elements (CE) DNA vaccine is immunogenic in cART treated SIV infected macaques, but does not improve viral control following analytical treatment interruption.

“*Potential is not an endpoint but a capacity to grow and learn.”* – Eileen Kennedy Moore

Paul Munson, Hillary Tunggal, Yi Liu, Debra Bratt, James T. Fuller, Xintao Hu, George N. Pavlakis, Barbara K. Felber[3] James I. Mullins, Deborah Heydenburg Fuller

Abstract:

The primary goal of therapeutic HIV vaccine research is to induce durable viral remission in the absence of combination anti-retroviral therapy (cART). The main obstacles of a successful therapeutic HIV vaccine are the enormous viral genetic diversity and propensity of the virus to mutate and thereby escape HIV-specific immune responses. To address these issues, we have designed DNA immunogens that encode conserved elements (CE) of functionally constrained regions of the SIV Gag capsid (p27), co-formulated with the genetic adjuvant "LT", and delivered to the epidermis via the gene gun. While these p27CE immunogens have previously demonstrated immunogenicity in SIV uninfected or SHIV infected macaques with low to undetectable viremia, their ability to elicit CE responses in the context of cART treatment of a highly pathogenic SIV infection remained unknown. Here we show for the first time that therapeutic CE vaccination increased the magnitude and breadth of CE-specific cellular and humoral immune responses, when compared to full-length (FL) and mock vaccines, in SIV∆B670 infected cART treated macaques. Additionally, we found that viral replication during cART was associated with increased frequencies of programmed death-1$^+$ (PD-1$^+$) CD8$^+$ T-cells which in turn were inversely correlated with the magnitude of SIV-specific T-cellular responses by ELISpot, and polyfunctional CE responses specifically, in both the CE and FL vaccine groups. These data demonstrate that therapeutic CE vaccination via gene gun is immunogenic in cART treated SIV-infected macaques, but the immunogenicity may be dampened by PD-1 expression. Lastly, neither the CE or FL vaccines limited viral replication post-ATI when compared to the Mock vaccine group.

Introduction:

Early initiation of cART can potently reduce HIV replication, restore CD4$^+$ T-cell counts, and prolong the lifespan of HIV-1 infected people[369]. However, cART is unable to fully reverse the immune dysfunction induced by HIV or simian immunodeficiency virus (SIV) infection, cannot deplete the latent viral reservoir, and therefore must be taken for life to maintain control of viral replication. Furthermore, cART for life is expensive and can have side-effects that may limit compliance[370,371]. cART's limitations have incentivized the development of novel cure based strategies, including the development of therapeutic HIV vaccines[372,373]. Therapeutic immunization could augment HIV-specific T-cellular and humoral immune responses that provide durable control of viremia in the absence of cART.

A variety of vaccine modalities have been evaluated for HIV therapy including subunit[119,334], attenuated[204], dendritic cells[209,216], viral vectors[247], and DNA vaccines[48,374,375]. A majority of these strategies involve immunogens with full-length (FL), or near FL viral genes or proteins that contain immunodominant sequences that interfere with the ability to direct immune responses to subdominant and functionally important regions of the viral proteome[105]. Given that the viral reservoir is enriched with proviral sequences that contain CTL escape mutations in CD8$^+$ T-cell epitopes[10], strategies to direct immune responses to conserved amino acid sequences that if mutated impose severe decreases in viral fitness may be necessary to provide a sustained block from viral recrudescence following cART cessation[10].

Towards this end, several conserved only HIV immunogens have been designed with the goal of eliciting potent cross-reactive immune responses that inhibit all viral variants and limit immune escape[134,129,127,128,126,104,247]. Previously, we developed conserved elements (CE) SIV/HIV DNA vaccines and demonstrated their ability to enhance CE-specific cellular and humoral immune responses in SIV uninfected macaques[129,134]. Furthermore, we demonstrated that CE DNA vaccines delivered via gene gun co-formulated with the heat-labile *E. coli* enterotoxin (LT) adjuvant potently increased CE-specific cellular immune responses in cART naïve SHIV-infected macaques with low to undetectable viremia[374]. Our previous study demonstrated the CE vaccination could overcome the “original antigenic sin” induced by SHIV viral infection. However, these macaques had previously received a prophylactic vaccination and following challenge exhibited viral loads ranging from undetectable to 10^4 copies per ml of plasma[374]. The small number of macaques used in the previous study as well as their controller phenotype made it unclear if CE vaccination would also be immunogenic and of clinical benefit in SIV infected cART treated macaques. To address this question, we tested mock, FL, and CE DNA vaccines delivered via gene gun in SIVB670 infected macaques on cART.

Here we report for the first time that CE DNA vaccination in the context of cART treatment of a highly pathogenic SIV infection could refocus cellular and humoral immune responses from immunodominant sequences primed by viral infection towards subdominant conserved epitopes. Specially, we found a greater frequency of CE-specific IFN-γ^+/CD8$^+$ T-cells as well as broader CE-specific ELISpot and ELISA responses in CE vaccinees when compared to the mock and FL groups. Additionally, we found that responsiveness to cART was positively associated with improved vaccine immunogenicity among both CE and FL vaccinated animals. We observed an inverse relationship between programmed death-1 (PD-1) expression on CD8$^+$ T-cells prior to vaccination and the ability to respond to vaccination by ELISpot or polyfunctional CE-responses. Lastly, there were no differences in viral load post-ATI among the CE, FL and Mock vaccine groups. In summary, these finding demonstrate that gene gun delivery of CE DNA vaccines can overcome the immunodominance induced by viral infection and boost or elicit *de novo* CE-specific immunity and warrants further development as a treatment for HIV infection.

Methods:

Ethics Statement and Animal Care

Animal care is consistent with Chapter 2.

MHC-I and TRIM5 typing

Macaques were typed for 32 major histocompatibility class I (MHC-I) alleles (including A*01, A*02, B*08, B*17) by PCR using DNA extracted with the Roche© MagnaPure™ system by Dr. David Watkins and the MCH Genotyping Service at the University of Miami, as previously described (NIH Grant: 5R24RR016038)[349,350]. TRIM5 haplotypes including TFP, Q, and CypA were determined by PCR of genomic DNA by Dr. David O'Connor at the Wisconsin National Primate Research Center (WNPRC).

Viral Challenge and AIDS Monitoring

Viral challenge and AIDS monitoring was consistent with Chapter 2.

Plasma Viral Load Quantification. The Virology Core at the WaNPRC quantified viral RNA in the plasma for the SIVΔB670 infected animals with a quantitative real time PCR (q-RT-PCR) assay utilizing previously described primers [48,345].

Combination Antiretroviral Therapy: 9-(2-Phosphoryl-methoxypropyly) adenine (PMPA; Gilead Sciences, Foster City, CA) was suspended in PBS at a concentration of 120 mg/mL. To completely dissolve the PMPA, 1 molar NaOH was added dropwise until a pH of 7.4-7.8 was reached, then filter purified, injected into sterile glass vials, and placed at -20C for long term storage. PMPA was given subcutaneously at a dose of 20 mg/kg once daily.

2',3'-dideoxy-5-fluroro-3'-thiacytidine (FTC, Gilead Sciences, Foster City, CA) was suspended in PBS at a concentration of 120 mg/mL and heated at 37°C with stirring until completely dissolved. FTC was prepared fresh and stored at 4°C. FTC was given subcutaneously at a dose of 30 mg/kg once daily the first month of cART (weeks 6-10) and a dose of 20 mg/kg once daily for the remainder of cART.

Raltegravir (Merck & Co., Kenilworth, NJ) was delivered orally at 250 mg/animal twice daily for the first month and at 150 mg/animal twice daily for the remainder of cART.

All drugs were administered by trained animal technicians and adverse side effects were closely monitored by veterinary staff and treated immediately. One animal (A16152) experienced elevated chloride/phosphate ratios and discontinued cART.

Percent ART Suppression of Viral Load

ART suppression was defined by the following equation:

$$Log\left(\frac{Post\ ART\ Viral\ Load}{Pre\ ART\ Viral\ Load}\right) \Big/ Log\left(\frac{30\ copies}{Pre\ ART\ Viral\ Load}\right) \times 100$$

$$= \%\ ART\ Suppression\ of\ Viral\ Load$$

This % ART suppression of viral load was used to determine if responsiveness to cART was correlated with vaccine immunogenicity.

Complete Blood Counts (CBC's) & Serum Chemistries: Complete blood counts were determined by a Beckman Coulter® AC*T™ 5diff hematology analyzer as previously described[374].

DNA Vaccinations: CE, Gag, and LT plasmids were constructed [101,129] and plasmid DNA formulated onto gold particles [48] as previously described. Using the PowderJect® XR1 gene delivery device (PowderJect Vaccines, Inc., Middleton, WI), each macaque received 32 μg of the CE or FL DNA vaccine co-formulated with 3.2 μg of plasmid expressing the LT genetic adjuvant and administered over 16 skin sites (2ug CE or FL DNA + 0.2 μg LT per site) along the abdomen and over the inguinal lymph nodes. The CE vaccinated animals received a 4th vaccination comprising both CE and FL DNA. Skin sites were clipped of fur and swabbed with alcohol prior to PMED administration.

ELISpot

ELISpot analysis to quantify the frequency of SIV-specific IFN-γ spot forming cells (SFC) was performed as previous described[374].

ELISA

Plasma was purified from blood samples collected in EDTA by centrifugation then kept at -80°C for long term storage. ELISA plates (Corning® EIA/RIA) were coated with **1)** DMSO as a negative control in triplicate, or **2)** 100 μL of 15mer and 10mer peptide pools corresponding to individual CE pools (10μg/mL) **3)** 15mer peptides corresponding to non-CE Gag peptides (AIDS Research and Reference Reagent Program, Germantown, MD) **4)** a 1:50 dilution of macaque plasma as a positive control. Wells were coated overnight at 4°C. The following day, plates were washed 5 times with PBS supplemented with 0.05% Tween®20 with an AquaMax 4000™ plate washing system and blocked with 200μL blocking buffer (PBS, 5% milk powder, 0.05%, and Tween®20) for 1.5 hours. Subsequently, blocking buffer was aspirated from plates and coated with 100μL of heat inactivated plasma from experimental animals diluted 1:50 in blocking buffer for 1.5 hours. Plates were then washed and coated with 100μL goat-anti-monkey-HRP antibody (1:15,000 in blocking buffer) for 1.5 hours, then washed and developed using the SureBlue™ TMB Microwell Peroxidase Substrate Kit (KPL Inc.) and neutralized with hydrocholic acid. Reactivity was scored by measuring optical density (OD) using an EMax® ELISA Microplate Reader with SoftMax® Pro software (Molecular Devices©, Sunnyvale, California). Samples were background subtracted from wells with peptides and secondary antibody alone in the absence of macaque plasma. All samples were analyzed in duplicate; a positive response was defined as greater than 3 standard deviations above the mean OD of DMSO containing wells in both duplicates.

Intracellular cytokine stain (ICS):

Cryopreserved PBMCs were thawed at rested for 4 hours and stimulated with DMSO, PMA (Sigma-Aldrich®) / Ionomycin (Life Technologies®), or peptides (1 μg/mL) for 1 hour with CD107a PECy5 (eBioH4A3, BioLegend) in R10 media prior to adding 1 mg/mL of Brefeldin A. Cells were stimulated in the presence of Brefeldin A (Sigma-Aldrich®) overnight at 37C and 5% CO_2. Viable cells were stained using a LIVE/DEAD® Yellow (ThermoFisher®) amine dye then surface stained with CD3 Brilliant Violet (BV) 711 (Sp34-2, BD Biosciences), CD4 PerCPCy5.5 (L200, BD Biosciences), CD8 APC-Cy7 (RPA-T8, BD Biosciences), CD28 PE-CF594 (CD28.2, BD Biosciences), CD95 BV421 (Dx2, BD), PD-1 BV605 (EHI12.2H7, BioLegend), and TIGIT PerCP-eFluor710 (MBSA43, ThermoFisher®). Cells were permeablizied with Cytofix/Cytoperm (BD Biosciences) and stained with an intracellular antibody cocktail containing IFNγ FITC (B27, BD Biosciences), TNFα PE-Cy8 (Mab11, BD Biosciences), IL-2 PE (MQ1-17H12, BD Biosciences), GranzymeB APC (GB12, ThermFisher), Ki67 AF700 (B56, BD Biosciences) in Perm/Wash^{TM} Buffer (BD Bioscinces). Cells were then wased with Perm/Wash^{TM} Buffer (BD Biosciences) then fixed with 1% paraformaldehyde (Sigma) and collected on an LSR II (BD Biosciences). Flow cytometry was analyzed in FlowJo (Version 9.7.6, Treestar Inc., Ashland, Oregon). Samples were considered positive if peptide-specific responses were at least twice that of the negative control plus at least 0.01% after background subtraction.

Statistical Analyses. Statistical differences between the two groups were calculated using a two-sided Mann-Whitney test and differences between multiple groups by a one-way ANOVA test in GraphPad Prism (Version 7, GraphPad Software, San Diego, CA). Differences in viral load or CD4$^+$ T-cell counts before and after cART were determined by a Wilcoxon matched-pairs signed rank test. Correlations between immune responses and viral loads were determined by a Spearman's rank correlation test. A P value ≤ 0.05 was considered significant for each test.

Results:

Study Design

An ideal therapeutic vaccine for HIV will need to be effective in HIV vaccine naïve individuals, who are not elite controllers, and will likely require targeting of conserved epitopes to limit immune escape[10]. We hypothesized that CE therapeutic vaccination, in conjunction with ART, will more potently boost or induce *de novo* CE-specific immunity when compared to FL or mock vaccinated macaques. Indian origin male rhesus macaques (*Macaca mulatta*) were intravenously infected with the highly pathogenic primary isolate SIVΔB670 and began combined antiretroviral therapy (cART) consisting of PMPA, FTC, and Raltegravir (**Figure 3.1**) six weeks post-infection. After 3 months of ART (Week 18), animals received 4 DNA immunizations via particle mediated epidermal delivery (PMED, or gene gun) spaced one month apart. The control vaccination cohort (N=7) included mock (N=3), consisting of a plasmid without an insert, and mock plus the genetic mucosal adjuvant the heat-labile *E. coli* enterotoxin LT (N=4). Next, the FL cohort received antigen encoding DNA consisting of coequal parts of a plasmid encoding Gag-Pol- Env and forms virus like particles as described previously[376] and a plasmid encoding p57 Gag, alongside LT (N=6, **Figure 3.1B**). Finally, the conserved element (CE) group received for three immunizations consisting of a p27 CE plasmid (CE1) and a p27 CE containing toggled versions of CE (CE2), as described previously[129], co-formulated with LT and a final CE + p57Gag + MCP3-p39[129] Gag immunization boost (N=6, **Figure 3.1C**). We used a CE + FL co-immunization boost because this demonstrated superior ability to elicit CE responses compared to a FL only boost[129]. Macaques were stratified into different vaccination groups based on TRIM5 haplotype and MHC genetics (**Table 3.1)**.

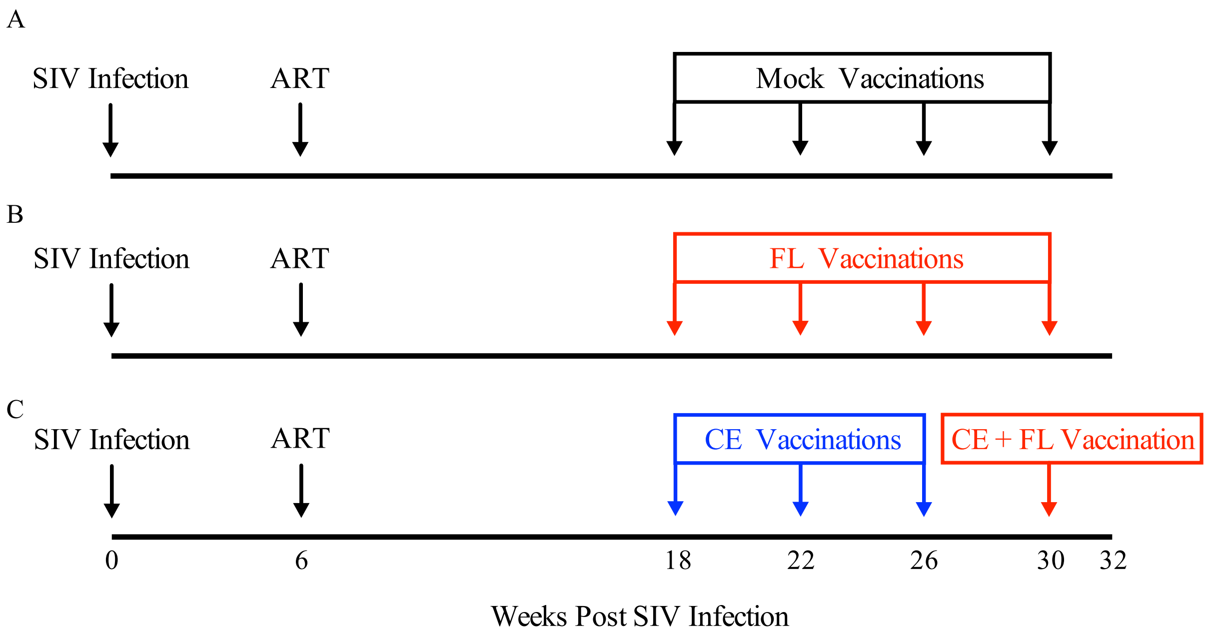

Figure 3.1. Therapeutic Vaccination Study Design. Indian origin rhesus macaque began anti-retroviral therapy (ART) consisting of subcutaneous Emtricitabine (FTC) and Tenofovir (PMPA) and oral Raltegravir. 6 weeks after they were intravenously infected with SIVΔB670 (A-C). Arrows indicate 4 DNA immunizations spaced 4 weeks apart. SIV Infection was an intravenous challenge with 100 $TCID_{50}$ of SIVΔB670. Vaccinations were DNA delivered by particle mediated epidermal delivery (PMED; or gene gun) co-formulatd with the mucosal genetic adjuvant LT.

Animal #	Vaccine (DNA)	TRIM5	MHC
A14041	Mock	TFP/TFP	A*01
A14043	Mock	TFP/CYPA	A*01, A*08
A14046	Mock	TFP/Q	B*01
A14044	Mock + LT	TFP/Q	B*01
A14045	Mock + LT	TFP/TFP	-
A14047	Mock + LT	TFP/TFP	A*08
A14048	Mock + LT	TFP/CYPA	A*01, A*08
A15353	FL + LT	Q/TFP	-
A15354	FL + LT	Q/TFP	-
A15355	FL + LT	TFP/CYPA	A*02
A15359	FL + LT	TFP/TFP	-
A15360	FL + LT	TFP/CYPA	A*02
A15122	CE + LT	TFP/TFP	A*01, A*08
A15123	CE + LT	TFP/TFP	A*02
A15124	CE + LT	TFP/Q	A*01
A15125	CE + LT	TFP/TFP	A*02, B*01
A15358	CE + LT	TFP/CYPA	A*02
A15365	CE + LT	Q/TFP	-

Table 3.1. Vaccine Groups with TRIM5 and MHC. Macaques were stratified into various vaccination groups to balance TRIM5 haplotype and MHC-I genes.

Viral loads and CD4 counts in PMPA, FTC and RAL treated SIV infected macaques

In our previous therapeutic vaccine study, our cART regimen consisted of PMPA and Kaltetra and only half (20/41) of the animals achieved viral loads less than $5x10^4$ and less than a 1 log reduction in mean viral load[48]. Since our group and others have shown that therapeutic vaccination is ineffective in cART non-responders[298,377] we sought to improve upon this regimen as a mechanism of increasing therapeutic vaccine efficacy. Towards this end, we utilized a regimen consisting of non-nucleoside reverse transcriptase inhibitors (NNRTI's) PMPA and FTC, as well as the integrase inhibitor RAL. To balance suppression of viremia and management of clinical symptoms, we began a high dose phase for 1 month (Weeks 6-10) consisting of 20 mg/kg PMPA and 30 mg/kg FTC delivered subcutaneously (s.c.) once daily, and twice daily 265 mg of Raltegravir orally, followed by a low dose phase for the remainder of cART consisting of 20 mg/kg PMPA and 20 mg/kg of FTC delivered s.c. once daily, and twice daily 165 mg of Raltegravir delivered orally. With this new regimen, all animals 19/19 controlled viremia to less than $5x10^4$ copies (**Figure 3.2A**). Furthermore, 10/19 animals achieved robust control of viremia (<100 viral copies) (**Figure 3.2B**). The degree to which an animal suppressed viremia post-cART (Week 16) was positively correlated ($P = 0.0084$, $r = 0.5859$, **Figure 3.2C**) with the amount of virus before initiating cART (Week 6).

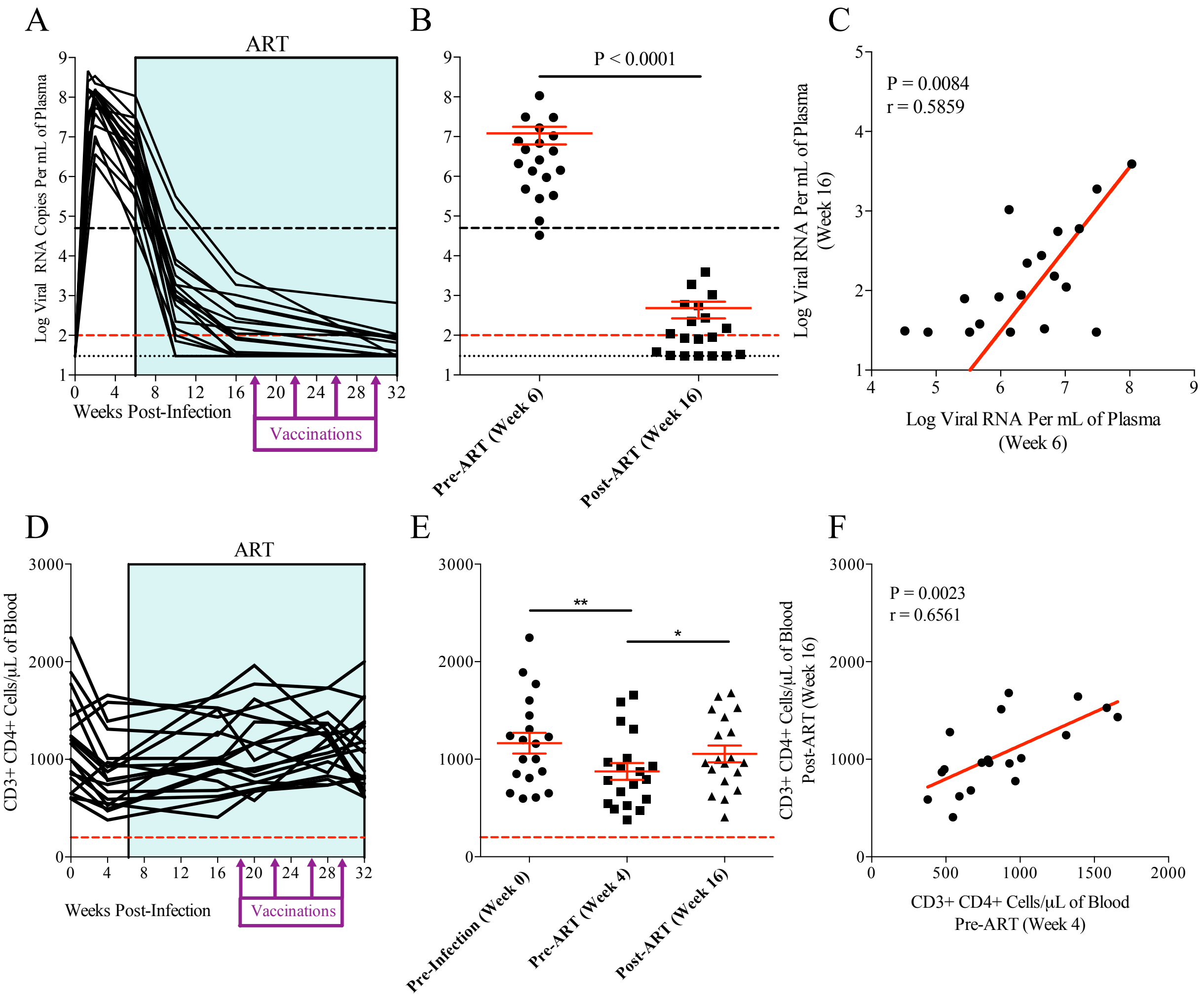

Figure 3.2. PMPA, FTC, and Raltegravir reductions in SIVΔB670 infected macaques. Viral RNA was measured in the plasma of SIV infected macaques by q-RT-PCR with a limit of detection of 30 copies (black dotted line). Shown are (**A**) viral loads throughout the study, (**B**) at pre-ART (Week 6) and post-ART (Week 16), and (**C**) correlations between viral loads pre-ART (Week 6) and pre-vaccine (Week 16). The black dashed line (5x10^4 viral copies) and red dashed line (100 viral copies) were used to categorize levels of viral control. Red bars show mean +/- SEM. (**D**) Absolute CD4$^+$ T-cells in the blood as measured by a complete blood count (CBC) throughout the study, (**E**) at select time points pre-infection (Week 0), pre-ART (Week 4), and post-ART (Week 16), and (**F**) correlations between pre and post-ART CD4 T-cell

frequencies. The red dashed line (200 CD4 T-cells per μL of blood) indicates a simian-AIDS defining criteria. Differences in viral load at Week 6 and Week 16 were determined by a Wilcoxon matched-pairs signed rank test. The P and r values indicated are from Spearman rank correlation tests. Differences in CD4 T-cell counts among multiple time points were determined by a one-way ANOVA with a Tukey's multiple comparisons test. An asterix (*) indicates an adjusted *p* value of less than 0.01, and a (**) indicates a *p* value of less than 0.001.

SIVΔB670 infection induces a rapid decline in CD4$^+$ T-cells, thus causing immune dysfunction which may interfere with vaccine immunogenicity[48]. The ability of more potent cART to restore CD4$^+$ T-cell frequencies, and thereby partially blunting or reversing immune dysfunction, may be an important prerequisite for therapeutic vaccine immunogenicity. Similar to our previous work, we observed a rapid, statistically significant 20% decline in CD4$^+$ T-cells in the blood by 4 weeks post-infection (**Figure 3.2D,E**). However, the decline in peripheral CD4$^+$ T-cell counts at week 4 was reversed after 10 weeks of cART (Week 16) (**Figure 3.2E**) to magnitudes similar to pre-infection levels. Comparable to the viral loads, the pre-and post-ART CD4$^+$ T-cell numbers were positively correlated (P = 0.0023, r = 0.6561) (**Figure 3.2F**). These findings potentially indicating that innate and or adaptive host factors that limit CD4$^+$ T-cell depletion may play a role in ART mediated restoration of CD4$^+$ T-cells.

TRIM5 and MHC Associations with Viral Load

TRIM5 is an innate sensor of retroviral capsids and is important for restricting HIV/SIV replication[378–380] as well contributing to prophylactic protection from SIV in macaques[381]. Previously, we did not observe an association between TRIM5 haplotype and acute viral load, responsiveness to ART, or post-ART viral control [48]. Similarly, we did not observe associations between MHC commonly associated with viral control (A*01, A*02, B*08, or B*17) at any time point. However, our previous study utilized a sub-optimal drug regimen (PMPA & Kaletra), compared to the three-drug regimen in this study, that may have obscured our ability to detect more subtle associations between drug responsiveness and TRIM5 and/or MHC. In this study, we excluded TRIM5 animals with a Q/Q phenotype due to one animal that progressed to AIDS within 8 weeks of infection (data not shown)[382]. Among the remaining animals, and

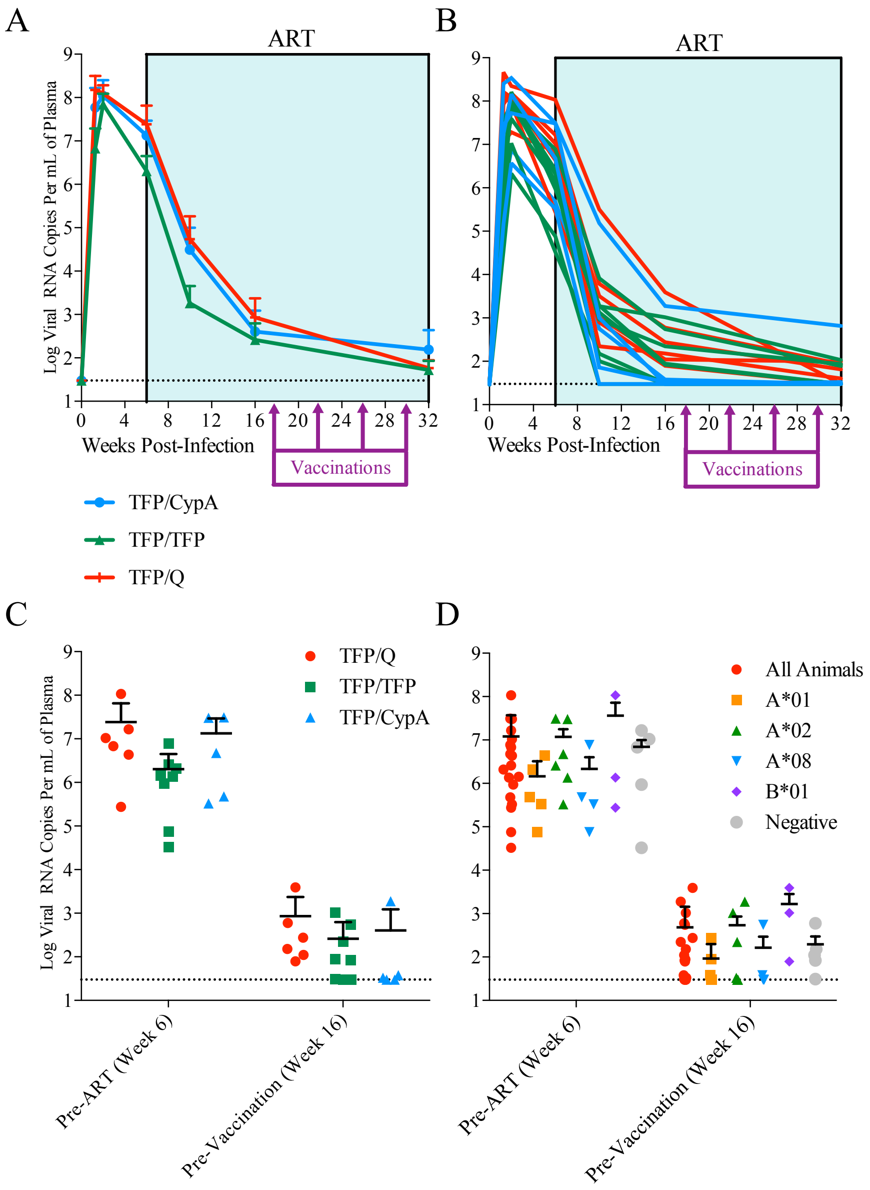

Figure 3.3. Relationship between MHC and TRIM5 with viral replication. Viral RNA copies were quantified in the plasma by q-RT-PCR deviation and are shown grouped by TRIM5 haplotype(**A**) and individual viral load curves (**B**). Individual viral loads before ART (Week 6) and vaccination (Week 16) corresponding to TRIM5 haplotype (**C**) and major-histocompatibility complex-I (MHC-I) (**D**). Error bars indicated are standard deviations. One way ANOVA's were performed at each time point to determine viral load associations with either TRIM5 or MHC.

in agreement with our prior study[48], we did not observe any statistical differences in viral loads at any time point among TRIM5 haplotypes (TFP/CypA, TFP/TFP, or TFP/Q) (**Figure 3.3A-C**) or MHC genes (**Figure 3.3D).**

ART reduces viremia and restores CD4$^+$ T-cells in the blood

HIV and SIV can induce immune dysfunction and stimulate immune-regulatory mechanisms that may interfere with vaccine immunogenicity and confound our ability to compare the mock, CE, and FL vaccine groups. To avoid this, we measured viral loads and CD4$^+$ T-cell counts throughout the study and found they were similar among vaccine groups at all time points measured (**Figure 3.4A-D**). Both pre-cART (Week 6) and post-cART (Week 16) viral load levels were not statistically different by ANOVA (**Figure 3.4E**). CD4$^+$ T-cell counts in the blood were similar among vaccine groups over the course of the study, with none of the animals experiencing an AIDS defining depletion at any time point, in the blood as measured by complete blood counts.

Therapeutic CE vaccination increases cellular and humoral responses to CE sequences.

To determine the immunogenicity of the FL and CE DNA vaccines, IFN-γ responses were quantified by ELISpot throughout the study in the PBMC, using peptide pools spanning p57^{Gag} (Gag), non-CE portions of Gag (Non-CE Gag), and only CE sequences (**Figure 3.5 A**). As expected, both the CE and FL vaccinated groups tended to have greater overall Gag-specific ELISpot responses compared to the mock group, whereas the CE group tended to have a greater magnitude of CE-specific ELISpot responses in the PBMC (**Figure 3.5 A)**. To further characterize the immune responses, non-CE Gag and CE-specific CD4$^+$ and CD8$^+$ T-cells were quantified by ICS in the PBMC 2 weeks following the 4th vaccination (Week 32). Consistent with

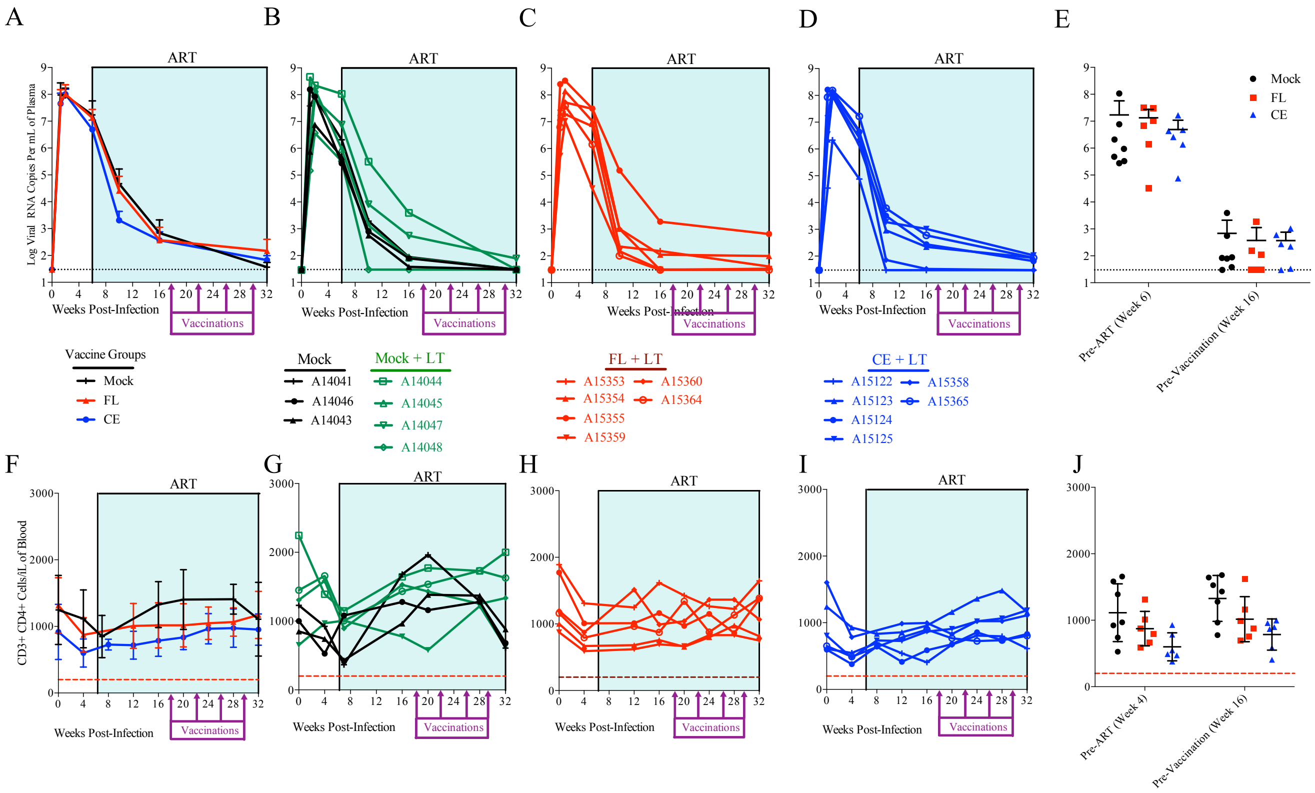

Figure 3.4. Viral load and CD4$^+$ T-cell counts stratified by vaccination group. Shown are plasma viral loads are measured q-RT-PCR throughout the study grouped by vaccine cohort (**A**), individual curves by vaccination group (**B-D**), and at pre-ART (Week 6) and pre-vaccination (Week 16) (**E**). The dotted line represents the limit of detection at 30 RNA copies per mL of plasma. CD4 T-cell counts were determined in the blood by complete blood counts (CBC) on fresh blood and are shown grouped by vaccination cohort (**F**), individual curves by vaccination group (**G-I**) and pre-ART (Week 4) and pre-vaccination (Week 16) (**J**). Error bars are averages +/- standard deviations. The red dashed line (200 CD4 T-cells per μL of blood) indicates a simian-AIDS defining criteria.

ELISpot data, the FL group had a greater frequency of non-CE Gag-specific IFN-γ^+ T-cells, that was driven primarily by elevated CD8$^+$ and not CD4$^+$ T-cell responses, relative to the CE and mock groups (**Figure 3.5 B**). In contrast, the CE group had greater CE-specific total T-cells and CD8$^+$, but not CD4$^+$, T-cells compared to the mock and FL vaccine groups (**Figure 3.5 C**). In the CE group, the 4th vaccination of CE + FL immunogens increased non-CE Gag-specific IFN-γ^+ T-cells (**Figure 3.5C**). Taken together, these data indicate that the FL and CE vaccines elicited cellular immunity in the PBMC to non-CE and CE portions of Gag respectively in cART treated SIV-infected macaques.

CE vaccination broadens immune targeting of CE sequences in SIV-infected ART treated macaques

Broadening immune responses targeting multiple conserved sequences will likely be essential to blocking viral escape pathways and thus providing durable control of viremia in the absence of cART. To determine if CE vaccination broadened cellular targeting of multiple CE, we measured CE-specific T-cells in PBMC by ELISpot. The number of CE targeted by ELISpot in the blood were few and similar among vaccine groups prior to vaccination (Week 0, Week 18) (**Figure 3.6A**), with a range of CE responses from 0-3 and 3/7 Mock, 2/6 FL, and 1/6 CE animals responding to any CE sequence. In contrast, following the fourth vaccination (Week 32) there was significantly broader CE-specific T-cellular immune responses in CE vaccinated animals with 5/6 animals responding to CE with a range of 0-4 responses, as compared to 1/7 mock and 2/6 FL animals responding to 0-1 CE (**Figure 3.6A**). The mock and FL groups only targeted CE3 or CE5, whereas the CE vaccinated macaques targeted CE3,4,5 and 6 (**Table 3.2**).

We next asked whether a p27CE therapeutic vaccine could boost or induce *de novo* CE-specific antibody responses to linear epitopes in SIV-infected cART treated macaques. Previously, we demonstrated in uninfected macaques that a p24CE, but not the full-length

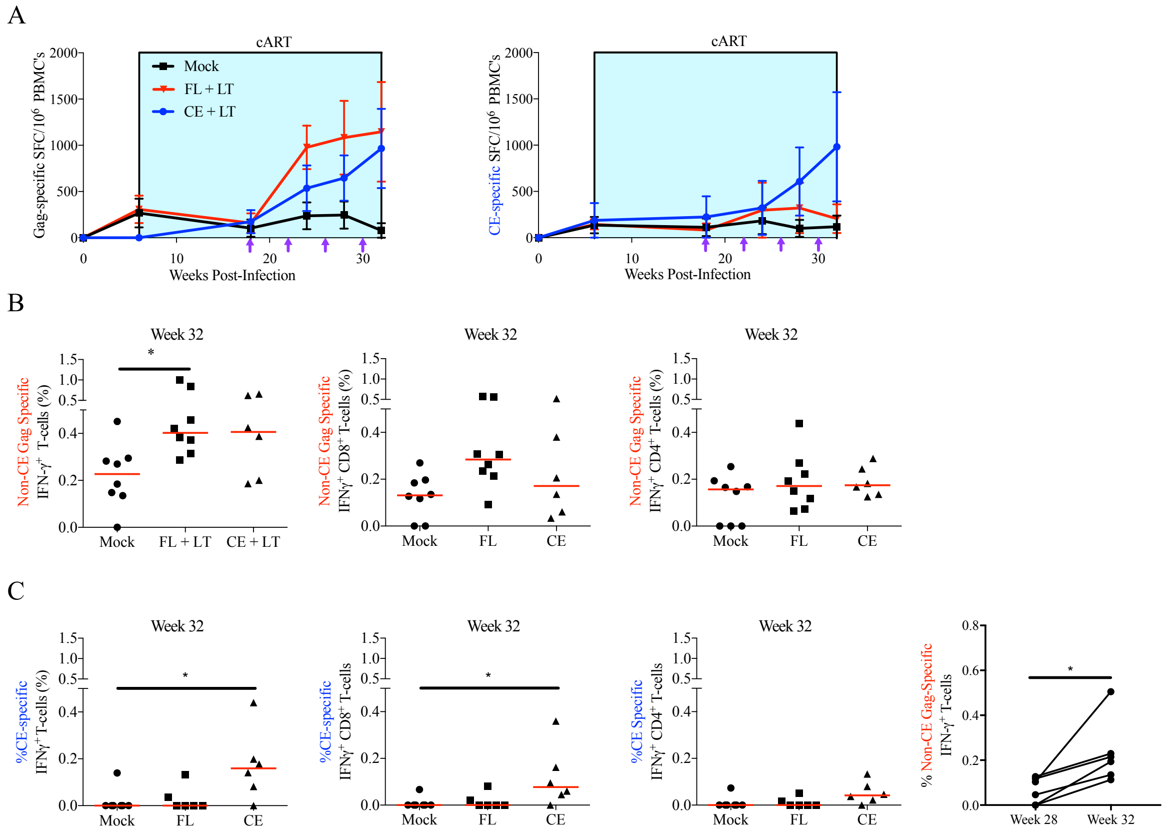

Figure 3.5. Therapeutic CE+LT, but not FL+LT, vaccination increases cellular and humoral CE-specific immunity. Peripheral blood mononuclear cells (PBMC's) were stimulated with Gag or CE peptides and specific IFN-γ T-cell responses (mean ± SEM) throughout the study that are above background DMSO stimulated levels by ELISpot (**A**). PBMC at Week 32 (2 weeks post 4th vaccination) were stimulated with non-CE Gag peptides and displayed are non-CE (**B**) or CE (**C**) Gag-specific total T-cell $CD8^+$, and $CD4^+$ T-cell responses. Shown are Non-CE Gag specific IFN-γ T-cell in the CE group at Week 28 and 32. Red lines indicate median values. Statistical differences between immune responses were determined by a one-way ANOVA. Differences in Non-CE Gag specific T-cells in the CE vaccine group between weeks 28 and Week 32 was determined by a Wilcoxon text.

p55^{Gag} DNA vaccine was able to induce broad antibody responses to several linear epitopes by ELISA[127]. In this study, the breadth of CE-specific antibodies was similar among all vaccine groups prior to infection or vaccination (Week 0, Week 18) with 3/7 mock vaccinated animals responding to a range of 0-1 CE whereas 0/6 FL and 0/6 CE had detectable CE specific antibodies responses prior to vaccination (**Figure 3.6B**). CE vaccination significantly increased the breadth of CE-specific antibodies, with 5/6 CE vaccinated animals having detectable CE specific responses with a range of 0-5, compared to 1/7 Mock and 2/6 FL responding with a range of 0-1 CE targeted. CE vaccinated animals had the most broadly specific CE specific antibody responses with 50% of CE vaccinated animals developing humoral responses to CE7 (**Figure 3.7**).

CE-specific cellular and humoral immune responses are biased toward different CE sequences

Previously, we have demonstrated that different CE tend to elicit either cellular or humoral immunity in SIV uninfected rhesus macaques[126,127,129]. However, it was unclear if these observations would extend to therapeutic CE DNA vaccination. To address this, we created a table of CE responses to determine if SIV-infected cART treated animals have a different specificity of CE targeting prior and after vaccination (**Figure 3.7**). Pre-vaccine 11/19 animals did not have any detectable CE responses and CE responders had a range of 0-3 CE targeted. After vaccination, Mock vaccinated macaques, only a single macaque had humoral immune responses to CE3 and another had cellular immune responses to CE5. In FL vaccinated macaques, only targeted CE1, 3, and 5. Conversely, CE vaccinated had the broadest CE-specific immune repertoire and were uniquely able to target CE2, 4, 6, and 7 at Week 32. Furthermore, only CE vaccinated animals, A15123 and A15365, had both cellular and antibody responses to CE3 and CE5 respectively. Additionally, the increase in CE targeting whether cellular and/or humoral was significantly increased relative to Mock or FL vaccinated macaques. Taken together, these data indicate the CE responses tend to be biased towards either cellular or humoral immunity.

Viral load and PD-1 expression on CD8$^+$ T-cells limit therapeutic vaccine immunogenicity.

Macaques exhibited a range in their ability to respond to cART or to therapeutic FL or CE DNA vaccines that was not clearly associated with TRIM5 or MHC genetics. Therefore, we sought to better understand the immunological correlates of therapeutic vaccine immunogenicity.

		Pre-vaccine (Week 18) : SFC per million PBMC							Post-Vaccine (Week 32) : SFC per million PBMC						
Animal #	Vaccine (DNA)	CE1	CE2	CE3	CE4	CE5	CE6	CE7	CE1	CE2	CE3	CE4	CE5	CE6	CE7
A14041	Mock														
A14043		116													
A14046															
A14044															
A14045						656							680		
A14047															
A14048		64													
A15353	FL + LT														
A15354								55					124		
A15355															
A15359															
A15360				385		55					265				
A15364															
A15122	CE + LT										1790	380	1360	180	
A15123											190				
A15124											200				
A15125											125		135		
A15358															
A15365						775	505	65					1010	520	

Color	SFC per million PBMC
🟥	>1000
🟧	500-999
🟩	200-499
🟦	1-199
⬜	0

Table 3.2. Specificity and magnitude before and after therapeutic DNA vaccination. Shown is a heat map of the spot forming cells (SFC) per million PBMC from cryopreserved PBMC stimulated with individual CE peptide pools by ELISpot at time points before (Week 18) and after vaccination (Week 32). The numbers in each box reflect the SFC/10^6 stimulated cells, and undetectable responses are blank.

First, we sought to determine if responsiveness to cART could predict therapeutic vaccine immunogenicity. We defined cART responsiveness by a greater absolute or relative magnitude of CD4$^+$ T-cells as well as limiting viremia. To more fully assess the magnitude of the immune responses we calculated the area under the curve (AUC) of the SIV-specific ELISpot responses over the course of vaccinations (Weeks 18-32). There was no correlation between the absolute magnitude of CD4$^+$ T-cells measured prior to vaccination (Week 18) and the ELISpot AUC (r = -0.1576, P = 0.6231; **Figure 3.8A**). Similarly, there was no significant correlations between relative magnitude of CD4$^+$ T-cells measured as a percent (%) of pre-infection levels and immunogenicity by ELISpot AUC (r = -0.1506, P = 0.6389; **Figure 3.8B).**

Next, we examined the relationship between viremia and vaccine immunogenicity. Plasma viral load prior to vaccination, as measured by q-RT-PCR, was inversely correlated with ELISpot AUC (r = -0.6380, P = 0.0287; **Figure 3.8C**). Additionally, the percent decrease in viremia pre-vaccine, or the % ART suppression, was strongly positively correlated with ELISpot AUC (r = 0.7337, P = 0.0087; **Figure 3.8D**). These data demonstrate that the ability to respond to cART therapy prior to vaccination was positively correlated with better therapeutic vaccine immunogenicity.

Previously, viral replication has been demonstrated to increase cellular exhaustion via upregulation of programmed death-1 (PD-1)[66]. We sought to explore if viral replication induced T-cellular exhaustion may explain the connection between viral load and vaccine immunogenicity. Viral replication pre-vaccination (Week 18) was positively correlated with the frequency (%) of PD-1$^+$ of CD8$^+$ T-cells (r = 0.6303, P = 0.0022; **Figure 3.8E**) but not on CD4$^+$ T-cells pre-vaccination (Week 18) (r = 0.2693, P = 0.2378; **Figure 3.8F**). As expected, therapeutic immunogenicity as measured by SIV-specific ELISpot area under the curve (AUC) responses were inversely correlated with PD-1 frequencies (%) on CD8$^+$ (r= -6670, P = 0.0110; **Figure 3.8G**) but not CD4$^+$ (**Figure 3.8H**) T-cells. In summary, these findings may indicate the viral

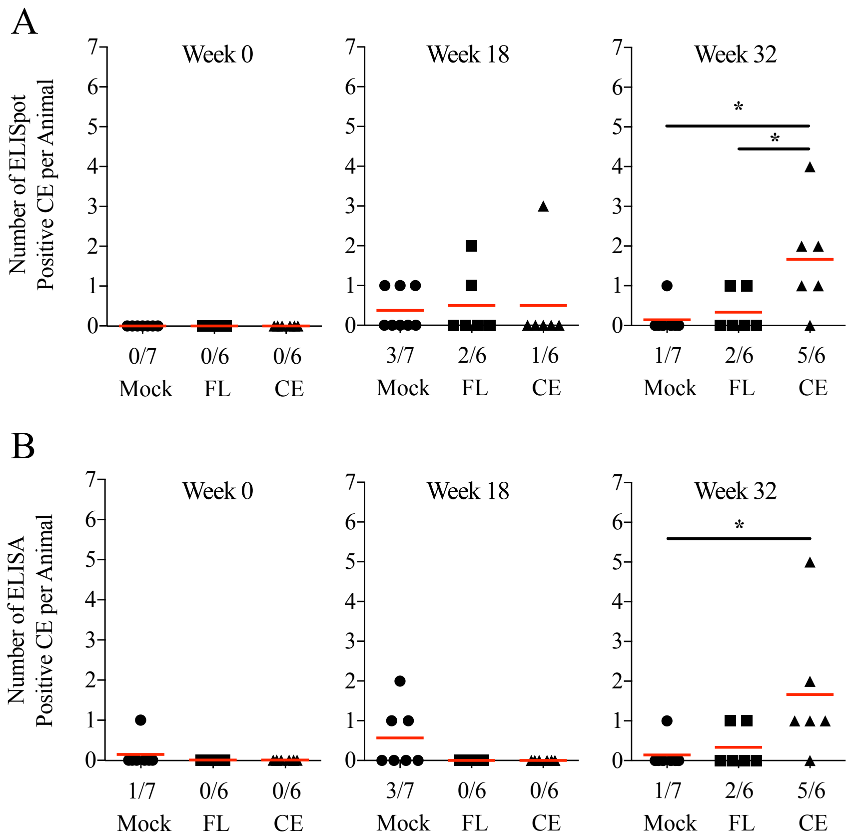

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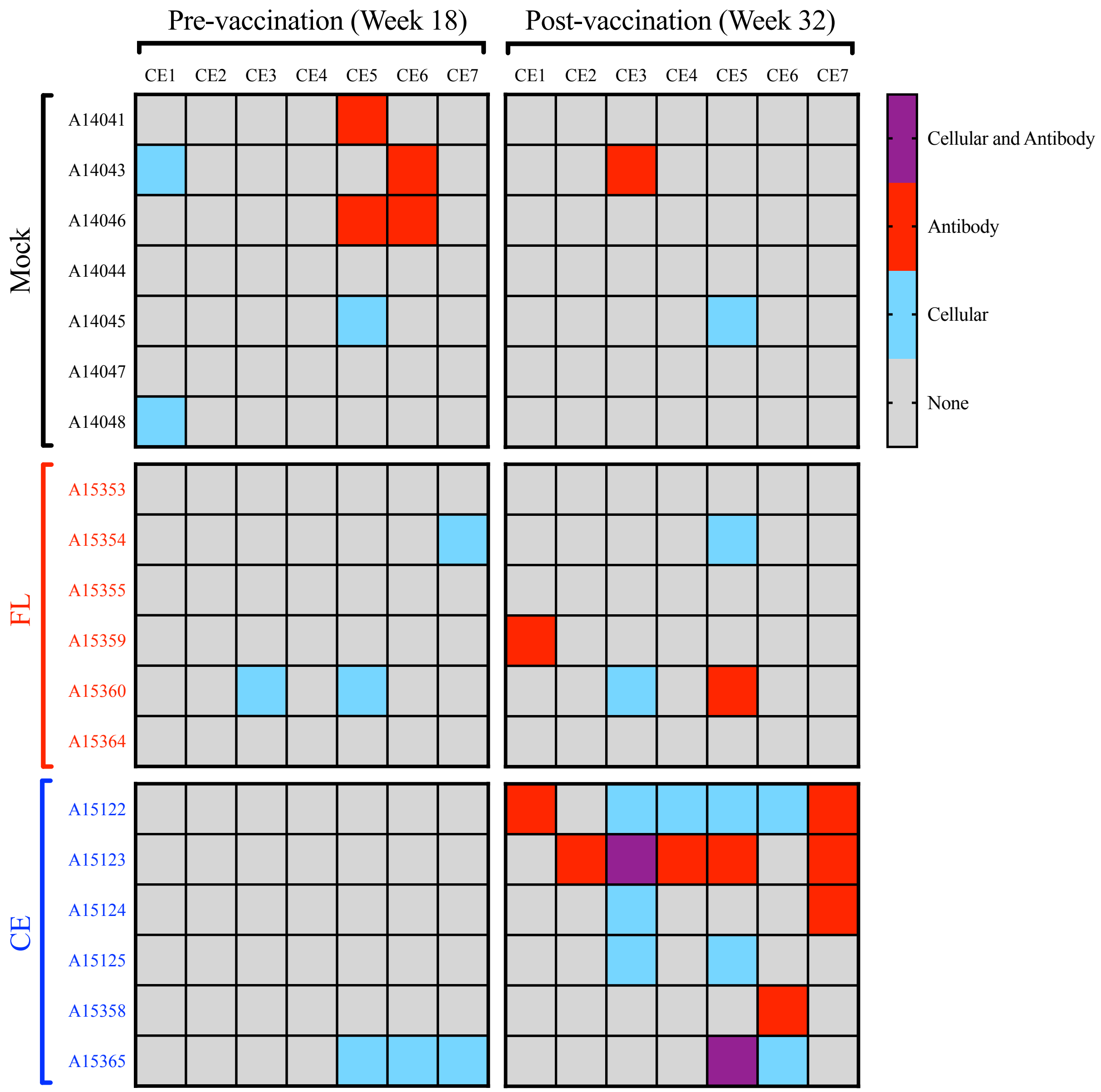
Figure 3.6. Breadth of CE-specific cellular or humoral responses. The breadth of CE-specific T-cellular responses were measured from cryopreserved peripheral blood mononuclear cells (PBMC's) through stimulation with peptides (15mers & 10mers) corresponding to CE amino acid sequences by ELISpot at time-points before SIV infection (Week 0), vaccine baseline (Week 18), and two weeks after the 4th vaccination, (Week 32). The breadth of CE-specific IgG antibody responses in the plasma were measured by a Pepscan ELISA against each CE pool, (1-7, 15mers overlapping by 11 amino acids) with responses considered positive if the OD_{450} was above 0.05 as well as 3 standard deviations above DMSO controls. Red lines indicate median values for cellular and humoral CE responses. Differences between CE-specific immune responses between vaccine groups were determined by a one-way ANOVA with a Tukey's multiple comparisons test with an alpha of 0.05.



		Pre-vaccination (Week 18)							Post-vaccination (Week 32)						
		CE1	CE2	CE3	CE4	CE5	CE6	CE7	CE1	CE2	CE3	CE4	CE5	CE6	CE7
Mock	A14041	⬜	⬜	⬜	⬜	🟥	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜
	A14043	🟦	⬜	⬜	⬜	⬜	🟥	⬜	⬜	⬜	🟥	⬜	⬜	⬜	⬜
	A14046	⬜	⬜	⬜	⬜	🟥	🟥	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜
	A14044	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜
	A14045	⬜	⬜	⬜	⬜	🟦	⬜	⬜	⬜	⬜	⬜	⬜	🟦	⬜	⬜
	A14047	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜
	A14048	🟦	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜
FL	A15353	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜
	A15354	⬜	⬜	⬜	⬜	⬜	⬜	🟦	⬜	⬜	⬜	⬜	🟦	⬜	⬜
	A15355	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜
	A15359	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟥	⬜	⬜	⬜	⬜	⬜	⬜
	A15360	⬜	⬜	🟦	⬜	🟦	⬜	⬜	⬜	⬜	🟦	⬜	🟥	⬜	⬜
	A15364	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜
CE	A15122	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟥	⬜	🟦	🟦	🟦	🟦	🟥
	A15123	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟥	🟪	🟥	🟥	⬜	🟥
	A15124	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟦	⬜	⬜	⬜	🟥
	A15125	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟦	⬜	🟦	⬜	⬜
	A15358	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟥	⬜
	A15365	⬜	⬜	⬜	⬜	🟦	🟦	🟦	⬜	⬜	⬜	⬜	🟪	🟦	⬜

🟪 Cellular and Antibody
🟥 Antibody
🟦 Cellular
⬜ None

Figure 3.7. Specificity of CE-specific cellular or humoral responses. CE-specific T-cell and antibody responses were mapped from cryopreserved PBMC and plasma by ELISpot and ELISA respectively before (Week 18) and after vaccination (Week 32). Each animal's CE responses were color coded as having no response (gray), cellular (blue), antibody (red), or cellular and antibody positive (purple).

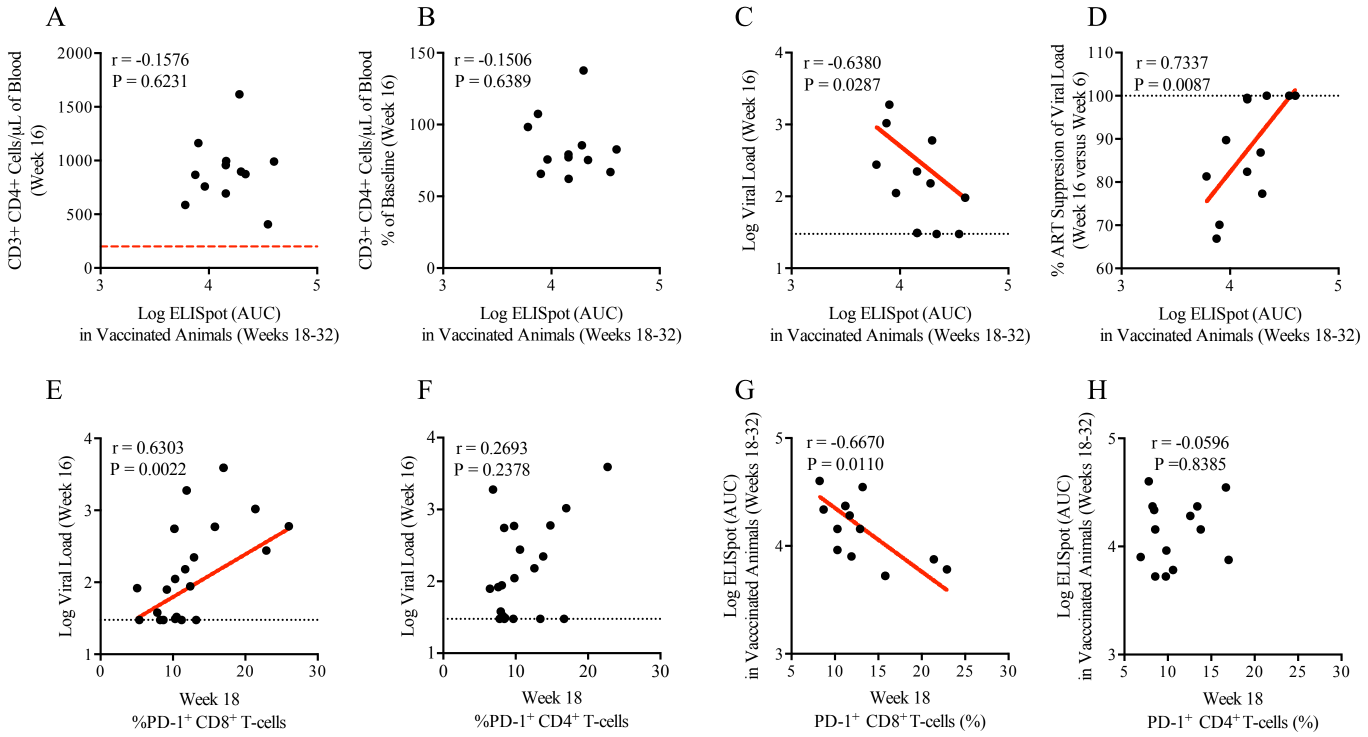


Figure 3.8. PD-1 expression on CD8$^+$ T-cells prior to vaccination is inversely associated with vaccine immunogenicity. Shown are correlations between absolute (**A**) and percent from baseline (**B**) of CD4$^+$ T-cells in the blood versus area under the curve (AUC) of the SIV-specific SFC/10^6 PBMC responses as measured by ELISpot in FL+LT and CE + LT vaccinated animals over the course of vaccination (Weeks 18-32). The dashed red line at 200 CD4$^+$ T-cells in the blood denotes a simian AIDS defining CD4 depletion. Plasma viral loads measured by q-RT-PCR prior to vaccination (Week 16) (**C**), as well as the percent decrease in plasma viral load from pre-ART (Week 6) to pre-vaccination (**D**), versus ELISpot AUC immunogenicity. The dotted line in panel C and D indicate the limit of detection of 50 copies of RNA per mL of plasma and the maximal viral suppression respectively. Plasma viral load versus the frequency of PD-1 on CD8$^+$ (**E**) and CD4$^+$ (**F**) T-cells prior to vaccination (Week 18). The ELISpot AUC versus the PD-1 frequencies (%) on CD8$^+$ (**G**) and CD4$^+$ (**H**) T-cells prior to vaccination (Week 18).

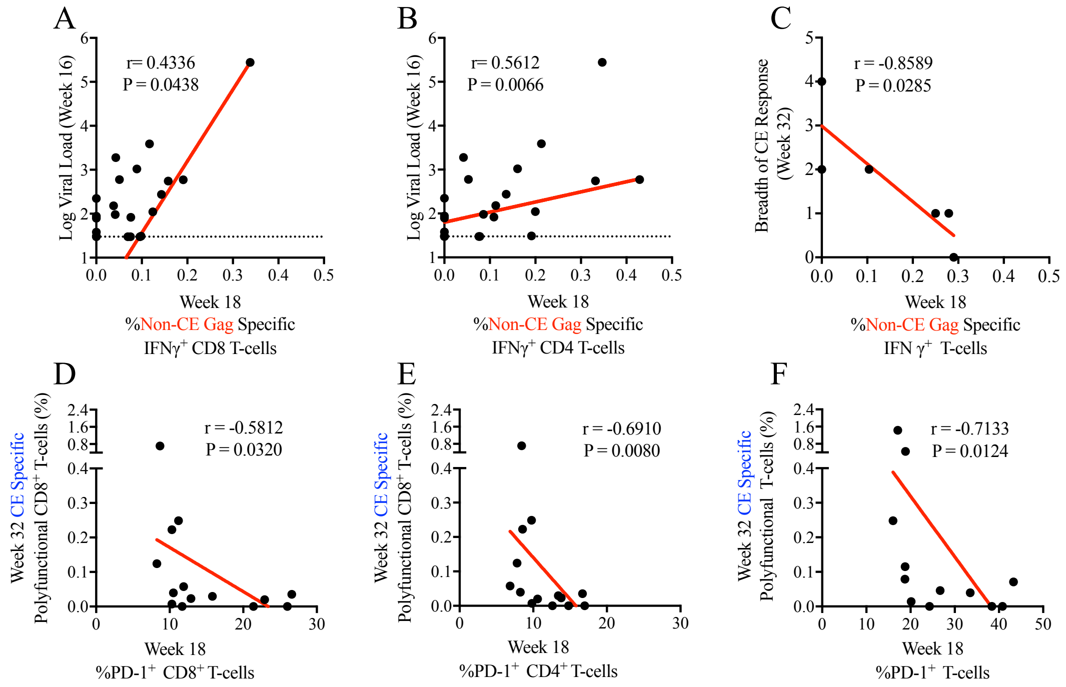


Figure 3.9. Immune exhaustion via PD-1 expression limits therapeutic vaccine immunogenicity.

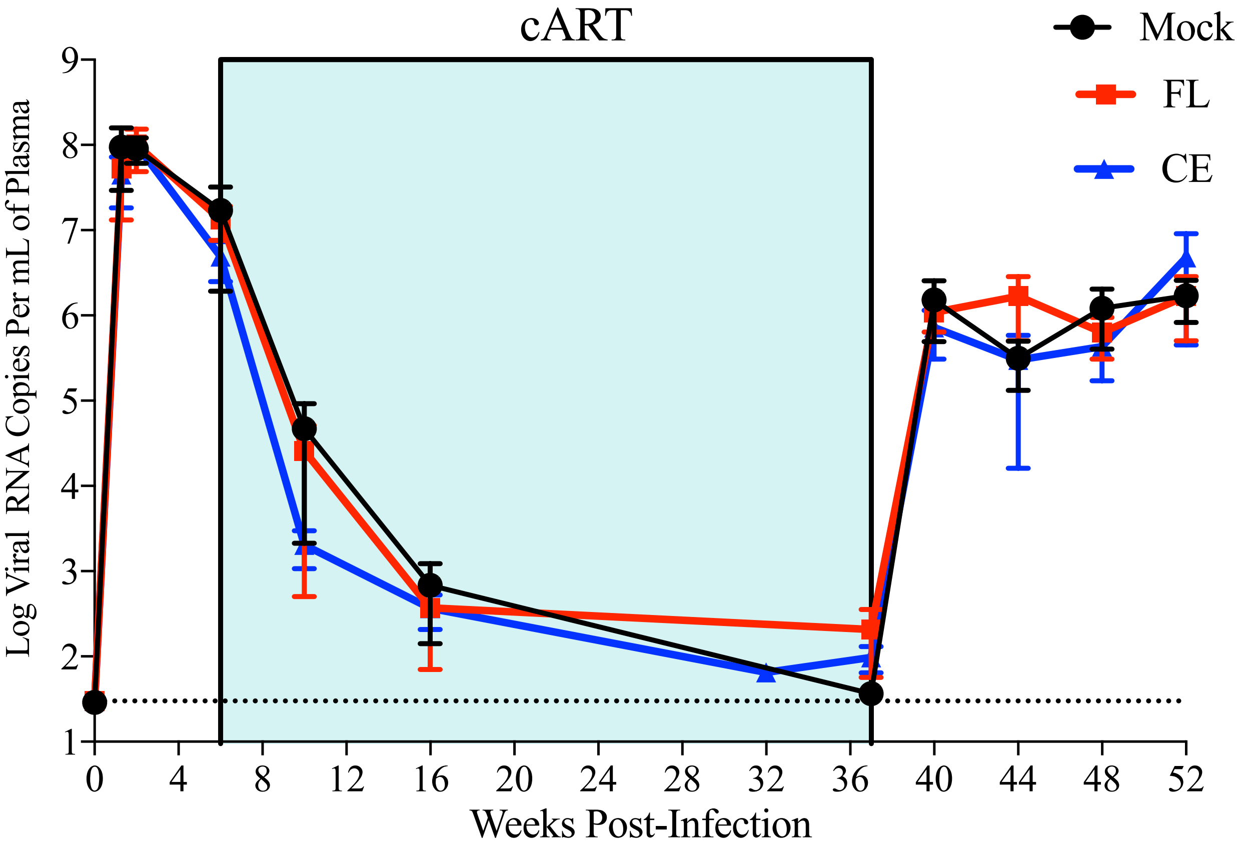
Correlations between plasma viral load and non-CE Gag specific IFN-γ$^+$ CD8$^+$ (**A**) and CD4$^+$ T-cells (**B**), measured from cryopreserved PBMC stimulated with non-CE Gag peptides in an intracellular cytokine staining assay. The dotted line indicates the viral load limit of detection (30 RNA copes per mL of plasma). The correlation between the breadth of CE specific T-cellular immune responses measured by ELISpot and pre-vaccination (Week 18) frequencies of non-CE Gag specific IFN-γ$^+$ T-cells (**C**). Frequency of PD-1$^+$ CD8$^+$ (**D**), CD4$^+$ (**E**), and total T-cells (**F**) pre-vaccination (Week 18) and CE-specific poly-functional T-cells two weeks post the fourth vaccination, measured by stimulating PBMC's with CE peptides in an intracellular cytokine staining assay (**L**). All r and P values displayed are the results of Spearman rank correlation tests.

replication induced PD-1 expression on $CD8^+$ T-cells that limits therapeutic vaccine immunogenicity.

Immunodominant responses and PD-1 expression limit vaccine induced CE vaccine induced breadth and polyfunctional T-cell responses

A potential hurdle to re-directing T-cell responses to conserved and subdominant regions of the proteome may be the presence of immunodominant non-CE Gag-specific T-cells that may preferentially expand upon CE + FL vaccine co-immunization. We sought to determine if these immunodominant responses were detectable after 3 months of cART and whether these responses may have limited CE vaccine breadth and polyfunctional immunity. Viral load on 3 months of cART was positively correlated with non-CE Gag $CD8^+$ (r = 0.4336, P = 0.0438; **Figure 3.9A**) and $CD4^+$ (r = 0.5612, P = 0.0066; **Figure 3.9B**) T-cell responses. Importantly, these immunodominant responses prior to vaccination were inversely correlated with CE vaccine induced CE breadth, suggesting they may limit the ability of the vaccine to broaden CE-specific immunity (r = -0.8589, P = 0.0285, **Figure 3.9C**).

Polyfunctional HIV/SIV-specific T-cellular immune responses have been implicated in the control in viral replication and may be important for vaccine induced viral control[60]. Therefore, we sought to determine if the frequency of $PD\text{-}1^+/CD8^+$ T-cells prior to vaccination may limit polyfunctional CE-specific T-cell responses. Post-vaccination (Week 32) polyfunctional CE-specific $CD8^+$ T-cells were inversely correlated with $PD\text{-}1^+$ $CD4^+$ (r=-0.5812, P = 0.0320, **Figure 3.9D**) and $CD8^+$ (r=-0.6910, P = 0.0080, **Figure 3.9E**). Additionally, there was an inverse association between pre-vaccination $PD\text{-}1^+$ T-cells and post-vaccination polyfunctional CE-specific T-cells (r=-0.7133, P=0.0124, **Figure 3.9F**). These findings suggest that while the CE vaccination regimen was immunogenic, it may be encumbered by immunodominant T-cell responses and PD-1 expression.



Supplemental Figure 3.1. CE vaccination does not alter viral load post-ATI. Plasma viral RNA levels were determined by a q-RT-PCR with a limit of detection of 30 copies per 1 mL of plasma (dashed line).

To determine the efficacy of the vaccines, all animals underwent an ATI at 37 wpi. Two weeks following the ATI (39 wpi) viremia increased to similar concentrations in the mock, FL, and CE vaccine groups (Supplemental Figure 3.1). Furthremore, the plasma viral loads were similar among all treatment groups for months following the ATI. These findings indicate that neither the FL or CE vaccine groups had therapeutic efficacy.

Discussion:

The data reported here are the first to show that a therapeutic SIV CE DNA vaccine can broaden cellular and humoral targeting of immunologically subdominant viral Gag sequences in SIV-infected cART treated macaques. Additionally, we observed that although the FL immunogens contain CE sequences in the vaccine they tend to bias the immune responses to immunodominant non-CE portions of the proteome. These divergent T-cell specificities between CE and FL immunogens are consistent with data from our prophylactic CE vaccine studies and findings from chronically SHIV infected macaques[129,374]. However, we identified that viral replication, immunodominant responses, and PD-1 expression may be factors that limit therapeutic vaccine responses generally, and CE immunogenicity specifically.

These findings build upon our previous therapeutic vaccine studies employing an epitope-based PMED DNA vaccine in SIVB670 infected macaques on a PMPA monotherapy[383]. However, the immunogen used previously primarily contained epitopes to immunodominant regions of the proteome that may select for CTL escape variants and reduce the long-term efficacy of these vaccines. In contrast, the CE immunogen contains 124 amino acids among 7 conserved sequences and therefore contains larger sequences than epitope based “beads on a string” type immunogens.

Our previous therapeutic CE vaccine studied employed SHIV infected controller macaques with low to undetectable viremia and demonstrated a substantial broadening of CE-specific immune responses[374]. Our present study also indicates an enhanced capacity of CE immunogens to direct T-cellular immunity to subdominant and conserved sequences in the immunodominant setting of viral infection. However, the CE vaccinated SHIV infected macaques[374] generally had more robust vaccine induced CE-specific T-cell responses relative to the SIV infected cART treated macaques. The superior CE vaccine immunogenicity in the SHIV infected animals compared to the SIV infected animals may be due to greater CE specific responses prior to immunization.

Encouragingly we found broader cellular and humoral CE-specific responses in CE vaccinated subjects when compared to the mock and FL groups. Our findings demonstrating enhanced Gag CE-specific antibody responses is consistent with our findings in un-infected macaques[127,134]. While Gag-specific antibodies of any kind are unlikely to have an antiviral effect, these findings are an important proof of concept that CE immunogens can elicit antibody responses in a therapeutic setting. Importantly, we have recently demonstrated the immunogenicity of an HIV Env derived CE in uninfected macaques[134]. Future experiments will be necessary to determine if CE vaccination can induce CE-specific Env antibodies in SIV/SHIV infected macaques, and whether such antibodies are broadly neutralizing or can mediate antibody-dependent cellular cytotoxicity (ADCC).

Importantly, we identified correlates of immunogenicity including viral load, immunodominant responses, and PD-1 frequencies on $CD8^+$ T-cells. While the triple drug therapy of PMPA, FTC, and Raltegravir was more effective at reducing SIVΔB670 replication compared to our previous studies[48], many of the macaques still had detectable viremia at the time of vaccination. Our findings that viral load was inversely correlated with vaccine immunogenicity is consistent with initial therapeutic HIV immunization studies in HIV infected humans[89]. While the exact mechanisms of viral interference with vaccine immunogenicity are not fully known, we observed positive correlations between viremia and immunodominant responses as well as $PD\text{-}1^+/CD8^+$ T-cell frequencies. PD-1 expression has been associated with limited T-cell function and exhaustion[66,71,72], however its expression can also be associated with activation or differentiation[384,385]. Our findings are correlative and limited to a relatively small number of animals and thus should be interpreted with caution, however they do support the concept that cellular exhaustion may play a role in limiting T-cell based vaccine responses. Further intervention based studies will be required to determine if there is a causal relationship between PD-1 expression and vaccine immune responses. In support of this concept, SIV-infected macaques at various stages of infection have been treated with anti-PD-1 antibodies that enhanced SIV-specific immunity and reduced viremia, microbial translocation, and inflammation[77,79]. Encouragingly, Gay *et al* recently reported that treatment of HIV infected people with an anti-PD-L1 was safe and enhanced Gag-specific $CD8^+$ T-cell responses[386]. Furthermore, treatment of SIV uninfected macaques with an anti-PD-1 antibody improved immune responses to prophylactic SIV vaccination[81]. Reversal of exhaustion may be of particular importance to boosting CE-specific responses because other studies have indicated that subdominant responses may be more susceptible to immune exhaustion[360]. In all, these findings strongly support the use of anti-PD-1 antibodies to improve the immunogenicity and efficacy of therapeutic SIV/HIV vaccines.

Immunodominant responses prior to therapeutic CE vaccination may interefere the FL or CE + FL boost. I observed the pre-vaccination frequencies of non-CE-specific T-cells inversely correlated with CE breadth after vaccination. Furthermore, these immunodominant (non-CE) responses were increased after the CE+FL boost. These findings suggest that efforts to reduce the frequency of immunodominant responses, such as extending the duration of cART prior to immunization, may improve the immunogenicity of CE vaccines.

Follwing an ATI, the similar viral loads between all of the vaccine groups indicate neither the FL or CE vaccines had a therapeutic benefit. While the exact mechanisms for this lack of benfit are not known, it may be due to CE-specific T-cellular exhaustion, lack of CE-specific T-cells in relevant anatomical viral reservoirs (lymph node or gut), or a re-establishment of the canonical immunodominance hierarchy. However, additional analyses will be required to determine what mechanism(s) may be contributing to a lack of anti-viral efficacy.

In summary, these findings demonstrate for the first time that gene gun delivery of Gag CE DNA can elicit potent cellular and humoral immune responses in the context of cART treatment of a highly pathogenic SIV infection. Incorporating CE from other viral proteins, including Env, may be necessary to fully block viral reemergence following antiretroviral therapy and provide a functional cure of HIV. Additionally, adding immune checkpoint inhibitors, such as anti-PD-1 antibodies, prior to therapeutic immunization may more potently reinvigorate the immune responses and provide and enhancement of CE vaccine immunogenicity and efficacy. These promising immunogenicity findings warrant the further development of gene gun delivery of CE DNA vaccines to treat HIV infection.

Chapter 4: T-cellular exhaustion is associated with gut disruption and diminished therapeutic DNA vaccine immunogenicity in SIV infected, ART treated macaques.

“The first virtue in a soldier is endurance of fatigue; courage is only the second virtue.”

-Napoleon Bonaparte

Paul Munson, Megan O’Connor, Hillary Tunggal, James Fuller, Deborah Heydenberg Fuller

Abstract:

A major barrier to T-cell based therapeutic HIV vaccines is the inability of combination antiretroviral therapy (cART) to fully reverse the immune dysfunction induced by viral infection. T-cellular “exhaustion”, is typically caused by antigen overstimulation and results in T-cells with a limited functional capacity. Various exhaustion markers are increased in bulk $CD4^+$ and $CD8^+$ T-cell populations upon HIV/SIV-infection that can remain elevated in the setting of low to undetectable viremia during cART. In this study, we evaluated frequencies of exhaustion markers (TIGIT/PD-1/SLAM/CTLA-4/LAG-3) on T-cells in the blood of SIV infected rhesus macaques to determine if gut disruption may underlie elevated exhaustion marker frequency, and if T-cellular exhaustion may limit therapeutic DNA vaccine immunogenicity. We found that exhaustion markers remained elevated above pre-infection levels on $CD4^+$ and $CD8^+$ central memory T-cells (T_{CM}) in the PBMC in SIV∆B670 infected macaques, cART treated macaques. These exhaustion frequencies in the blood were correlated with disruption of the Th17 and Treg subsets in the colon, and microbial translocation (plasma sCD14) was correlated with exhausted $CD4^+$ T-cells. Additionally, we found that exhausted (TIGIT/PD-1^+) $CD8^+$ T-cells prior to therapeutic immunization were inversely correlated with vaccine immunogenicity. In all, these findings suggest that gut disruption may underlie peripheral T-cellular exhaustion that limits therapeutic HIV/SIV vaccine immunogenicity.

Introduction:

While cART is effective at reducing HIV/SIV replication and preventing AIDS[387], it cannot deplete the viral reservoir[373], or fully reverse T-cellular exhaustion[388]. Immune “exhaustion” refers to a limited functional capacity of immune cells that is mainly driven by antigen over-stimulation[389]. In particular, the exhaustion marker TIGIT has been correlated with disease progression and remained elevated on $CD8^+$ T-cells in HIV-infected ART suppressed humans when compared to un-infected individuals[68,390]. Additional surface markers including PD-1[66,71,72,391], SLAM[392], CTLA-4[393], and LAG-3[394] have been implicated in T-cell exhaustion generally, and can remain elevated on bulk T-cell populations in lentiviral infections. Given that cART results in a profound reduction in viral replication, the increased frequency of exhaustion markers on bulk T-cells during cART is likely maintained through viral antigen-independent mechanisms, such as chronic inflammation, however the exact mechanisms remained unknown[395].

Microbial translocation from the gut to the lumen is a major contributor to the sustained inflammation observed in HIV and SIV infection[30]. Th17's are an important $CD4^+$ T-cell subset for maintenance of the gut homeostasis and are preferentially depleted during HIV[396] and SIV infection[397], leading to loss of gut integrity, and translocation of microbes and microbial products into the periphery[30]. Prior work has demonstrated that cART does not fully restore the gut Th17 frequency or function in the SIV_{mac239} macaque model[398] or HIV positive men on long-term cART[399]. We sought to explore the hypothesis that T-cellular exhaustion could be driven by declines in the Th17/Treg ratio and Th17 polyfunctionality in the colon, and would impede therapeutic DNA vaccine immunogenicity. Herein, we demonstrate for the first time that T-cellular immune exhaustion is correlated with dysregulation of the Th17's in the gut mucosa, and exhausted (TIGIT/$PD\text{-}1^+$) $CD8^+$ T-cells are inversely associated with therapeutic DNA vaccine immunogenicity in ART treated, SIVΔB670 infected macaques. Understanding the factors contributing to T-cell exhaustion in HIV/SIV infection, and how exhaustion can limit therapeutic HIV/SIV vaccine immunogenicity could lead to better T-cell based strategies to treat or cure HIV.

Methods:

Ethics Statement and Animal Care

The animal care is consistent with Chapter 2 of this thesis.

Viral Challenge and AIDS Monitoring

Macaques were challenged with SIV ΔB670 and monitored for AIDS as described in Chapter 2.

Plasma Viral Load Quantification. Viral load quantification is described in Chapter 3..

Antiretroviral Therapy

The ART protocol of PMPA, FTC, and Raltegravir is the same as described in Chapter 3.

$CD4^+$ and $CD8^+$ T-cell Blood Immunophenotyping

Blood immunophenotyping was described in Chapter 3.

DNA Vaccinations

DNA vaccines were delivered by gene gun as described previously in Chapter 3.

Exhaustion markers assay

Cryopreserved PBMCs were thawed, washed with PBS, and were stained using a LIVE/DEAD® Aqua (ThermoFisher®) amine dye. Next, cells were washed with PBS then stained with a surface stain antibody cocktail including: CD3 Brilliant Violet (BV) 650 (Sp34-2, BD Biosciences), CD4 BV605 (OKT4, BD Biosciences), CD25 APC-R700 (2A3, BD), CD8 BV710 (RPA-T8, BD Biosciences), CD28 PE-CF594 (CD28.2, BD Biosciences), CD95 PerCP-eFluor710 (Dx2, ThermoFisher), PD-1 BV785 (EHI12.2H7, BioLegend), TIGIT FITC (MBSA43, ThermoFisher), SLAM BV421 (A12, BD), CTLA-4 PECy5 (BNI3, BD), and LAG-3 PE (polyclonal, R&D). Cells were then washed then fixed with 1% paraformaldehyde (Sigma) and collected on an LSR II (BD). Flow cytometry was analyzed in FlowJo (Version 9.7.6, Treestar Inc., Ashland, Oregon). First, doublets were excluded based on area and height gating and lymphocytes were identified by size (forward scatter) and granularity (side scatter). Next viable T-cells were identified using a Live/Dead amine dye as well as $CD3^+$. CD4 and CD8 subsets including naïve (T_N), effector memory (T_{EM}), and central memory (T_{CM}) by CD28, CD95, and CCR7 staining. Tregs ($CD3^+CD4^+CD25^+CTLA\text{-}4^+$) cells were defined by Boolean analysis and analyzed separately from non-Treg $CD4^+$ populations. Florescence minus one (FMO) controls were included for CD25, PD-1, TIGIT, SLAM, CTLA-4, and LAG-3 to accurately draw gates.

Exhaustion Index

The exhaustion index (adapted from "polyfunctionality index") was calculated using the Funky Cells software package using the following equation as previously described[400,401]:

$$Exhaustion\ Index = \sum_{i=0}^{n} F_i \cdot \left(\frac{i}{n}\right)^q \qquad (1)$$

$$100 = \sum_{i=0}^{n} F_i \qquad (2)$$

$$F_i \geq 0\ for\ all\ i \qquad (3)$$

n = number of exhaustion markers measured (5 in our study: TIGIT/PD-1/SLAM/CTLA-4/LAG-3)

i = number of exhaustion markers expressed (0, 1, 2, 3, 4, or 5)

F_i = the percentage (%) of cells expressing i number of exhaustion makers

Th17/Treg & Th17 Polyfuncitonality:

Colonic lymphocytes and were stimulated overnight with 10 ng/ml PMA (Sigma) and 1 ug/ml Ionomycin (Life Technologies) in supplemented RPMI-1640, an unstimulated sample served as the negative control. Both PMA/Ionomycin and unstimulated samples were treated overnight with 1 ug/ml Brefledin A (Sigma). The following day, cells were washed with PBS and viability was assessed using a LIVE/DEAD stain (Life Technologies) and subsequently cells were stained with a cocktail of surface markers including: BioLegend: CD4 (OKT4), CD8 (RPA-T8), BD Biosciences: CD25 (M-A251), CD3 (Sp34-2), CD45 (D058-1283). Next, cells were washed and permeabolized with True-Nuclear Transcription Buffer Set (Biologend) and stained for the intranuclear transcription factor FoxP3 (206D, BioLegend) and for intracellular cytokines: BioLegend: IL-2 (MQ1-17H12); BD Biosciences: IFNγ (B27), Ki67 (B56), TNFα (Mab11); eBioscience: IL-17A (eBio64CAP17), IL-22 (IL22J0P). Samples were acquired on a LSRII (BD Biosciences), and analyzed using FlowJo software version 9.9.4 (FlowJo, LLC). Cells were identified by excluding doublets using forward light scatter and side scatter (of light), gating on

$CD45^+$ cells, and removing dead cells with an Aqua Live/Dead viability dye, then $CD3^+$ T-cells and then subsequent gating for T-cell subsets, as described in figure legends. Th17 polyfucntionality was determined by Boolean gating for all possible combination of functions, and defined as the sum total of cells expressing 3+ cytokines in addition to IL-17. (**Methodology adapted from Dr. Megan O'Connor)**.

sCD14 ELISA.

The sCD14 quantification was determined in plasma using the Quantikine ELISA Human CD14 Immunoassay kit (R&D Systems) per the manufacture's instruction. Results were analyzed using a four parameter logistic (4-PL) function for fitting standard curves (**Methodology provided by Dr. Megan O'Connor, assay performed by Hillary Tunggal)**.

ELISpot

ELISpot analysis to quantify the frequency of SIV-specific IFN-γ spot forming cells (SFC) was performed as previous described[373]. The ELISpot area under the curve (AUC) was calculated over the time course from the time of vaccination to two weeks following the last vaccination, using GraphPad Prsim (Versioni 7, GraphPad Software, San Diego, CA).

Statistical Analyses. Statistical differences between the two groups were calculated using a two-sided Mann-Whitney and differences between multiple groups by a one or two-way ANOVA test in GraphPad Prism (Version 7, GraphPad Software, San Diego, CA). Correlations between immune responses, viral loads, and sCD14 were determined by a Spearman's rank correlation test. A P value ≤ 0.05 was considered significant for each test.

Results:

Study design, viral load, and $CD4^+$ T-cell kinetics

Rhesus macaques (N=19) were infected with SIVΔB670 intravenously as previously described[48], initiated antiretroviral therapy (ART) at 6 weeks post infection (wpi), and beginning at 30 wpi they received 5 DNA vaccinations spaced one month apart via particle mediated epidermal delivery (PMED) (**Figure 4.1A**). After SIV inoculation, macaques experienced viremia at 9 days and 2 wpi that was reduced after 6 wpi, and further reduced by cART ($p<0.0001$, **Figure 4.1B**). Within 4-6 weeks of infection $CD4^+$ T-cells declined in both the blood ($p<0.0001$, **Figure 4.1C**) and colon ($p<0.0001$, **Figure 4.1C**), and were partially restored by 22 weeks of cART (blood: $p = 0.0053$, colon: $p<0.0001$, **Figure 4.1C**). CD4/CD8 ratio declines in HIV-infected, cART treated humans has been correlated with non-AIDS morbidity [402], and we observed a decline in the CD4/CD8 ratio in the blood and colon that was partially restored by cART (**Figure 4.1D**). In summary, SIVΔB670 infection recapitulates several important features of HIV infection in humans such as robust viremia as well as declines in the $CD4^+$ T-cell count and CD4/CD8 ratio in the blood and gut within 6 wpi, that were partially reversed by cART.

Exhaustion parameter kinetics in central memory T-cells (T_{CM})

TIGT, PD-1, SLAM, CTLA-4, and LAG-3 were measured on $CD4^+$ and $CD8^+$ central memory T-cells (T_{CM}) from cryopreserved peripheral blood mononuclear cells (PBMC) by flow cytometry at -4, 6, 8 and 28 wpi. Naïve (T_N), effector memory (T_{EM}), and central memory (T_{CM}) T-cell subsets express varying levels of certain exhaustion markers, therefore analyzing bulk populations can lead to spurious findings[385], to avoid this we focused primarily on the T_{CM}

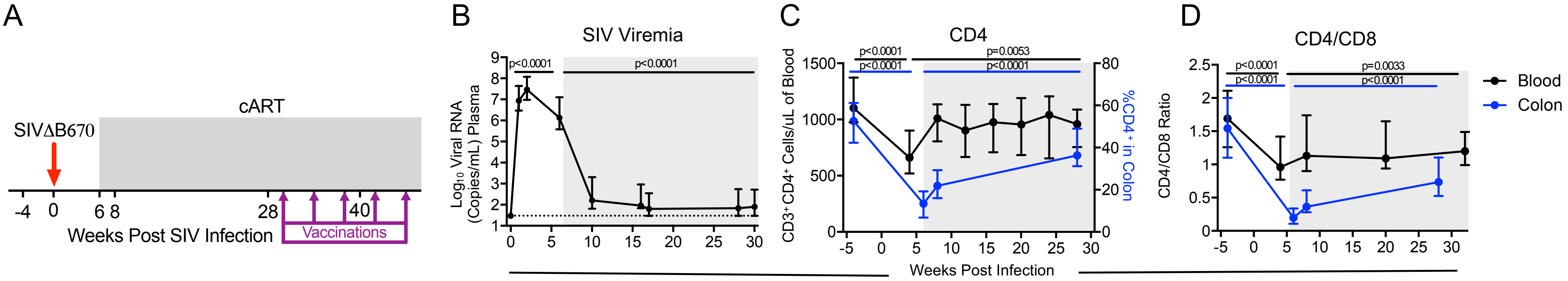


Figure 4.1. ART reduces plasma viremia and restores CD4$^+$ T-cell frequencies and the CD4/CD8 ratio in the blood and colon. (A) Indian origin male rhesus macaques (*Macaca mulatta*) were infected with SIVΔB670 intravenously (i.v.) at 0 wpi. At 6 wpi, macaques initiated ART therapy consisting of PMPA, FTC, and Raltegravir. Colon tissue was collected via a pinch biopsy at -4, 6, 8, and 28 wpi. DNA vaccinations via gene gun were performed beginning at 30 wpi, for a total of 5 vaccinations spaced one month apart. (B) Copies of SIV viral RNA in the plasma were quantified by q-RT-PCR with a limit of detection of 30 copies/mL (dashed line). (C) CD4 T-cells in the blood (black) and colon (blue) are shown throughout the study. (D) The ratio of CD4/CD8 T-cells absolute counts in the blood or percentages within the CD3$^+$ population in the colon. Shown are the medians +/- interquartile ranges. Differences between time points were determined by a Wilcoxon rank sum test. *P* values shown are adjusted for multiple comparisons.

***The SIV viral load figure and CD4 T-cell counts in the blood and colon were provided by Dr. Megan O’Connor.**

population, a subset implicated in vaccine induced prevention of AIDS in monkeys[403]. At 6 wpi, TIGIT$^+$ and TIGIT/PD-1$^+$ CD4$^+$ and CD8$^+$ T$_{CM}$ frequencies increased approximately three-fold above pre-infection levels ($p<0.0001$, **Figure 4.2A,B**). Following 2 weeks of ART, (8 wpi) TIGIT$^+$ and TIGIT/PD-1$^+$ frequencies were modestly reduced on CD8$^+$, but not CD4$^+$, T$_{CM}$ ($p<0.0001$, **Figure 4.2A,B**). After 22 weeks of ART (28 wpi.), TIGIT$^+$ and TIGIT/PD-1$^+$ frequencies were decreased on both CD4$^+$ (TIGIT : $P < 0001$, TIGIT/PD-1: $P < 0.001$) and CD8$^+$ T$_{CM}$ (TIGIT : $P < 0001$, TIGIT/PD-1: $P < 0.001$) compared to 6 wpi. However these frequencies remained elevated compared to baseline, with the exception of TIGIT/PD-1$^+$ CD8$^+$ T$_{CM}$ (**Figure 4.2A,B**). TIGIT/PD-1/SLAM$^+$ and TIGIT/PD-1/SLAM/CTLA-4$^+$ frequencies were markedly increased on CD4 ($P <0.0001$, $P <0.0001$), and minimally increased on CD8 ($P = 0.0247$, $P = 0.0301$), T$_{CM}$ following at 6 wpi, and were decreased after 22 weeks of cART, although TIGIT/PD-1/SLAM$^+$ CD4$^+$ T$_{CM}$ remained slightly elevated above baseline ($P = 0.0194$) (**Figure 4.2C,D**). CD4$^+$ T$_{CM}$ expression all five of the exhaustion markers measured (TIGIT/PD-1/SLAM/CTLA-4/LAG-3$^+$) were increased after 2 weeks of ART, ($P = 0.0037$) and returned to baseline levels after 22 weeks of cART (**Figure 4.2E**). Interestingly, CD8 T$_{CM}$ expression positive for all 5 markers were not increased above pre-infection frequencies at any time point measured and were slightly decreased after extended cART ($P = 0.0019$), **Figure 4.2E**). To more fully assess every combination of exhaustion marker expression, we measured the exhaustion index (EI), based on previously described methods[400,401]. The EI of CD8$^+$ T$_{CM}$ was increased at 6 wpi ($P < 0.0001$, **Figure 4.2F**), that was partially reduced by 2 weeks of ART ($P = 0.0123$, **Figure 4.2F**). Consistent with the EI kinetics of CD8$^+$ T$_{CM}$, the EI's of CD4$^+$ T$_{CM}$ increased at 6 wpi ($P < 0.0001$, **Figure 4.2F**). Interestingly, the EI of CD4$^+$ T$_{CM}$ continued to increase after 2 weeks of ART ($P < 0.0001$), and then 22 weeks of ART reduced the EI of CD4$^+$ T$_{CM}$ to pre-infection levels (**Figure 4.2F**). In all, SIV infection increased the expression of various exhaustion markers on CD4$^+$ and CD8$^+$ T$_{CM}$ that were partially reversed by cART.

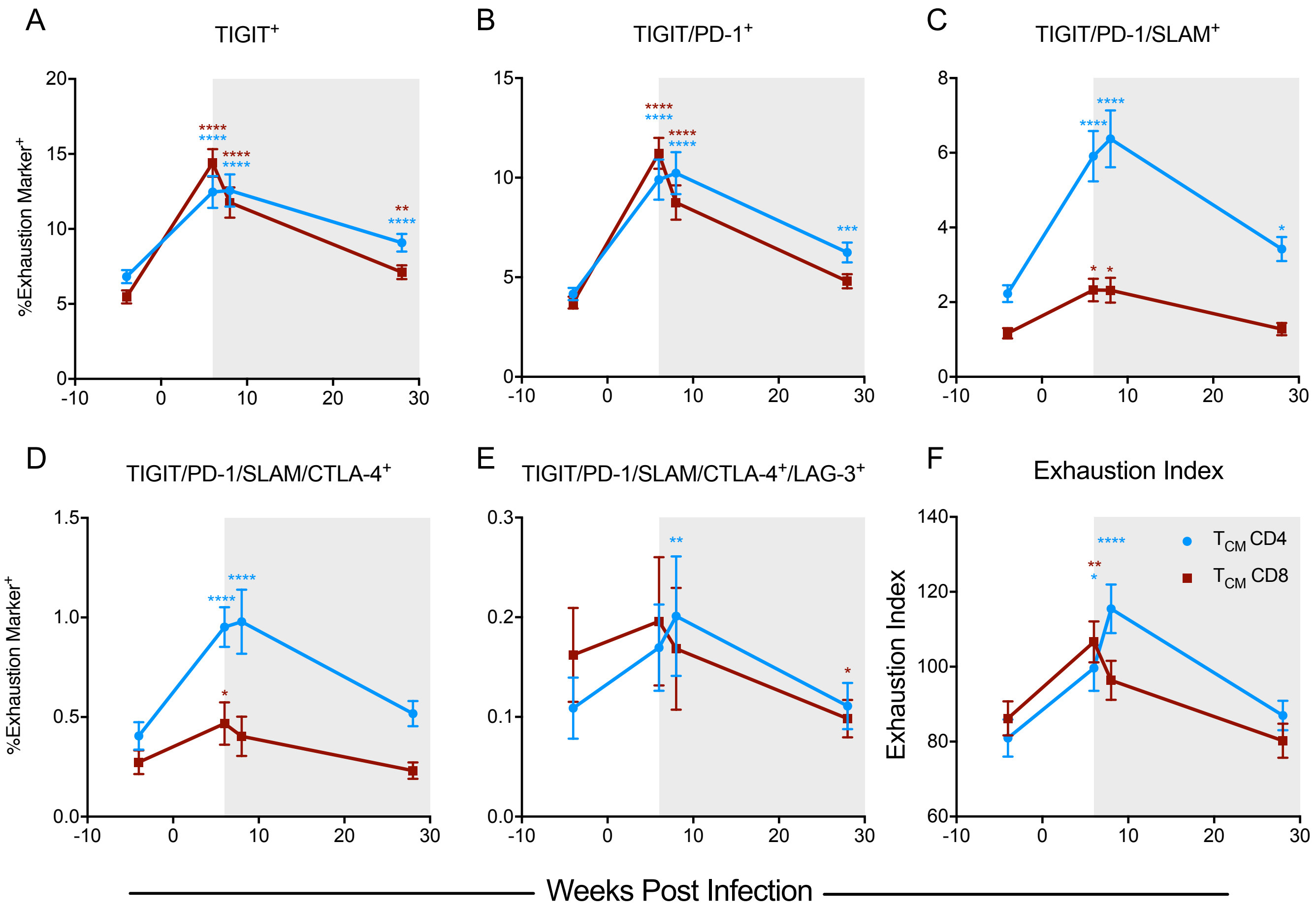


Figure 4.2. Exhaustion markers on central memory T-cells are increased after SIV infection and partially reduced by ART. Cryopreserved PBMC were thawed at time points corresponding to -4, 6, 8, and 28 wpi from rhesus macaques and immediately stained for exhaustion marker expression by flow cytometry. The gray box indicates ART treatment. The percentage of the exhaustion markers TIGIT (**A**), TIGIT/PD-1 (**B**), TIGIT/PD-1/SLAM (**C**), TIGIT/PD-1/SLAM/CTLA-4 (**D**), TIGIT/PD-1/SLAM/CTLA-4/LAG-3 (**E**), and Exhaustion Index (**F**) on CD4 and CD8 central memory T-cells (T_{CM}). T regulatory ($CD25^+CTLA\text{-}4^+$) were excluded from the CD4 population. Shown are the mean +/- SEM. Differences between time points were determined by a repeat measures two-way ANOVA with a Tukey's multiple comparisons test. *P* values indicated are adjusted and **** indicates a *P* < 0.0001.

Exhaustion marker expression in the blood is associated with declines in the Th17/Treg ratio and Th17 polyfunctionality in the colon

Loss of Th17 frequency and functionality in the colon has been correlated with chronic inflammation and activation[398], and we sought to determine if the loss of these cells correlated with exhausted T-cells in the blood. The ratio of Th17 to T-regulator (Treg) cells, as well as the frequency of polyfunctional Th17's, were quantified in the colon mucosa pre-infection (-4 wpi, red circles), prior to cART initiation (6 wpi, blue squares), 2 weeks of ART (8 wpi, green triangles), and 22 weeks of ART (28 wpi, purple triangles) (**Figure 4.3**). A decline in the Th17/Treg ratio was inversely associated with %TIGIT$^+$ / CD4$^+$ (r = -0.3551, P = 0.0016, **Figure 4.3A**) and CD8$^+$ (r = -0.4475, P < 0.0001, **Figure 4.3B**) T_{CM} in the blood. Similarly, %TIGIT/PD-1$^+$ /CD4$^+$ (r = -0.4013, *P* = 0.0003, **Figure 4.3C**) and CD8$^+$ (r = -0.4369, *P* = 0.0001, **Figure 4.3D**) T_{CM} expression in the blood inversely correlated with the Th17/Treg ratio in the colon. The frequency of polyfunctional Th17's in the colon was strongly negatively associated with increased TIGIT/PD-1/SLAM$^+$ CD4$^+$ (r = -0.3756, P = 0.0008, **Figure 4.3E**) and to a modest extent on CD8$^+$ (r = -0.2430, P = 0.0344, **Figure 4.3F**) T_{CM}. Lastly, there was a modest negative correlation with the frequency of TIGIT/PD-1/SLAM/CTLA-4$^+$ / CD4$^+$ (r = -0.2565, P = 0.0253, **Figure 4.3G**) but not CD8$^+$ (r = -0.097, P = 0.4026) T_{CM}. Taken together, these observations show that there are inverse associations between exhaustion markers on CD8$^+$ and particularly CD4$^+$ T_{CM} in the blood and a decreased Th17/Treg ratio and a loss of Th17 polyfunctionality in the colon.

Plasma soluble sCD14 is associated with elevated frequency of exhausted T_{CM} CD4 T-cells in the blood

The Th17/Treg ratio[404] and polyfunctionality[398] are important for maintaining gut homeostasis, and limiting microbial translocation. To better understand the connection between gut disruption and exhaustion markers in the blood we measured plasma concentration of soluble CD14 (sCD14) which is an LPS binding protein[405] , and has been used as a surrogate marker for

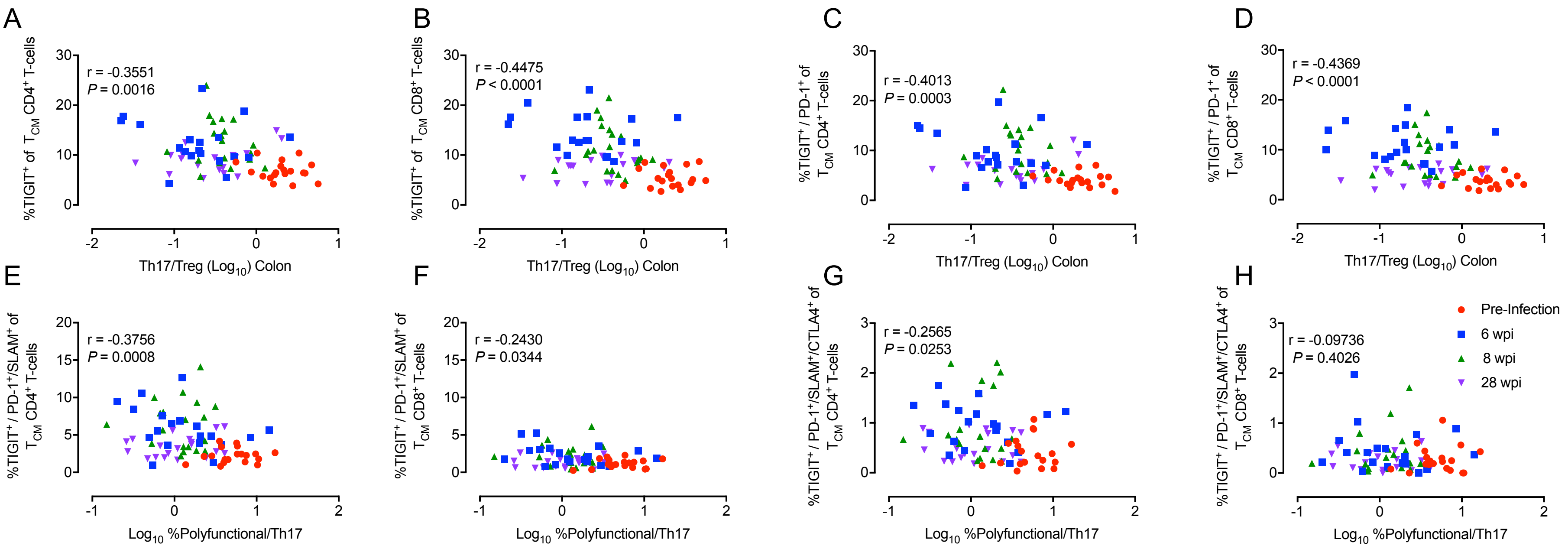


Figure 4.3. Declines in the Th17/Treg ratio and Th17 polyfunctionality in the colon correlate with elevated exhaustion markers on central memory T-cells in the blood. Cryopreserved PBMC were thawed from time points corresponding to -4 (red dots), 6 (blue squares), 8 (green triangles) and 28 (purple triangles) wpi and CD4 and CD8 T_{CM} expressing various combinations of exhaustion markers including TIGIT (**A,B**), TIGIT/PD-1 (**C,D**), TIGIT/PD-1/SLAM (**E,F**) and TIGIT/PD-1/SLAM/CTLA-4 (**G, H)** were quantified by flow cytometry. Colon biopsies were samples from rhesus macaques and stimulated overnight with PMA/ionomycin or unstimulated in R10 media and evaluated by flow cytometry. Th17 cells were measured by the production of IL-17 in PMA/ionomycin conditions after subtraction from unstimulated controls. Tregs were defined as FoxP3 and CD25 positive cells in the unstimulated samples. The ratio was defined by dividing the frequency of Th17s to Tregs (**A-D**). Th17 polyfucntionality was defined as cells producing 3 or more of the cytokines IL-22, IFN-γ, TNF-α, and/or IL-2 as well as IL-17 (**E-H**). P and r values shown are the results of Spearman's rank correlation tests.

***Th17/Treg ratio and Th17 polyfunctionality data were provided by Dr. Megan O'Connor.**

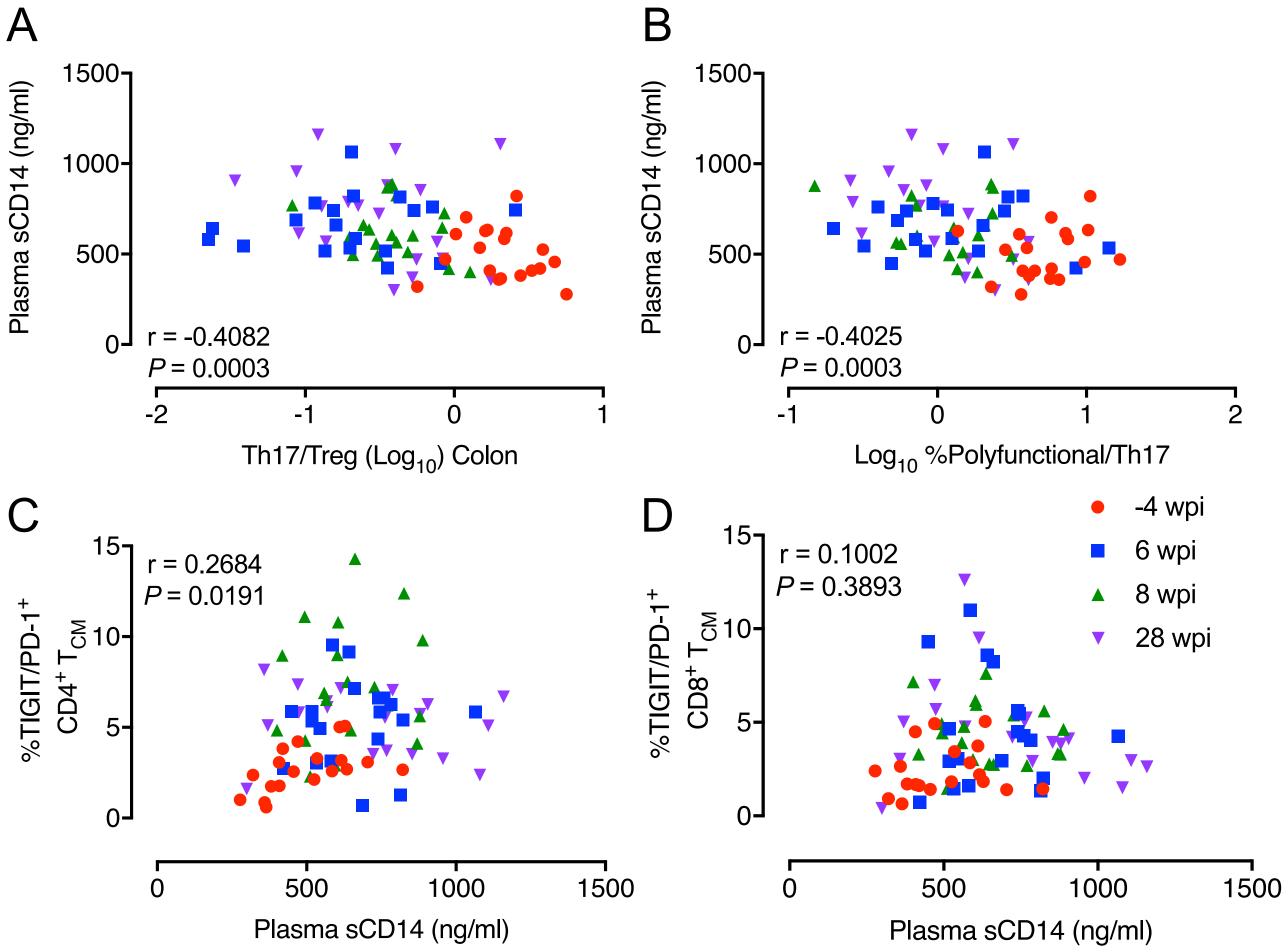


Figure 4.4. Plasma sCD14 is correlated with the frequency of TIGIT/PD-1$^+$ central memory CD4$^+$ T-cells. Shown are the plasma sCD14 concentrations as measured by ELISA (**A-D**). Correlations are shown between sCD14 and the Th17/Treg ratio (**A**) and the percent of polyfunctional Th17's (**B**) in the colon, TIGIT/PD-1$^+$ CD4 (**C**) and CD8 (**D**) T_{CM} in the PBMC. P and r values shown are the results of Spearman's rank correlation tests.

***Plasma sCD14 data were provided by Hillary Tunggal.**

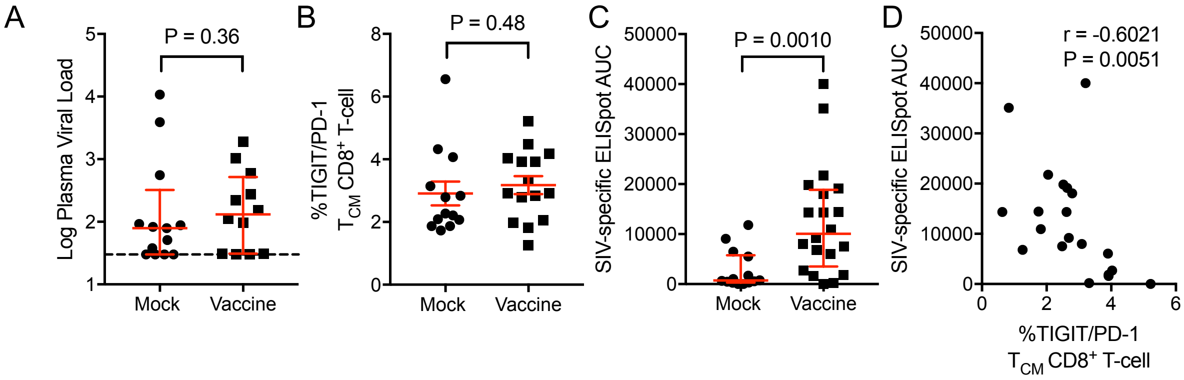


Figure 4.5. The frequency of TIGIT/PD-1$^+$ central memory CD8$^+$ T-cells prior to vaccination are inversely correlated with DNA vaccine immunogenicity.

(**A**) SIVΔB670 plasma viral loads as determined by q-RT-PCR prior to immunization (week 18 for short ART, week 32 for long ART) are shown for the mock and DNA vaccine groups delivered via the gene gun. The dotted line indicates the limit of detection (30 viral RNA copies per mL of plasma). (**B**) Shown are the frequencies of TIGIT/PD-1$^+$ CD8$^+$ T$_{CM}$ as determined by flow cytometry in the PBMC prior to DNA immunization. (**C**) Cryopreserved PBMC were thawed and rested overnight, then plated on IFN-γ ELISpot plates and stimulated with 15mer peptides overlapping by 11 amino acids corresponding to the entire SIV proteome for 24 hours, developed, and spot forming cells were enumerated. Displayed are he area under the curve (AUC) of the SIV-specific IFN-γ ELISpot responses for each animal over the course the vaccination regimen. (**D**) Shown is the correlation between the SIV-specific ELISpot AUC and the frequency of TIGIT/PD-1$^+$ CD8$^+$ T$_{CM}$ prior to therapeutic immunization in the vaccine group. Differences between viral load, TIGIT/PD-1$^+$ CD8$^+$ T$_{CM,}$ and SIV-specific ELISpot AUC between the mock and vaccine groups were determined by a two-sided Mann-Whitney test. Error bars (red) are the mean +/- the SEM. P and r values shown are the result of a Spearman rank correlation test.

translocation[30]. As expected, we observed plasma sCD14 was inversely correlated with the Th17/Treg ratio (r = -0.4082, P = 0.0003, **Figure 5A**) and Th17 polyfunctionality (r = -0.4025, P = 0.0003, **Figure 5B**). Plasma sCD14 was positively correlated with TIGIT/PD-1$^+$ T$_{CM}$ CD4$^+$ (r = 0.2684, P = 0.0191, **Figure 5C**) and not CD8$^+$ (r = 0.1002, P = 0.3893, **Figure 5D**) T$_{CM}$. These correlations suggest that changes in the Th17/Treg subsets in the colon may underlie microbial translocation and subsequent inflammation and cellular exhaustion in the blood.

T-cellular exhaustion is associated with diminished therapeutic DNA vaccine immunogenicity

Next, we sought to examine if lingering immune exhaustion on cART may limit DNA vaccine immunogenicity. Macaques that were on ART from 3-6 months prior to receiving therapeutic DNA vaccination via particle mediated epidermal delivery. The vaccine group received Gag and/or Env encoding DNA co-formulated with an adjuvant, while the mock group received non-coding plasmid as a control. Prior to therapeutic vaccination, the mock and vaccine groups had similar plasma viral loads (*P* = 0.36, **Figure 6A**) and frequencies of exhausted TIGIT/PD-1$^+$ T$_{CM}$ CD8$^+$ T-cells (*P* = 0.48, **Figure 6B**) in the PBMC. As expected, vaccinees had increased frequencies of SIV-specific IFN$^+$ cells as measured by the ELISpot area under the curve (AUC) when compared to the mock group (*P* = 0.0010, **Figure 6C**). Consistent with our previous findings, we observed an inverse relationship between vaccine immunogenicity and levels of TIGIT/PD-1$^+$ T$_{CM}$ CD8$^+$ T-cells (r = -0.6021, P = 0.0051, **Figure 6D**). While the DNA vaccines were immunogenic, these findings suggest their immunogenicity may be limited in part by cART's inability to fully reverse SIV induced cellular exhaustion.

Discussion

HIV/SIV infection preferentially depletes the frequency and functionality of Th17 cells that are important in maintenance of the gut barrier leading to microbial translocation. Microbial translocation is a major contributor to the chronic immune activation during HIV and SIV infection. However, the factors contributing to elevated frequencies of exhaustion markers such as TIGIT/PD-1^+ on T-cells in the blood of cART suppressed HIV-infected humans remained poorly understood. Prior studies examining the inability of ART to fully restore gut homeostasis or reduce inflammation in HIV/SIV, gave important clues that these factors may contribute to T-cellular exhaustion in the blood. In support of this concept, cytokines such as type-I interferons[406] or IL-6[407] have been associated with increased expression of certain exhaustion markers, and are increased in HIV infected patients. However, detailed time course studies evaluating the mucosal correlates of T-cellular exhaustion on ART, and if exhaustion may limit therapeutic vaccine immunogenicity have not been reported.

To address these questions, we utilized the SIV/macaque model to measure cellular exhaustion by the surface markers TIGIT, PD-1, SLAM, CTLA-4, and LAG-3 on central memory $CD4^+$ and $CD8^+$ T-cells, gut disruption by Th17/Treg ratio and Th17 functionality in the colon, as well as measuring sCD14, a surrogate marker for microbial translocation, in the plasma. The SIV/macaque model allows for extensive control over the timing and sampling that would be difficult to achieve in HIV infected humans. Our study is further strengthened by the number of exhaustion markers measured, matched mucosal time points at various stages of infection to characterize Th17 and Tregs, as well as determining how exhaustion may limit therapeutic vaccine immunogenicity.

SIV∆B670 is a highly pathogenic primary isolate that replicates to high titers in rhesus macaques and depletes $CD4^+$ T-cells in the blood and gut within weeks of infection. Consistent with our previous studies, we observed robust plasma viremia during the first 2 weeks of infection that diminished at 6 wpi. The triple drug regimen of PMPA, FTC, and Raltegravir was more effective at reducing plasma viral RNA when compared to our previous study using only PMPA and FTC[48]. Our previous therapeutic vaccine study demonstrated a strong correlation between plasma viral load and viral RNA in the lymph node and gut mucosa suggesting that our cART was effective at reducing viral replication systemically[48], however we cannot rule out the possibility that antigen production in the tissue may contribute to cellular exhaustion. Additionally, consistent with data from HIV infected husmans we found that ART partially restored CD4$^+$ T-cell counts and the CD4/CD8 ratio in the blood and gut mucosa[408]. The ability of our model to recapitulate viral load and CD4 kinetics observed in humans suggests our findings may translate to cART treated HIV infected humans.

We next examined how SIV infection and ART therapy alters the frequency of exhaustion markers on central memory CD4$^+$ and CD8$^+$ T-cells. Various exhaustion markers can also be upregulated during activation or during differentiation with naïve, effector memory and central memory cells inherently express varying levels of exhaustion markers. Therefore, measuring exhaustion markers on total T-cells may be confounded by shifts in naïve, effector, and memory populations. Consistent with previous studies by in HIV infected humans by Chew *et al* we observed elevated frequencies TIGIT$^+$ of T_{CM} CD4$^+$ and CD8$^+$ during cART therapy[68]. While cART was effective at reducing expression of multiple exhaustion markers on CD8's, in contrast CD4$^+$ T-cells had elevated frequencies of TIGIT/PD-1 and TIGIT/PD-1/SLAM above pre-infection levels. These findings suggest that factors other than antigen overstimulation may be contributing to elevated frequencies of exhaustion markers during cART.

This study has some limitations in our ability to discern cART's reversal of cellular exhaustion, for example we did not include a group of macaques that did not receive cART. Therefore, we cannot definitively conclude that cART reduces exhaustion parameters, or rather these decreases represent normal variation in exhaustion parameter expression during HIV/SIV infection. Additionally, the latest time point cellular exhaustion was measured on cART was 22 weeks, which may not be representative of HIV infected humans receiving fully suppressive ART for years or decades. On a related point, we only reported on global levels of T-cellular exhaustion and not on HIV-specific T-cellular exhaustion. cART may have a different effect on bulk CD4$^+$ and CD8$^+$ populations versus HIV-specific T-cellular exhaustion.

To better understand the factors contributing to exhaustion markers in the blood we evaluated Th17 to Treg ratio in the colon, as well as Th17 polyfunctionality at matched time points. Previous studies have demonstrated that the Th17/Treg ratio was an important predictor of clinical outcome and the ability to mount robust HIV-specific $CD8^+$ T-cell immunity[409]. Our study builds upon these findings by measuring the Th17/Treg ratio in the colon mucosa and we found their depletion is associated with increased exhaustion marker expression in the blood, particularly on $CD4^+$ T-cells. The positive correlation between sCD14 and TIGIT/PD-1^+ T_{CM} $CD4^+$ and not $CD8^+$ T-cells, suggests different mechanisms may be driving exhaustion marker expression on CD4's versus CD8's. This is in partial agreement with prior longitudinal study of ART naïve HIV-infected Ugandan children that initiated ART, PD-1 expression on $CD8^+$ T-cells correlated most strongly with plasma viremia where PD-1^+ $CD4^+$ T-cells were correlated with $CD4^+$ T-cell decline and activation[410]. However, PD-1 expression is also indicative of activation or differentiation and not necessarily exhaustion, making it unclear if they were truly measuring exhausted cells. While consistent with these findings, the exact mechanisms underlying the differential regulation of exhaustion markers on $CD4^+$ and $CD8^+$ T-cells are not fully understood.

Additionally, we measured the surrogate marker for microbial translocation sCD14 in the plasma, while this isn't a direct measurement of microbial translocation it has been strongly associated with microbial translocation[30]. Consistent with other studies, we observed an increase in plasma sCD14 concentrations that was not reduced by cART, a likely result of cART's inability to restore the Th17 frequency and function in the colon. The correlation between plasma sCD14 and TIGIT/PD-1^+ $CD4^+$ T-cells and not $CD8^+$ T-cells, further supports the concept that different mechanisms may contribute to $CD4^+$ and $CD8^+$ exhaustion. The elevated frequency of exhaustion on CD4's is likely not a direct effect of sCD14 on $CD4^+$ T-cells, but rather due to increases in inflammatory cytokines induced by microbial translocation. While we did not measure plasma cytokines, sCD14 has been shown to increase type I interferons[411], which have been correlated with cellular exhaustion[412]. Additional studies and analyses will be required to determine the relationship between microbial translocation and exhaustion marker expression on CD4$^+$ T-cells.

Finally, we sought to determine if cellular exhaustion limits therapeutic DNA vaccine immunogenicity. We previously reported that %PD-1$^+$/CD8$^+$ T-cells prior to vaccination inversely correlated with therapeutic vaccine immunogenicity. Our findings here build upon those initial observations by including an additional exhaustion marker TIGIT, as well as examining specific cellular subsets (naïve, effector memory, and central memory). These findings are the first to report on correlations between cellular exhaustion and limiting responsiveness to therapeutic HIV/SIV DNA vaccination. In our study, we only employed DNA vaccination making it unclear if these findings would extend to other vaccine modalities such as viral vectors, *ex vivo* pulsed dendritic cells, or subunit vaccines.

In all, these findings suggest a connection between SIV induced gut disruption and T-cellular exhaustion in the blood, and may provide strategies for improving therapeutic vaccine immunogenicity and efficacy. For example, strategies that better restore homeostatic T-cell populations in the gut, such as increasing Th17 polyfunctionality and the Th17/Treg ratio, may limit T-cellular exhaustion and improve therapeutic HIV/SIV vaccine immunogenicity. Encouragingly, IL-21 and probiotic therapy has been shown to improve Th17 frequencies and decrease microbial translocation in SIV infected ART treated macaques, and thus could improve responsiveness to therapeutic vaccines[413]. Alternatively, anti-PD-1 antibodies have been shown to both augment SIV-specific CD8$^+$ T-cell responses[77] and in a separate study reinvigorate CD4 T-cells in the gut mucosa and limit microbial translocation[79] in SIV infected macaques. These findings suggest that combinations of immune checkpoint inhibitors and/or homeostatic cytokines or probiotics, could be viable strategies to improve HIV/SIV therapeutic vaccines.

Chapter 5

Conclusions & Future Directions

"It always seems impossible until it's done."
-Nelson Mandela

Taken together, I discussed the progress towards a therapeutic HIV vaccine (**Chapter** 1) have demonstrated that CE-specific T-cellular immune responses are associated with control of SIV viremia and that I could improve these responses by therapeutic DNA vaccination. Particularly, I demonstrated that CE, and not FL, vaccination broadened CE-specific immune responses in cART naïve long term non-progressor SHIV infected macaques (**Chapter 2**) (**Figure 5.1**). While the viral replication was too low in these animals to assess vaccine efficacy, this was the first demonstration of therapeutic vaccination boosting immunity to subdominant SIV/SHIV epitopes. Additionally, I demonstrated, in a highly pathogenic SIV/macaque model, that CE vaccination in ART treated macaques significantly broadened cellular and humoral CE specific immunity when compared to Mock or FL vaccinated macaques (**Chapter 3**). Importantly, these findings demonstrate that gene gun delivered CE encoding DNA, in conjunction with the adjuvant LT, is an effective platform for increasing CE specific immunity. Furthermore, these data demonstrate that the immunodominant setting of viral infection is not an insurmountable barrier to improving subdominant immune responses to conserved regions of the viral proteome.

Additionally, I have defined factors that limit HIV therapeutic vaccine immunogenicity generally, as well as CE vaccination specifically. Particularly, I found that better responses to cART was positively correlated with both FL and CE vaccine immunogenicity. I hypothesized that viral replication may be driving immune exhaustion, in part by upregulation of PD-1, that may limit therapeutic vaccine immunogenicity (**Figure 5.2**). In support of this hypothesis, I revealed that viral replication was correlated with greater frequencies of PD-1$^+$ / CD8$^+$, but not

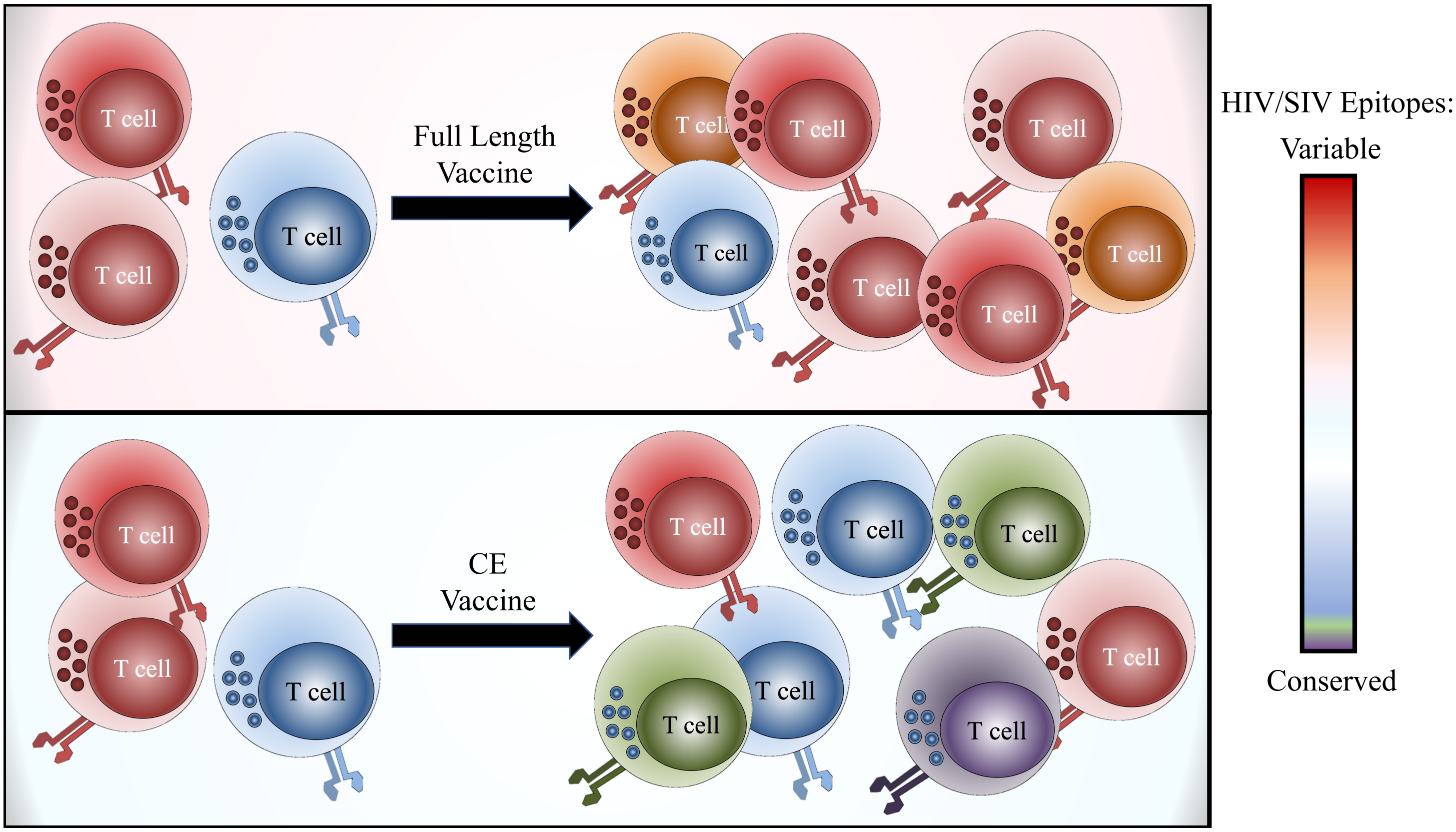


Figure 5.1 CE and FL immunogens bias T-cell responses to CE and non-CE epitopes respectively. Shown is a depiction of immune responses to prior to vaccination to immunodominant variable epitopes (red & orange) as well as a low frequency of conserved element (CE) responses (blue, green, and purple). A full length vaccine expands responses to non-CE epitopes

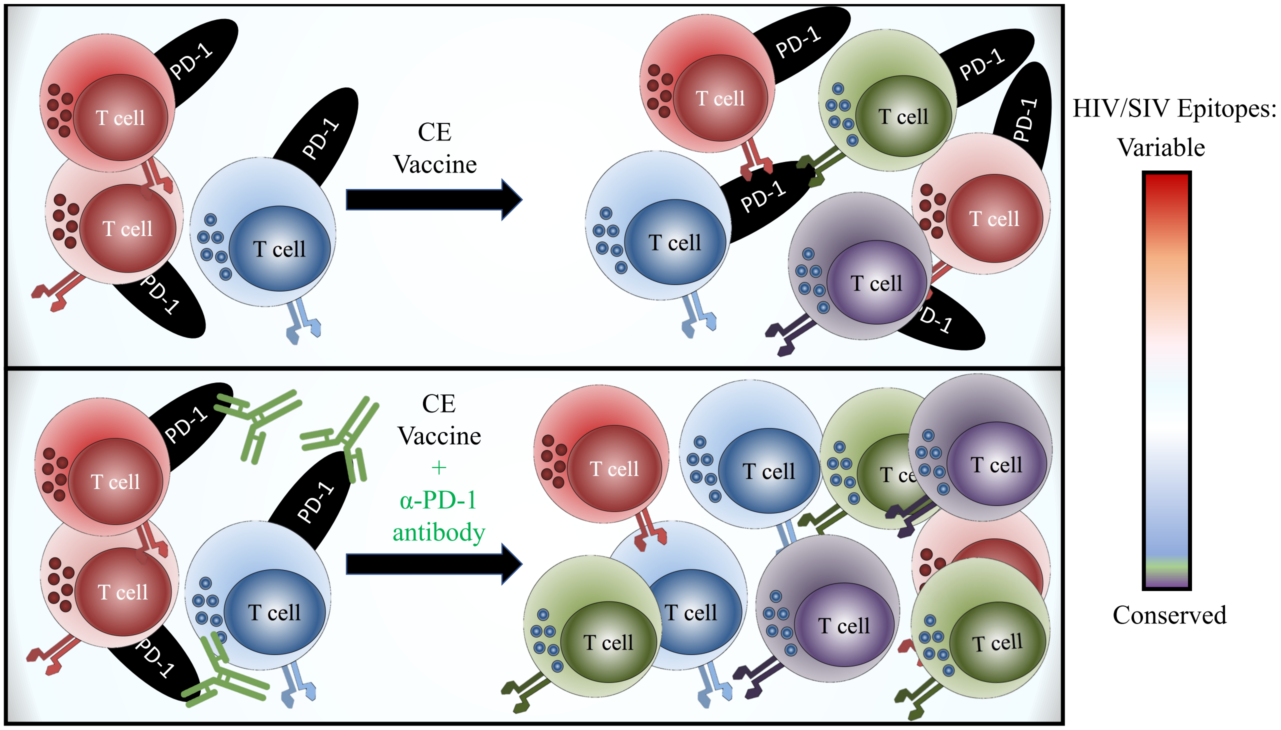


Figure 5.2. Anti-PD-1 antibodies could enhance CE vaccine immunogenicity. Shown is a theoretical representation of how an anti-PD-1 antibody could reverse cellular exhaustion and lead to a more potent expansion of CE-specific T-cells.

$CD4^+$, T-cells. Importantly, pre-vaccine frequencies of PD-1^+ $CD8^+$ T-cells were inversely correlated with post-vaccination T-cellular immune magnitudes. Furthermore, PD-1 expression on both $CD4^+$ and $CD8^+$ T-cells was inversely correlated with CE-specific polyfunctional T-cell responses. These findings suggest that strategies to decrease to immune exhaustion, such as administering α-PD-1 antibodies prior to vaccination, may reinvigorate the immune system thereby enhancing the immunogenicity of CE vaccines.

Expanding on the initial observations of PD-1 (**Chapter 3**), I expanded on the exhaustion analysis and sought to identify immunological factors contributing to lingering exhaustion during cART treatment of SIV infection (**Chapter 4**). Towards this end, I found that expression of multiple exhaustion markers (TIGIT, PD-1, SLAM, CTLA-4, LAG-3) increased during therapy and were incompletely reversed by ART. Lingering gut disruption and microbial translocation was correlated with increased expression of exhaustion markers on T-cells in the blood, particularly TIGIT/PD-1^+ cells. Specifically, a loss of the frequency and functionality of Th17's and an expansion of T-regulatory cells in the gut corresponded with elevated exhaustion marker expression on T-cells in the blood. Consistent with my prior data, I found that exhausted T-cells prior to immunization was inversely correlated with vaccine immunogenicity. These findings suggest that multiple immune checkpoint inhibitors, in addition to anti-PD-1 antibodies, may be required to more fully reverse cellular exhaustion and enhance therapeutic vaccine immunogenicity.

In addition to immune checkpoint inhibitors immunotherapies that can more fully restore gut immune homeostasis may be an effective way to augment therapeutic DNA immunization. Specifically, anti-inflammatories and probiotics may limit global inflammation and immune dysfunction thereby increasing vaccine induced responses while limiting the maintenance of the reservoir.

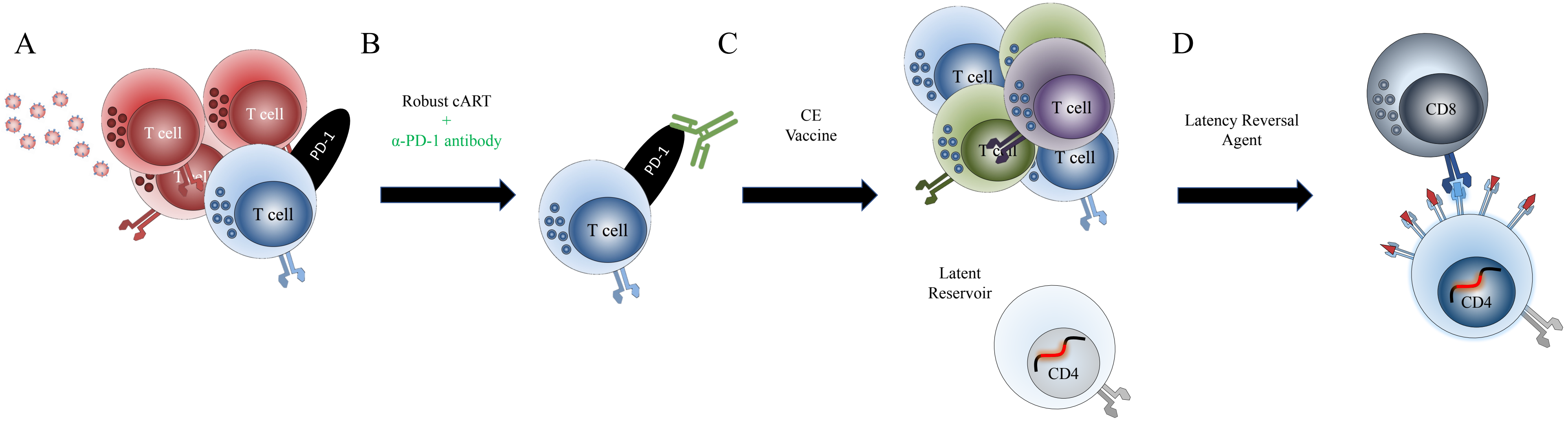


Figure 5.3. The role of CE vaccines for an HIV cure. A) Prior to cART, viral replication drives immunodominant responses and cellular exhaustion. B) Potent antiretroviral therapy limits viral replication and an anti-PD-1 antibody reverses cellular exhaustion. C) CE vaccination potently expands CE-specific T-cell responses. D) Administration of a latency reversal agent induces cells harboring proviral DNA to present MHC-I restricted peptides that can be recognized by CE-specific CTL responses leading to a profound reduction in the viral reservoir.

Reducing microbial translocation could improve normal immune function and increase responsiveness to vaccination. HIV infection leads to alterations in the microbiome and is correlated with immune activation[414]. Probiotic therapies have proven successful, in some cases, in reversing the inflammation/gut damage in the setting of a lentiviral infection[415,416]. Additionally, probiotic treatment was shown to improve mucosal immunity in SIV negative macaques[417]. Probiotic treatment may provide a novel way to improve therapeutic HIV vaccine responses in cART suppressed subjects. Overall, my findings suggest that immunomodulatory agents that can better reverse SIV/HIV-induced gut dysfunction may be a viable strategy to improve therapeutic vaccine immunogenicity and efficacy.

Broadly speaking, these experiments provide a proof-of-concept that CE-specific immune responses can be induced, even in the presence of immunodominant responses, by gene gun delivery of CE DNA in conjunction with the LT adjuvant. The compelling nature of this platform is its flexibility to be adapted to other variable viruses and pathogens. Of particular interest, is influenza, because antigenic drift requires annual vaccine reformulation, and antigen shift requires a new vaccine entirely[418]. Importantly, a “universal” influenza vaccine that provides lifelong protection from infection has not been developed. It has previously been demonstrated that $CD8^+$ and $CD4^+$ T-cells specific to conserved epitopes in influenza contribute to protection from infection[297,419–421]. Importantly, our laboratory demonstrated that a multi-antigen (MA) DNA vaccine, with the genetic adjuvant LT, induced cross-reactive NP-specific T-cell responses that correlated with reduced viral replication and inflammation after a heterologous influenza challenge in cynomolgous macaques (*Macaca fascicularis*), when compared to mock vaccinees. However, these studies have primarily focused on influenza naïve animals that do not have immune responses primed to immunodominant and variable regions of the influenza proteome[297,421]. Theoretically, an influenza CE DNA vaccine could redirect cellular immune responses, even in the setting of immunodominant influenza immunity to variable epitopes, towards highly conserved and functionally constrained sequences that may provide more sustained protection from infection.

We are proceeding with further testing of therapeutic CE DNA vaccines in conjunction with anti-PD-1 antibodies with and without the use of latency reversal agents, a strategy often referred to a “shock-and-kill” (**Figure 5.3**). Robust antiretroviral therapy in conjunction with anti-PD-1 antibodies could robustly diminish immunodominant responses and re-invigorate CE-specific T-cells (**Figure 5.3A&B**). CE vaccination would then increase the frequency and breadth of CE-specific T-cell responses, but may not efficiently target the latent reservoir due to little to no presentation of antigen (**Figure 5.3C**). Latency reversal agents could induce the latent reservoir to present CE epitopes, leading to clearance by CE-specific CTL, causing a profound reduction in the viral reservoir, and viral remission post ART withdrawal (**Figure 5.3D**).

In summary, this dissertation demonstrates that CE DNA vaccination can induce potent CE-specific T-cell response in SHIV and SIV-infected macaques. While immunogenic, the CE vaccine did not limit viral rebound during an ATI compared to the Mock or FL vaccine groups, which may be due to immune exhaustion that diminished T-cellular functionality. In addition, I explored the mechanisms contributing to immune exhaustion during SIV-infection, and how immune exhaustion limits therapeutic vaccine immunogenicity. These findings may lead to better strategies to for therapeutic HIV vaccines and developing CE vaccines for other variable viruses and pathogens.

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421. Eliasson, D. G. *et al.* M2e-tetramer-specific memory CD4 T cells are broadly protective against influenza infection. *Mucosal Immunol.* **11,** 273–289 (2018).

CURRICULUM VITAE

Paul Veness Munson

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EDUCATION

09/2011-Spring 2018

Doctor of Philosophy, Microbiology
University of Washington, Seattle, WA
Advisor: Dr. Deborah Fuller
Thesis: Conserved element vaccine and
cellular responses in SIV positive macaques

09/2007-06/2011

Bachelor of Science in Microbiology &
Bachelor of Science in Chemistry
Oregon State University, Corvallis, OR

HONORS

2007	Rosenberg-Ibarra Scholar, Boise, ID
2007-2010	Oregon State University Provost Scholarship, Corvallis, OR
2010	Howard Hughes Medical Institute Undergraduate Research Fellowship, Corvallis, OR
2012	National Institutes of Health Interdisciplinary Training Grant, Seattle, WA
2014	Best Poster in Category of Vaccines/Immunizations Conference on Cell Gene Therapy for HIV Cure, Seattle WA
2015	International AIDS Society Travel Scholarship, Vancouver, Canada
2016	Best Poster Award at the 34th Annual Symposium on NHP Models for AIDS, New Orleans, LA
2017	Cell and Gene Therapy for HIV Cure Scholarship, Seattle, WA
2017	35th Annual Symposium on Nonhuman Primate Models for AIDS Scholarship, Madison, WI
2018	1rst Annual HIV Mucosal Systems Meeting Travel Scholarship, Santa Rosa, CA

TEACHING & STEWARDSHIP

Courses and Lectures:

Graduate:

Spring Quarter 2011	MICRO302: General Microbiology Laboratory, Teaching Assistant
Fall Quarter 2012	MICRO302: General Microbiology Laboratory, Teaching Assistant
Winter Quarter 2015	CONJ 539: Viral Vaccines and Immunity, Guest Lecturer and Teaching Assistant
Winter Quarter 2016	MICRO 431: Prokaryotic Recombinant DNA Techniques, Guest Lecturer

Undergraduate Trainees:

2013-2014	Ken Steckler (currently pursuing a Doctor of Pharmacy at the University of Washington)
2015-2016	Nika Hajari (currently pursuing a PhD in Pathobiology at the University of Washington)
2017-Current	Alizan Rosen (pursuing a B.S. at the University of Washington)

Professional Societies

American Society for Microbiology
Out in Science Technology Engineering and Math (oSTEM)

RESEARCH

Peer-reviewed Articles:

1. **Paul Munson,** Deborah Fuller. (2018) *Progress towards a therapeutic HIV DNA vaccine. Human Gene Therapy* (invited review, in preparation)

2. **Paul Munson**, Yi Liu, Debra Bratt, James T. Fuller, Xintao Hu, George N. Pavlakis, Barbara K. Felber, James I. Mullins, Deborah Heydenburg Fuller. (2018). Therapeutic conserved elements (CE) DNA vaccine induces strong T-cell responses against highly conserved viral sequences during simian-human immunodeficiency virus infection. Human Vaccines & Immunotherapeutics.

3. Koday MT, Leonard JA, **Munson P**, Forero A, Koday M, Bratt DL, Fuller JT, Murnane R, Qin S, Reinhart TA, Duus K, Messaoudi I, Hartman AL, Stefano-Cole K, Morrison J, Katze MG, Fuller DH. (2017). Multigenic DNA vaccine induces protective cross-reactive

T cell responses against heterologous influenza virus in nonhuman primates. *PloS One,* 12:e0189780. PMID: 29267331

4. Sandra E. Dross, **Paul V. Munson**, Se Eun Kim, Debra L. Bratt, Hillary C. Tunggal, Ana L. Gervassi, Deborah H. Fuller and Helen Horton (2017). Kinetics of Myeloid-Derived Suppressor Cell Frequency and Function during Simian Immunodeficiency Virus Infection, Combination Antiretroviral Therapy, and Treatment Interruption. *J Immunol*, 757-766 PMID: 27974456

5. Smedley J, Macalister R, Wangari S, Gathuka M, Ahrens J, Iwayama N, May D, Bratt D, O'Connor M, **Munson P**, Koday M, Lifson J, Fuller DH (2016). Laparoscopic Technique for Serial Collection of Para-Colonic, Left Colic, and Inferior Mesenteric Lymph Nodes in Macaques. *PLoS ONE*, PMID: 27309717

Abstracts and Conference Presentation

1. **Paul Munson**, Megan O'Connor, Hillary Tunggal, Nika Hajari, Debra Bratt, Deborah Fuller. (2018) Mucosal Th17 dysfunction and microbial translocation are associated with elected PD-1$^+$TIGIT$^+$ frequencies on peripheral T-cells in SIV infected macaques. 1rst Annual HIV Mucosal Systems Meeting. (*Oral presentation*)

2. **Paul Munson**, Hillary Tunggal, Nika Hajari, Megan O'Connor, Debra Bratt, James T. Fuller, Drew May, Solomon Wangari, Brian Agricola, Jeremy Smedley, Xintao Hu, Barbara K. Felber, George N. Pavlakis, James I. Mullins, Deborah Heydenburg Fuller. (2017) Conserved Elements (CE) DNA Vaccination Induces CE Responses in SIV Infected, cART Treated Macaques. Non-Human Primate Models for AIDS 2017. (*Oral presentation)*

3. **Paul Munson**, Hillary Tunggal, Nika Hajari, Megan O'Connor, Debra Bratt, James T. Fuller, Drew May, Solomon Wangari, Brian Agricola, Jeremy Smedley, Xintao Hu, Barbara K. Felber, George N. Pavlakis, James I. Mullins, Deborah Heydenburg Fuller. (2017) T-cell Exhaustion Limits Therapeutic DNA Vaccine Immunogenicity in SIV Infected, cART Treated Macaques. Cell and Gene Therapy for HIV Cure 2017. (*Oral presentation)*

4. **Paul Munson,** Debra Bratt, Andrew May, Solomon Wangari, Brian Agricola, Jeremy Smedley, James Fuller, Viraj Kulkarni, Barbara K. Felber, George N. Pavlakis, James I. Mullins, Deborah Fuller. (2016) Conserved Elements (CE) DNA Vaccination Induces CE Responses in SIV Infected, cART Treated Macaques. Non-Human Primate Models for AIDS 2016. (*Poster presentation*)

5. **Paul V. Munson,** Debra Bratt, Brian Agricola, Mike Koday, Merika Treants, Jim Fuller, Viraj Kulkarni, Barbara K. Felber, George N. Pavlakis, Yi Liu, James I. Mullins, Deborah Fuller. (2015) Conserved Element Responses Induced by SIV Infection and Vaccination. Cell and Gene Therapy 4 HIV Cure. (*Oral presentation)*

6. **Paul Munson,** Debra Bratt, Brian Agricola, Mike Koday, Merika Treants, Jim Fuller, Viraj Kulkarni, Barbara K. Felber, George N. Pavlakis, Yi Liu, James I. Mullins, Deborah Fuller. (2015) Therapeutic conserved elements (CE) DNA vaccine induces strong T-cell

responses against highly conserved viral sequences during chronic viral infection. International AIDS Society. (*Poster presentation*)

7. **Paul Munson,** Debra Bratt, Brian Agricola, Mike Koday, Merika Treants, Jim Fuller, Viraj Kulkarni, Barbara K. Felber, George N. Pavlakis, Yi Liu, James I. Mullins, Deborah Fuller. (2014) Therapeutic conserved elements (CE) DNA vaccine induces strong T-cell responses against highly conserved viral sequences during chronic viral infection. Cell and Gene Therapy 4 HIV Cure (CTG4HIVCure). (*Poster presentation*)

8. **Paul Munson,** Debra Bratt, Brian Agricola, Mike Koday, Merika Treants, Jim Fuller, Viraj Kulkarni, Barbara K. Felber, George N. Pavlakis, Yi Liu, James I. Mullins, Deborah Fuller. (2013) Therapeutic conserved elements (CE) DNA vaccine induces strong T-cell responses against highly conserved viral sequences during chronic viral infection. Non-human Primate Models for AIDS. (*Poster presentation*)

Research support

1R43AI140784-01 2018-2020
SBIR
Design and Optimization of Clinical Gene Gun for Delivery of Nucleic Acid Vaccines
Paul Munson (PI), Deborah Fuller (MPI)
The primary focus of this grant is to develop engineering enhancements to the research grade gene gun to improve its clinical viability. These improvements will focus on novel a disposable barrel, a “spinner” to better distribute DNA coated gold particles, and the inclusion of novel nucleic acid formulations. We foresee these modifications working synergistically to dramatically improve gene expression and immunogenicity.

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NIH/NIAID
Interdisciplinary graduate training fellowship to Paul Veness Munson
The goal of this training grant is to foster research combining two or more distinct disciplines to address biological questions. My project combined virology and immunology to investigate how simian immunodeficiency virus (SIV) derived conserved element (CE) DNA vaccines can limit viremia and progression to AIDS after cessation of antiretroviral therapy (ART).

VITA

Paul Munson was born in Boise, Idaho in 1989. He graduated from Timberline High School in 2007 and Oregon State University in 2011 with a B.S. in microbiology and a B.S. in chemistry. The summer prior to his senior year in college he received an undergraduate research fellowship to complete a research project at SIGA Technologies® under the mentorship of Dr. Robert Jordan studying inhibitors of alphaviruses. He joined the University of Washington Microbiology Graduate Program in 2011. After rotating in several virology labs, he joined the laboratory of Dr. Deborah Fuller in the summer of 2012. His graduate work in the Fuller lab focused on conserved element (CE) specific T-cell responses in SIV/SHIV infected macaques.