Designing Synthetic Dna Encoded Immunotherapeutics And Nanoparticle Vaccines For Enhanced Immunity-Mediated Protection

Ziyang Xu
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Designing Synthetic Dna Encoded Immunotherapeutics And Nanoparticle Vaccines For Enhanced Immunity-Mediated Protection

Abstract
Through introduction of antigenic materials into hosts, vaccines can elicit adaptive immunity which confers protection to the hosts from subsequent exposure to pathogens and cancerous cells. As active immunizations require immune-competent hosts, passive immunotherapy can be a complementary treatment in which protective biologics are directly administered for more immediate protection. However, GMP production of both vaccines and immuno-therapies can be costly and time-consuming, impeding translation and deployment of promising therapeutic candidates. Prior work has demonstrated the use of synthetic DNA and adaptive electroporation (EP) for in vivo delivery of vaccines and immunoglobulins. In this dissertation, I explored the use of synthetic DNA/EP for in vivo folding, assembly, and secretion of more complex anti-HIV-1 biologic eCD4-Ig, and simultaneous delivery of four distinct HIV bNAbs in a single host. Prolonged expression of both eCD4-Ig and HIV bNAbs were observed, demonstrating the host's own myocytes can be efficient bio-factories for the assembly of biologics. In the case of eCD4-Ig, the neutralization potency can be improved when the biologic is post-translationally modified in vivo through a co-administered DNA-encoded enzyme. Harnessing engineering lessons learnt for in vivo expression of complex protein domains, I studied if DNA/EP can be used to launch designed self-assembly nanoparticle vaccines in vivo. Using three complementary techniques, I directly demonstrated that DNA-launched HIV-1 priming antigen eOD-GT8-60mer can assemble in vivo and can induce significantly faster sero-conversion, higher setpoint antibody titers and CD8+ T-cell responses (CTL) in both mice and guinea pigs than a DNA-encoded GT8-monomer. Importantly, induction of CTL was unique to DNA-launched nano-vaccines and not observed for protein nano-vaccines, due to differences I observed regarding the mechanisms of antigen presentations for each. This observation was used to construct the next-generation DNA-launched nano-vaccines scaffolding melanoma tumor-associated antigens, which exerted strong protection to mice challenged with B16F10 melanoma cells. In summary, my work has demonstrated that DNA/EP can be a robust mechanism through which complex biologics and vaccines can be delivered to induce unique immunological features highly relevant in the treatment of cancer and other diseases and provide new tools for study for the betterment of human and animal health.

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DESIGNING SYNTHETIC DNA ENCODED IMMUNOTHERAPEUTICS AND
NANOPARTICLE VACCINES FOR ENHANCED IMMUNITY-MEDIATED PROTECTION

Ziyang Xu

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ABSTRACT

DESIGNING SYNTHETIC DNA ENCODED IMMUNOTHERAPEUTICS AND NANOPARTICLE VACCINES FOR ENHANCED IMMUNITY-MEDIATED PROTECTION

Ziyang Xu

Dr. David B. Weiner

Through introduction of antigenic materials into hosts, vaccines can elicit adaptive immunity which confers protection to the hosts from subsequent exposure to pathogens and cancerous cells. As active immunizations require immune-competent hosts, passive immunotherapy can be a complementary treatment in which protective biologics are directly administered for more immediate protection. However, GMP production of both vaccines and immuno-therapies can be costly and time-consuming, impeding translation and deployment of promising therapeutic candidates. Prior work has demonstrated the use of synthetic DNA and adaptive electroporation (EP) for \textit{in vivo} delivery of vaccines and immunoglobulins. In this dissertation, I explored the use of synthetic DNA/EP for \textit{in vivo} folding, assembly, and secretion of more complex anti-HIV-1 biologic eCD4-Ig, and simultaneous delivery of four distinct HIV bNAbs in a single host. Prolonged expression of both eCD4-Ig and HIV bNAbs were observed, demonstrating the host’s own myocytes can be efficient bio-factories for the assembly of biologics. In the case of eCD4-Ig, the neutralization potency can be improved when the biologic is post-translationally modified \textit{in vivo} through a co-administered DNA-encoded enzyme. Harnessing engineering lessons learnt for \textit{in vivo} expression of complex protein domains, I studied if DNA/EP can be used to launch designed self-assembly nanoparticle vaccines \textit{in vivo}. Using three complementary techniques, I directly demonstrated that DNA-launched HIV-1 priming antigen eOD-GT8-60mer can assemble \textit{in vivo} and can induce significantly faster sero-conversion, higher setpoint antibody titers and CD8+ T-cell responses (CTL) in both mice
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CHAPTER 1: INTRODUCTION

Adaptive immunity is an important branch of the immune system that helps prevent and clear viral infections (Braciale and Hahn, 2013), as well as mediating surveillance against tumor cells (Swann and Smyth, 2007). Humoral immunity refers to protection mediated by circulating antibodies which are secreted by B cells. These immunoglobulins, by direct interaction with their specific epitopes on the surfaces of pathogens, serve to neutralize these pathogens by blocking viral entry into the target cells (Burton and Hangartner, 2016). Alternatively, the constant portion of immunoglobulin heavy chain (Fc) can also interact with a host of downstream effector cells, including macrophages and NK cells, or serum proteins, such as the complements, to facilitate clearance of infected cells (Seidel et al., 2013). Cytotoxic T lymphocyte (CTL) responses, on the other hand, are mediated by CD8+ T-cells, which can directly kill target cells through T-cell receptor (TCR) peptide-MHC I complex interaction, which triggers the release of effector molecules such as IFNγ and perforin (Zhang and Bevan, 2011). Several clinical trials reported a direct correlation between tumor-infiltrating CD8+ T-cells and tumor regression or disease-free survival in the cancer patients, alluding to their important role in this context.

Induction of the adaptive immune responses through active vaccination is the most important approach to prevent human-to-human or animal-to-human transmission of infectious diseases and is also now being actively explored in the treatment of cancer (Guo et al., 2013). Vaccines have, over the past century, led to eradication of once lethal infectious agents such as smallpox (Voigt et al., 2016), and also significantly reduced the incidences of many diseases, including measles, mumps, rubella, diphtheria, tetanus, hepatitis B, Polio, and also cervical dysplasia caused by high risk HPV (Greenwood, 2014). Annual influenza vaccines, additionally, have also significantly lowered influenza-
related hospital admissions and mortality on a year-to-year basis, even though the seasonal inactivated vaccines currently licensed by the FDAs are still inadequate to deal with antigenic drift and shift in the virus (Ramsay et al., 2019).

While crucial, there are still significant limitations with regard to current approaches for active vaccination. For example, individuals who are immunocompromised, such as newborns, elderlies, chronic AIDS patients or patients who need to be on constant immunosuppressive regimen because of prior organ transplants, cannot mount effective immune responses upon vaccination (Arvas, 2014) and may not be allowed to take live attenuated vaccines. Additionally, time is required for the development of effective immune responses upon vaccination, limiting the initial utility of active vaccination in an outbreak situation (Bonanni et al., 2013). Prior studies demonstrate that seroconversion in vaccinated individuals may occur between 2 weeks and 2 months following their first immunization; and in some scenarios, a second dose may be required (Bonanni et al., 2013; Grassly, 2014). Finally, even in immune-competent individuals, breadth of the induced immune responses may not be sufficient to prevent viral infection (van Schooten and van Gils, 2018). For example, due to the error prone process of reverse transcription, tremendous sequence diversity has been observed for HIV-1 (Lee et al., 2019). The HIV-1 envelope proteins, which mediate viral entry through their interaction of CD4 receptor and CCR5/CXCR4 co-receptor on T cells and macrophages (Wilen et al., 2012), can be extremely different for different HIV-1 isolates, such that autologous neutralizing antibody response against a particular isolate may fail to prevent infection by another isolate (Overbaugh and Morris, 2012).

Passive transfer of immunotherapeutics has been explored as a complementary strategy to active vaccination (Overbaugh and Morris, 2012). These immunotherapeutics may include antibodies isolated from patients or animals, or maybe designed de novo with
various protein engineering techniques (Holliger and Hudson, 2005). Several clinical studies have already explored the utility of this concept in humans, for which intravenous/subcutaneous infusion of these immunotherapeutics may allow protective circulating levels of immunotherapeutics to be present for several weeks (Johnson et al., 2019), depending on the half-life and biodistribution of the construct (Mackness et al., 2019). As compared to active vaccination, passive transfer immunotherapeutics will confer protection in recipients within just hours of administration (Nasser et al., 2010). They have additionally been demonstrated to exert therapeutic benefits against diseases including HIV-1, for which broadly neutralizing antibodies may be hard to generate with active vaccination (Gruell and Klein, 2018).

While promising, manufacturing of the recombinant forms of vaccines and immunotherapeutics may be time-consuming and costly, impeding their clinical translation and broad usage (Nagasawa et al., 2012; Schlake et al., 2019). GMP grade materials need to be used for both production and purification with these immunotherapeutics. Therefore, a method to simplify manufacturing of these constructs through direct nucleic acid mediated direct in vivo production may be highly advantageous for the rapid global deployment of new approaches.

1.1 Passive Immunotherapeutics against HIV-1 and cancer

Passive transfer of immunotherapeutics has been explored in both preclinical and clinical trials for HIV-1 and cancer, generating promising results in several studies (Kruger et al., 2019; Puronen et al., 2019). In the case of HIV, passive transfer of broadly neutralizing antibodies (bNAbs), which target various epitopes on HIV envelope such as the CD4 binding site, V2-glycan patch, V3-glycan patch, gp120/gp41 interface, and MPER region have been isolated from HIV-infected individuals (Burton and Hangartner, 2016), has been demonstrated to confer protection to rhesus macaques from Simian-Human
Immunodeficiency Virus (SHIV) challenge (Julg et al., 2017a). In SHIV-infected anti-retroviral therapy (ART)-suppressed aviremic monkeys, latency reversal agent, such as TLR7 agonist, and passive transfer of bNAbs have been observed to eradicate HIV reservoir in several monkeys (Borducchi et al., 2018). Additionally, for both chronically infected viremic patients and ART suppressed individuals who went through temporary withdrawal of ART (analytical therapeutic interruption, ATI), passive transfer of bNAbs, especially the combination of 3BNC117 and 10-1074, can transiently suppress viremia or delay viral rebound (Bar-On et al., 2018; Scheid et al., 2016); and in some cases, help elicit endogenous CTL responses to facilitate maintenance of viral suppression (Mylvaganam et al., 2019; Yamamoto and Matano, 2016).

Other than bNAbs, de novo designed biologics have also been observed to potently neutralize HIV-1. eCD4-Ig is a promising example of such biologic. It consists of extracellular D1 and D2 domains of the CD4-receptor, fused to CH2 and CH3 domains of human IgG1 Fc, and incorporates a short peptide fragment that mimics human CCR5 co-receptor on the C-terminus. In principle, the CD4 domain on eCD4-Ig will interact HIV envelope to expose the co-receptor binding site on Env, and subsequent binding of CCR5 mimetic peptide with Env will potently neutralize the virion by blocking Env receptor co-receptor interaction (Gardner et al., 2015). On a diverse panel of HIV pseudoviruses evaluated, eCD4-Ig has been demonstrated to neutralize broadly with more potent IC\textsubscript{50} values than several well-characterized bNAbs. In addition, resistance mutation against eCD4-Ig is less likely to arise without imposing a fitness cost to the infectivity of the virus (Fellinger et al., 2019). It has been also previously demonstrated that post-translational modification, such as tyrosine sulfation, is important for the interaction of CCR5 co-receptor with HIV envelope (Farzan et al., 1999). As such, tyrosine sulfation of CCR5 mimetic peptide is also considered to be important for maximizing the potency of eCD4-Ig.
(Xu et al., 2018). In a non-human primate (NHP) study, co-administration of AAV-encoded eCD4-Ig with AAV-encoded TPST2, a Golgi enzyme which promotes tyrosine sulfation of eCD4-Ig, can protect rhesus macaques from escalating doses of SHIV-AD8 challenges (Gardner et al., 2015). In a separate NHP study, AAV-encoded eCD4-Ig also transiently suppressed viremia in SHIV-infected NHPs, demonstrating potency and utility of such engineered biologics (Gardner et al., 2019a).

Antibody-based therapy has also shown great promises in the treatment of cancer. Immune checkpoint inhibitors such as anti-PD1, anti-PDL1, and anti-CTLA4 antibodies used either singly or in combination has significantly improved survival of late-stage cancer patients, and are currently licensed for treatment of breast, colon, lung and liver cancers (Azoury et al., 2015; Darvin et al., 2018). Monoclonal antibody against tumor specific targets, such as Herceptin which binds to and blocks the signaling of HER2/neu receptor, is also efficacious in the treatment of some cancer types, including breast cancer (Nahta and Esteva, 2007).

However, administration of passive immunotherapies for both HIV and cancer can be limited by their cost of production. Frequent dosing may also be required due to limited half-lives of the constructs. For example, Herceptin is usually administered at an initial dose of 8mg/kg via 90-minute-long intravenous (IV) infusion. The patient subsequently needs to be re-dosed every three weeks via IV infusion until disease progression (Leyland-Jones, 2001). On average, each patient costs are approximately $70,000 per year for Herceptin and will need to return to the healthcare facility for each IV infusion (Aboutorabi et al., 2014). As such, financial and logistic hurdles associated with administration of recombinant protein form of these immunotherapeutics can impede broader use of these agents for patients’ benefits and limit their use globally.
1.2 DNA-encoded Monoclonal Antibody (DMAb) platform

Our lab, in collaboration with others, has established the method of using synthetic DNA to facilitate direct in vivo production of monoclonal antibodies. When coupled with adaptive electroporation, synthetic DNA plasmids will be directly taken up by muscle cells at the site of transfection (Flingai et al., 2013). Transcription and translation of the gene inserts by transfected myocytes will lead to in vivo expression of the DMAb transgene (Muthumani et al., 2013). Secretion of the gene product by myocytes will then lead to systemic expression of the construct. As DNA is extremely stable in host cells, encoded gene product is continuously expressed and can be detected in circulation for over a year (Wise et al., 2019).

Using this approach, in the infectious disease space, we have designed DNA-encoded bispecific antibody against pseudomonas and attained potent protection of mice challenged with the bacteria. Similarly, DMAbs encoding two broadly neutralizing antibodies against influenza head and stem domains effectively protected mice in both Influenza A and Influenza B challenges (Elliott et al., 2017; Patel et al., 2017). Co-administration of DNA-encoded vaccine and DMAb against Chikungunya enables both early protections, mediated by DMAb, and late protections, mediated by DNA vaccine, of virally challenged mice (Muthumani et al., 2016). Through several iterations, optimizations in construct sequences (codon and mRNA sequence optimizations), synthetic DNA formulation, adaptive electroporation parameters have contributed to significantly improved in vivo expression of DMAbs, allowing DMAb expression in NHP to improve from ng/mL range to ug/mL range. Recently, we have shown that DMAb encoding an anti-ZIKA antibody (ZK-190) can be expressed at potent level in NHPs to protect them from viral challenge (Esquivel et al., 2019).
In the cancer space, DMAb encoding anti-human CTLA4 antibody (ipilimumab) can be expressed at potent level \textit{in vivo} and leads to complete regression of tumors in both prophylactic and therapeutic treatment models (Duperret et al., 2018a). Recently, we have also reported the use of synthetic DNA to encode bi-specific T-cell engager (BiTE) for treating cancer. These BiTE constructs consists of a Fab fragment targeting human CD3+ on one arm and another Fab fragment targeting a tumor-associated antigen on the other arm. They serve to cross-link tumor target cell with effector T-cell, while simultaneously activating T-cell (with CD3 receptor binding) to enable potent killing of target cells (Slaney et al., 2018). However, clearance of the recombinant protein form of BiTE is extremely fast, with reported half-lives ranging from minutes to few hours, requiring patients to receive treatments in clinic very frequently (Huehls et al., 2015). The DNA BiTE (DBiTE) approach significantly prolonged \textit{in vivo} expression of BiTE even with a single round of injection, with observable expression in the mice sera, as assessed by the ability to facilitate effector-mediated killing of target cells, for several months. In NSG mice, single treatment of DBiTE, followed by passive transfer of human PBMCs, lead to complete response in 80% mice challenged with OVCAR3 tumors (Perales-Puchalt et al., 2019).

A potential drawback with the DMAb approach is the hosts’ anti-DMAb immune responses. Anti-drug antibodies (ADA) against the foreign transgene in this scenario can lead to rapid clearance of DMAb from systemic circulation, leading to significantly attenuated systemic expression of DMAb seven days post treatment when DNA-encoding human antibodies were administered to mice (Esquivel et al., 2019). It was, however, found that transient immune-modulation at the time of DMAb treatment, either with antibody-mediated depletion of CD4+ and CD8+ T cells or blocking of CD40L/CD40 signaling pathway, can abolish observed ADA response and enable prolonged \textit{in vivo} DMAb expression (Wise et al., 2019; Xu et al., 2018). Regardless, our work with DMAb
demonstrates that synthetic DNA may be a versatile tool for direct in vivo production, folding and assembly of complex biologics for passive immunotherapy or active vaccination.

1.3 Synthetic nanoparticle vaccines

In the past decade, advances in material engineering has allowed for the development of a nanoparticle vaccines for clinical use (Al-Halifa et al., 2019; Gregory et al., 2013; Zhao et al., 2014). It was observed that vaccine efficacy can be significantly enhanced through multivalent antigen display, presumably by cross-linking of B-cell receptors for enhanced signaling. Nanoparticles may come in several shapes and forms. Inorganic materials such as gold, silica and aluminum hydroxide (or alum), can be used to present multiple copies protein antigens electrochemically adsorbed to the materials (Cheng et al., 2012; Stone et al., 2013). Nontoxic phospholipids may be used to directly encapsulate antigens for presentation as liposomes (Giddam et al., 2012). Alternatively, virus-like particles (VLPs) or naturally occurring non-viral protein cages, de novo designed protein shells and coiled-coil assemblies can each be reengineered as nanoparticle scaffolds to display vaccine antigens as self-assembling protein nanoparticles (SAPN). These approaches have demonstrated remarkable utility in enhancing vaccine potency (Table 1.1).
Nanoparticle Source Shape Valency Size (nm) Decorated antigens

<table>
<thead>
<tr>
<th>Nanoparticle</th>
<th>Source</th>
<th>Shape</th>
<th>Valency</th>
<th>Size (nm)</th>
<th>Decorated antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferritin</td>
<td><em>Helicobacter pylori</em></td>
<td>Octahedral</td>
<td>24</td>
<td>12</td>
<td>HA stem (Yassine et al., 2015)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HA RBD (Kanekiyo et al., 2019)</td>
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<td></td>
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<td></td>
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<td></td>
<td>HIV BG505.MD39 (Tokatlian et al., 2019)</td>
</tr>
<tr>
<td>Lumazine synthase</td>
<td><em>Aquifex Aeolicus</em></td>
<td>Icosahedral</td>
<td>60</td>
<td>30</td>
<td>HIV eOD-GT8 (Jardine et al., 2016)</td>
</tr>
<tr>
<td>E2p</td>
<td><em>G. stearothermophilus</em></td>
<td>Icosahedral</td>
<td>20</td>
<td>23</td>
<td>HIV BG505.gp140 (He et al., 2016)</td>
</tr>
<tr>
<td>I3-01</td>
<td><em>De novo design</em></td>
<td>Icosahedral</td>
<td>20</td>
<td>25</td>
<td>HIV BG505.gp140 (He et al., 2018)</td>
</tr>
<tr>
<td>Encapsulin</td>
<td><em>T. maritima</em></td>
<td>Icosahedral</td>
<td>60</td>
<td>24</td>
<td>EBV gp350.D123 (Kanekiyo et al., 2015)</td>
</tr>
<tr>
<td>Major vault protein</td>
<td>eukaryotes</td>
<td>Barrel-shaped</td>
<td>78</td>
<td>40 × 67</td>
<td>HIV Gag (Ding et al., 2018)</td>
</tr>
<tr>
<td>Qb/AP205 coat proteins</td>
<td>bacteriophage</td>
<td>Icosahedral</td>
<td>180</td>
<td>26</td>
<td>Malaria Pfs25 (Brune et al., 2016; Leneghan et al., 2017)</td>
</tr>
<tr>
<td>Coiled-coil-based cages</td>
<td><em>De novo design</em></td>
<td>Octahedral</td>
<td>24</td>
<td>20</td>
<td>HA Helix C/M2e (Karch et al., 2017)</td>
</tr>
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**Table 1.1 Summary of self-assembly protein nanoparticle scaffolds used to present immunogens**

For example, several recent studies reported that ferritin nanoparticles decorated with engineered influenza hemagglutinin (HA) (Kanekiyo et al., 2013; Yassine et al., 2015) or HIV Env (Morris et al., 2017; Sliepen et al., 2015; Tokatlian et al., 2019) elicit stronger humoral responses than the corresponding monomers. Georgiev et al. recently demonstrated that two-component ferritin nanoparticles composed of both HA and HIV antigens could induce neutralizing antibodies to both viruses, demonstrating the feasibility of using nanoparticle vaccine cocktails (Georgiev et al., 2018). Kanekiyo et al. created ‘mosaic’ ferritins from HA receptor-binding domains of eight influenza isolates and reported that the mosaic nanoparticles induce broader responses than either a mixture of eight individual ferritin nanoparticles or equivalent sequential immunizations (Kanekiyo et al., 2019). The potency of these nanoparticle vaccines may be further enhanced. While the ferritin scaffold is capable of displaying 8 copies of a trimeric immunogen or 24 copies
of a monomeric immunogen, 60-subunit icosahedral protein assemblies can display 20 trimers or 60 monomers. For example, native-like HIV-1 Env trimers have been engineered to be displayed on E2p and I3-01 scaffolds (He et al., 2016; He et al., 2018). Lumazine synthase has been used to decorate 60 copies of HIV-1 priming antigen eOD-GT8 (Abbott et al., 2018; Jardine et al., 2015). The multivalency of GT-60mers is important for priming broadly neutralizing antibody responses in knock-in mice (Jardine et al., 2015) and activating rare bnAb precursors (Abbott et al., 2018). Encapsulin is a 60-mer scaffold that has been used to display Epstein–Barr Virus antigens (Kanekiyo et al., 2015). Coiled-coil driven assemblies, where homotrimeric and heterodimeric coiled-coils are fused together, were used to present two influenza epitopes (the ectodomain of the M2 protein and helix C of the HA protein) along with a TLR5 agonist (flagellin) to induce high titers of antibodies against both epitopes (Karch et al., 2017). Recent structures of an engineered major vault protein in fusion with HIV Gag consist of 78 subunits may pave the way for using this nanoparticle scaffold for future vaccine studies (Ding et al., 2018). Overall, using nanoparticles to scaffold and present immunogens has been demonstrated as a viable strategy for the induction of more potent functional humoral immunity.

However, some intrinsic production challenges with these inorganic nanoparticle vaccines or SAPN remain and have impeded their broader translation into the clinical space (Desai, 2012; Feng et al., 2019). VLP vaccines are often produced at low yields in mammalian cell lines and are difficult to purify, requiring complex reassembly processes and additional post-hoc characterization (Fuenmayor et al., 2017; Lua et al., 2014; Urakami et al., 2017). Production of HPV VLPs, for example, requires three sequential purification steps of strong cation exchange chromatography, size-exclusion chromatography, and hydroxyapatite chromatography (Jiang et al., 2011). Large-scale production of liposome-based nano-vaccines is challenging, as slight variations in the
methods of production result in heterogeneity of the liposomes produced (Desai, 2012). Production of nano-vaccines for a global market could therefore require specialized pipelines which raise costs. In addition, regulatory approval of drugs for use in humans can be complex for development of multicomponent nano-medicines (Eifler and Thaxton, 2011). Technologies that would allow de novo nanoparticle assemblies directly in the hosts from materials that are inexpensive, simple and stable, which bypass complex biochemical processes and downstream purifications, may be of interest.

1.4 DNA vaccines

DNA vaccines were first brought to the attention of scientific community in the early 1990s, when Tang et al. reported delivery of DNA-encoding human growth hormone with a gene gun as a gene therapy could induce anti-transgene antibodies unexpectedly (Tang et al., 1992). Coincident with that report, at the Cold Spring Harbor Vaccine meeting (September 1992), the first presentations on development of DNA vaccines against true pathogens or tumors were featured. Presentations by Dr. Liu (Merck) and Dr. Robinson (UMASS) reported that influenza vaccine antigens cloned into plasmids could induce immune responses in animals when the DNA was injected intramuscularly (IM) and Dr. Weiner (UPenn) reported that HIV antigens could be delivered as vaccines via the IM route to induce immune responses. Additionally, Dr. Weiner also reported that delivery of tumor antigens encoded into plasmids could impact tumor growth in a mouse challenge models. Their publications rapidly followed (Fynan et al., 1993; Ulmer et al., 1993; Wang et al., 1993). Within two years of this meeting, the first DNA vaccine human trial started and was led by MacGregor, Gluckman and co-workers at UPenn to evaluate DNA encoded HIV envelope constructs, initially developed at Dr. David Weiner’s laboratory, as immunotherapy (MacGregor et al., 1998). Soon after, Lessen, Rook, Williams and co-workers at UPenn examined DNA vaccine in the treatment of human CTCL cancer. By
1995, DNA vaccine encoding HIV Env immunogen was allowed to be evaluated in a healthy population (led by joint efforts amongst UPenn, Apollon and the NIH), which was followed rapidly by another effort to evaluate an influenza DNA vaccine in healthy individuals (led by Drs. Clemons, White and Liu at Merck and John’s Hopkins). Subsequently, a plethora of work on DNA vaccines that targeted a range of different diseases, from cancer, influenza, malaria, hepatitis B, to HIV-1, emerged (Giri et al., 2004; Jin et al., 2005; Kutzler and Weiner, 2008; Stachyra et al., 2014; Stevenson et al., 2004; Tuteja, 2002). As compared to other routes of vaccination (recombinant proteins and viral vector), DNA plasmids encoding antigen transgenes can be rapidly and cost-efficiently manufactured. Simple DNA plasmids do not provoke antigen specific immunity against the DNA backbone, enabling vaccine boosting in the same individuals with the same plasmid vector, and focusing the host immunity on the transgene. Synthetic DNA is highly stable, thereby obviating the need for cold chain transport/storage and facilitating global deployment of the vaccines during outbreaks (Hobernik and Bros, 2018; Saade and Petrovsky, 2012).

However, one major limitation with the first-generation DNA vaccine is that, as compared to recombinant protein or viral-vectored vaccine, immunogenicity of DNA vaccine was lower than optimal in larger mammals (NHPs) and in humans, impeding enthusiasm for this approach (Redding and Weiner, 2009; Suschak et al., 2017). Several strategies have been attempted to improve the overall immunogenicity of DNA vaccines. One such strategy is the co-delivery of DNA-vaccine with DNA-encoded cytokine adjuvant (such as IL-12) to enhance co-stimulation of antigen-presenting cells (APCs) (Kalams et al., 2012). It was observed that induced immune responses were significantly enhanced with the co-delivery of cytokines; in several NHP studies, cytokine-augmented DNA vaccination was observed to improve control of viremia in SHIV monkeys (Kulkarni et al.,
More importantly, it was observed that induced immune responses were significantly increased when DNA vaccines were delivered with adaptive electroporation (Mann et al., 2014; Todorova et al., 2017). Membrane electrochemical permeabilization and electric field created by applied voltages can significantly improve uptake of DNA plasmids by the transfected cells, improving transfection efficiency by up to 1000-fold in some scenarios (Donate et al., 2011; Roos et al., 2006). Additionally, as compared to recombinant protein vaccines, DNA vaccines adjuvanted by electroporation have been observed to uniquely elicit strong CD8+ T-cell responses, potentially through direct transfection of APCs at the site of transfection, or enhanced APC infiltration/ cross-presentation through the creation of unique pro-inflammatory environments (Cho et al., 2001; Rush et al., 2002; Walters et al., 2017). DNA responses, in conjunction with newer adaptive electroporation technologies, have now started to induce more potent and consistent responses in humans, and have been evaluated in a series of clinical trials against ZIKA, EBOLA, MERS and HIV-1 (Tebas et al., 2017; Kalams et al., 2013; Modjarrad et al., 2019; Tebas et al., 2019). Promising results have been obtained in recent ZIKA and MERS trials, for which both binding and neutralizing antibody responses, as well as CD8+ T-cell responses have been observed (Tebas et al., 2017; Modjarrad et al., 2019). The unique ability of DNA vaccines to induce CTL, additionally, has been explored in the treatment of cancer patients. In a Phase II study, it was observed that DNA vaccination of HPV E6/E7 antigens in patients with HPV-induced cervical dysplasia can lead to infiltration of CD8+ T-cells into cancer tissues and profound regression of pathology and infection in 50% of treated patients (Trimble et al., 2015).

With intramuscular administration of DNA vaccines, however, a relatively high plasmid dose of 2mg would be required during each vaccination for the induction of immune responses. While manufacturing of DNA is relatively cheap, high dose
requirement may still impede broader translation of this approach during global pandemics. Several attempts have been made to reduce the dose requirement for DNA vaccination (dose-sparing). One promising approach involves intradermal (ID) delivery of DNA plasmid in combination with electroporation (Smith et al., 2017). As there is a relatively higher abundance of APCs in the skin as compared to in the muscles, ID DNA vaccination may provide a more direct route for both plasmids and expressed antigens to be delivered to the relevant APC populations (Romani et al., 2012). For example, in a recent MERS Phase I trial, it was observed that ID DNA vaccination could confer dosing sparing by approximately 4-fold (Modjarrad et al., 2019). Strategies that could further reduce the dose requirement for DNA vaccines while simultaneously improving their immunogenicity will significantly improve utility and translatability of such approach.

1.5 Nucleic acid encoded nanoparticle vaccines for direct \textit{in vivo} production

Due to technical challenges and significant costs associated with \textit{in vitro} production and purification of nanoparticle vaccines, alternative strategies that prompt \textit{de novo} assembly of nanovaccines in the hosts have been explored. In the nucleic acid space, liposome-encapsulated mRNA has been used as a vehicle for \textit{in vivo} gene delivery (Michel et al., 2017). Pardi et al., for example, has reported the use of mRNA for \textit{in vivo} delivery of HIV bNAb VRC01. Single infusion of liposome-encapsualted mRNA encoding VRC01 has allowed for potent \textit{in vivo} expression of the bNAb in humanized mice, and which also confers them with protection from intravenous HIV-1 challenge (Pardi et al., 2017). More recently, mRNA has been explored for \textit{in vivo} expression of an HIV-1 priming nanoparticle vaccine eOD-GT8-60mer. A single immunization of mRNA-encoded GT8-60mer in mice elicited high titers of HIV-1 gp120-specific binding antibodies, as well as increased germinal center B cell responses. In transgenic mice harboring the germline VRC01 heavy chain allele, mRNA GT8-60mer immunization induced somatic mutations.
in the heavy chain locus consistent with VRC01-class antibody development (Melo et al., 2019). While the mRNA approach is extremely promising, however, it had been noted that both local and systemic administration of mRNA vehicle would result in expression of the transgene in the liver (Pardi et al., 2015). Additionally, elevations in liver enzymes (such as AST and ALT) have been observed to be associated with liposome-mRNA infusion (Sedic et al., 2018). Additional toxicology study with this approach is likely important while alternative safer approaches of biologic and nanovaccine delivery should be considered.

Synthetic DNA, which has demonstrated a strong safety profile in the clinic, has also been explored for direct in vivo production of these nano-vaccines. One example included the use of MLV gag and a modified MLV Env to display T-cell epitopes on in vivo produced VLPs (plasmo-retro VLPs) (Masavuli et al., 2017). In comparison to a control DNA plasmid harboring a point mutation in MLV gag which disrupts VLP assembly, DNA encoding intact plasmo-retro VLPs enabled superior induction of both CD4+ and CD8+ T-cell responses to the displayed peptides. Additionally, simple structural motifs have also been explored for in vivo multimerization of antigens. An example is IMX313P, a motif that closely resembles naturally occurring complement C4 binding protein (C4bp) to facilitate heptamerization of antigens (Ogun et al., 2008). More recently, DNA-vaccine encoding IMX313P scaffolded HIV-antigen Tat had been shown to elicit superior antigen-specific humoral and cellular responses (Tomusange et al., 2016). Similarly, DNA-vaccines encoding IMX313P scaffolded hepatitis C virus (HCV) envelope protein induced improved neutralizing antibody responses in mice, demonstrating nucleic acid can support in vivo formation of more complex immunogens to induce stronger responses (Masavuli et al., 2019). The main drawback for DNA-encoded VLPs is that encoded ancillary viral genes to facilitate VLP assembly may be immune-dominant, thereby distracting the immune responses away for intended target epitopes (Sant et al., 2007). The DNA heptamerization
strategy is promising. It can, however, be envisioned that DNA vaccines can be used to encode structurally designed, more potent nanoparticle scaffolds to further enhance vaccine efficacy. Since in vivo produced nano-vaccines cannot be further purified, a greater emphasis should be placed on ensuring the encoded nanoparticle antigen can assemble relatively homogenously in vitro through various protein engineering approaches and thereby bypassing the need for further downstream purification.

1.6 Dissertation Aims

As highlighted in the introduction, our lab and several others have provided a proof of principle that synthetic DNA alongside with adaptive electroporation (DNA/EP) can be used for in vivo delivery of not only vaccine antigens but also monoclonal antibodies against different disease targets. In my thesis, I have built on such foundation to further demonstrate DNA/EP can be used to fold highly structured biologics and macromolecular vaccines in vivo (Chapters 3, 4 and 5), and additionally, that functionalities of these proteins can be further modulated through post-translational modification by another DNA-encoded enzyme (Chapter 3). Functionalities of DNA-encoded biologics/ nanovaccines were evaluated with both in vitro assays and in vivo challenge models (Chapters 5 and 6). Finally, different vaccination modalities (protein nano-vaccine and DNA-encoded nano-vaccines) were compared head-to-head and mechanistic studies were pursued to elicit observed differences in terms of induced immune responses (specifically CTL) between protein and DNA vaccination (Chapters 5 and 6).

Aim 1: Determination of DNA/EP as a vehicle for in vivo folding and delivery of complex biologics (immunoglobulins and immunoadhesins) against HIV-1 and characterization of the strategy to modulate their in vivo functionalities with DNA-encoded enzymes for post-translational modifications.
While we have previously demonstrated that single treatment of DMAb encoding an influenza antibody can protect mice from lethal influenza A challenge, HIV-1 pose several unique challenges for DMAb to be viable as a possible preventative strategy. Due to the significant sequence diversity amongst different HIV-1 strains and the high likelihood for resistant clones to arise, a single anti-HIV antibody clone is unlikely to confer significant protection to treated individuals from infection on a population scale (Overbaugh and Morris, 2012). Two strategies were evaluated in this work: first, an immunoadhesin eCD4-Ig, which consisted of artificially designed CCR5 mimetic peptide and naturally occurring extracellular protein domain of CD4 fused to immunoglobulin Fc to improve its breadth of coverage, was re-engineered for in vivo expression with DNA/EP; and second, a cocktail of HIV bNAbS targeting four distinct epitopes on HIV Env have been optimized and encoded in separate plasmid vectors for simultaneous in vivo delivery. This study expands upon our existing platform by allowing us to evaluate whether a cocktail of different antibodies can be simultaneously delivered by DNA/EP for in vivo expression, and whether an artificially engineered biologic can also be expressed by the system.

Additionally, it may be highly desirable to devise strategies to further fine-tune and regulate the activities of in vivo produced biologics. While the variable fragment of an antibody can directly bind to its target epitope and inhibits the biological activity of its target (neutralization in the case of virus), the constant Fc portion of an immunoglobulin can mediate a range of activities, from antibody-dependent cellular cytotoxicity (ADCC) to complement dependent cytotoxicity (CDC) (Saunders, 2019). The Fc effector functions are in turn tightly linked to the post-translational modifications of Fc. Fc sialylation, for example, can significantly prolong the in vivo half-life of a monoclonal antibody, potentially through enhancing interaction between Fc and FcRn (Bas et al., 2019). Fc afucosylation, on the other hand, can significantly enhance the ADCC effector function of an antibody.
(Pereira et al., 2018). In the case of eCD4-Ig, tyrosine sulfation of CCR5 mimetic peptide may directly impact its neutralization potency (as the extracellular domains of CCR5 on CD4 T-cells are constitutively tyrosine sulfated (Farzan et al., 1999)). Therefore, we intend to use eCD4-Ig as a model system to investigate whether we can impact post-translational modification of in vivo produced biologics to influence their functions through co-delivery of DNA-encoded enzyme.

Aim 2: Determination of DNA/EP as a vehicle for in vivo folding and assembly of complex macromolecular nanoparticle vaccines, as well as characterization of induced immune phenotypes with in vitro assays and animal challenge models

I intended to harness insights obtained from Aim 1 to design DNA-cassettes encoding significantly more complex nanoparticle vaccines and evaluate whether they can be launched by adaptive electroporation delivery for direct in vivo assembly. Through different protein engineering techniques, I, in collaboration with Dr. Kulp’s group, have engineered several nanoparticle vaccines scaffolding up to 180 copies of an HIV-1 priming antigen eOD-GT8-60mer that expressed and assembled relatively homogenously in vitro, as characterized by various biophysical techniques. DNA-vaccines encoding simpler multimerized antigens have been investigated before. In this study, we intend to characterize in vivo assembly of significantly more complex nanoparticle vaccines both directly (direct observation of their in vivo formation) and indirectly (through interrogation of immune systems induced by these more advanced immunogens). Induction of both humoral and cellular responses will be examined. In addition, we will determine whether enhancement in responses, if any, will correlate to improved protection of mice from viral challenge, using influenza as a model.
Lastly, it has been previously observed that as compared to recombinant protein vaccine, DNA vaccination of an identical immunogen may result in significantly higher level of CTL responses (Zoller and Christ, 2001). We intend to examine if this observation also applies to DNA-launched nanoparticle vaccines in comparison to protein nanoparticle vaccines, and if so, we intend to examine both forms of vaccination in the cancer model, in which CTL serves as an extremely important correlate of protection. Upon completion of these aims, I intend to further immunologic understandings of using synthetic DNA/EP for *in vivo* delivery of complex and folded biologics or next-generation vaccines to further evaluate/advance the technology as a strategy to simplify manufacturing of highly needed therapeutics.
CHAPTER 2 Materials and Methods

2.1 DNA design and plasmid synthesis

Protein sequence for ReCD4-Ig was as previously reported (Gardner et al., 2015). Protein sequences for HIV-1 antibodies were obtained from NCBI GenBank. Protein sequences for human TPST2 and HS3SA were obtained from UniProt (accession numbers: O60704 and Q9Y663). Protein sequence for SIV\textsubscript{mac239} was obtained from GenBank (accession number M33262). Protein sequences for IgE Leader Sequence and eOD-GT8-60mer were as previously reported (Briney et al., 2016; Xu et al., 2018). Protein sequences for 3BVE-ferritin, PfV and HA\_CA09 were obtained from UniProt (accession numbers: Q9ZLI1, I6U7J4, and C3W5X2). Protein sequence for HA1\_NC99 was obtained from GenBank (accession number AY289929.1). DNA encoding protein sequences were codon and RNA optimized as previously described. (Elliott et al., 2017; Patel et al., 2017) The optimized transgenes were synthesized \textit{de novo} (GenScript, Piscataway, NJ) and cloned into a modified pVAX-1 backbone under the control of the human CMV promoter and bovine growth hormone poly-adenylation signal. Plasmids that encode HIV envelope gp160 for TRO11, 25710, 398F1, CNE8, X2278, BJOX2000, X1632, CE1176, 246F3, CH119, CE0217 and CNE55 were obtained from NIH-AIDS reagent.

2.2 Structure modeling and design of 3BVE, ferritin, LS, PfV and flu nanoparticles

The nanoparticle structures for ferritin (PDB ID: 3BVE), lumazine synthase (PDB ID: 1HQK) and PfV (2E0Z) were used to seed the modeling simulations. The structure of eOD-GT8 (PDB ID: 5IDL) and HA1 (PDB ID: 3GBN) were used to decorate the nanoparticles. N-linked glycans with missing density were added using glycan modeling modules of Rosetta (Labonte et al., 2017). Next, we wrote a new algorithm (simpleNanoparticleModeling) in the Molecular Software Library (Kulp et al., 2012). Briefly,
we aligned the appropriate number of immunogens at the nanoparticle surface using coordinate frames constructed by 3C atoms of the terminal positions of each protein. Immunogens were then tilted by random rotations around the x- and y- axes up to 30 degrees for the first ¾ of the simulation and up to 75 degrees for the last ¼ of the simulation, with a 120-degree rotation allowed for the z-axis. The immunogens were also translated by 10Å to 200Å along an axis projected away from the nanoparticle surface. Clashes were detected at each iteration and the models with the lowest number of clashes at each translation was written out as a potential structural model. The models were manually inspected and utilized to construct linkers as glycine-serine repeats using 30Å per 9 linker residues as a guide. The sequence of the HA isolate H1 NC99 (A/New Caledonia/30/1999 (H1N1)) from residues 65-276 was used to construct the flu nanoparticle.

2.3 Cell lines, transfection and ReCD4-Ig/HIV DMAb purification

HEK293T cells (ATCC Cat# CRL-3216) and TZM-bl cells (NIH-ARP Cat# 8129-442) were maintained in DMEM (Corning Cat# 10-013CV) supplemented with 10% fetal bovine serum (Atlas Biologicals Cat# EF-0500-A) and grown at 37°C and 5% CO₂. Expi293F cells (ThermoFisher Cat # A14527) were maintained in Expi293 expression medium (ThermoFisher Cat# A1435101) at 37°C and 8% CO₂. To determine in vitro sulfation of ReCD4-Ig, cells were seeded at a density of 0.5×10⁶ cells/mL in a 6-well plate and transfected with 1.0μg of p-ReCD4-Ig and varying doses of plasmid encoded enzymes with GeneJammer (Agilent Cat# A204130). Forty-eight hours after transfection, supernatants were collected and centrifuged at 1500g for 5 minutes to remove cellular debris. Adherent cells were lysed with cell lysis buffer (Cell Signaling Cat# A204130) modified with protease inhibitor cocktail (Roche Cat# 26733200). To obtain ReCD4-Ig standards for quantitative ELISA, Expi 293F cells were plated at a density of 2.5×10⁶
cells/mL in Expi293 expression medium, rested overnight and transfected with p-ReCD4-Ig and Expifectamine™ (ThermoFisher Cat# A14525) in OPTI-MEM (ThermoFisher Cat# 31985070). Transfection enhancers were added 20 hours after transfection, and supernatant was harvested 5 days after transfection. Magnetic protein G beads (GenScript Cat# L00673S) were used for purification of ReCD4-Ig, and purity was confirmed with Comassie staining of the SDS-Page gels (data not shown) (ThermoFisher Cat# NP0321).

2.4 HIV Trimer production

Expi293F cells were transfected with plasmid expressing the HIV-1 gp160 Env trimer BG505_MD39_His construct. Cell supernatants containing trimer were clarified by centrifuging (4000xg, 25mins) and filtering (0.2um Nalgene Rapid-Flow Filter). Trimers were then purified from supernatants by nickel affinity chromatography on a HIS-TRAP HP column (GE Healthcare). The trimers were then purified over a size-exclusion chromatography column (GE S200 Increase) in PBS. The molecular weight and homogeneity of the trimers were confirmed by protein conjugated analysis from ASTRA software (Wyatt Technology) with data collected from a size-exclusion chromatography-multi-angle light scattering (SEC-MALS) experiment run in PBS using a GE S6 Increase column, followed by DAWN HELEOS II and Optilab T-rEX detectors. The trimers were aliquoted at 1mg/ml and flash frozen in thin-walled PCR tubes prior to use.

2.5 Production of His-Tagged GT8-monomer and recombinant protein DLnanos

Expi293F cells were transfected with pVAX plasmid vector carrying the DLnano or His-Tagged GT8-monomer transgene with PEI/OPTI-MEM and harvested 6 days post-transfection. Transfection supernatant was first purified with affinity chromatography using the AKTA pure 25 system and an IMAC Nickel column (for His-tagged GT8) and gravity flow columns filled with GNL Lectin beads (for DLnanos). The eluate fractions from the
affinity purification were pooled, concentrated and dialyzed into 1X PBS buffer before being loaded onto the SEC column and then purified with size exclusion chromatography, for which the Superdex 75 10/300 GL column was used to purify His-tagged GT8-monomer and the Superose 6 Increase 10/300 GL column was used for DLnanos (run at 0.5 mL/min). Identified eluate fractions were then collected and concentrated to 1mg/mL in PBS.

2.6 Animals

2.6.1 Mice experiments

All animal experiments in Chapters 3 and 4 were carried out in accordance with animal protocol 112776 approved by the Wistar Institute Institutional Animal Care and Use Committee (IACUC) (Philadelphia, PA). Six-eight week old female BALB/c (Charles River Cat# 028) and B6.Cg-Foxn1nuJ (Jackson laboratory Cat# 000819, RRID:IMSR_JAX:000819) and housed in the animal facility. For transient immune-modulation for eCD4-Ig study, mice were given single intraperitoneal injection of 500µg of anti-mouse CD40L (Bio X Cell Cat# BE0017-1). Mice were then given 160µg (2 injections) or 320µg (4 injections) of DNA co-formulated with hyaluronidase (SigmaAldrich Cat# H4272). One minute after injections, IM-EP was performed at each injection site with the CELLECTRA® 3P device (Inovio Pharmaceutical, Plymouth Meeting, PA).

For mice experiments where a single HIV dMAb was delivered, mice were injected with 100µg (25µg of heavy chain and 25µg of light chain per site, 2 sites total) dMAb plasmids formulated with hyaluronidase (200U/L, Sigma Aldrich) and injected into the tibialis anterior muscles followed by intramuscular electroporation (IM-EP) using the CELLECTRA® 3P device (Inovio Pharmaceuticals). For experiments with two dMAb delivery, mice were injected with 100µg (25µg of heavy chain and 25µg of light chain per site, 2 sites total) of each dMAb plasmids formulated with hyaluronidase and injected into
the tibialis anterior and quadriceps muscles followed by intramuscular electroporation (IM-EP). For experiments with four dMAb delivery, single dMAb control mice were injected with 50µg (25µg of heavy chain and 25µg of light chain per site, 1 site total), formulated and injected into the tibialis anterior. Mice receiving four dMAb plasmids were injected with 50µg (25µg of heavy chain and 25µg of light chain per site, 2 sites total) of each dMAb plasmid, formulated and injected into the tibialis anterior and quadriceps muscles followed by IM-EP. Mice were serially bled to obtain serum for analysis.

All mouse experiments in Chapter 5 were carried out in accordance with animal protocols 1127760 and 112782 approved by the Wistar Institute Institutional Animal Care and Use Committee (IACUC) (Philadelphia, PA). For DNA-based immunization, 6-8 week old female C57BL/6, BALB/c and CD1 mice or 6-8 week old male BALB/c mice purchased from Jackson Laboratory or Charles River Laboratories were immunized one to three times (three-weeks apart) with DLmono_GT8, DLmono_HA_NC99, DLmono_HA_CA09, DNA-encoded LS_HA_CA09, DL_GT8_IMX313P or DLnano_LS_GT8, DLnano_CD4MutLS_GT8, DLnano_3BVE_GT8, DLnano_PIV_GT8, DLnano_LS_HA_NC99 and DLnano_3BVE_HA_CA09 via intramuscular injections into the tibialis anterior muscles (over two sites), followed by intramuscular electroporation with the CELLECTRA 3P device (Inovio Pharmaceuticals). For electroporation, 2 sets of 2 pulses (at 0.1 Amps) were delivered. Each set of 2 pulses lasts 52 milliseconds with a 1 second delay. For all DNA-encoded GT8-based immunizations (except for dosing studies), 25ug of plasmid DNA was used, a standard DNA dose as in prior study (Duperret et al., 2018b). For the control experiment to assess the importance of antigen decoration on nanoparticle, balb/c mice were immunized with 1:1 co-formulated (25ug each) DLmono_GT8 with pVAX, DLmono_GT8 with DLnano_LS (core), and DLnano_LS_GT8 with pVAX and followed for seven d.p.i for sero-conversion. For all DNA-encoded HA-
based immunizations, doses of 1ug were used for each immunization for studies of humoral responses and 10ug for studies of cellular responses. MBL knockout mice (B6.129S4-Mbl1tm1Kata Mbl2tm1Kata/J) and CR2 knockout mice (B6.129S7(NOD)-Cr2tm1Hmo/J) purchased from Jackson Laboratory were immunized in the same fashion.

For protein-based immunization in Chapter 5, 6-8 week old female C57BL/6, MBL knockout and CR2 knockout mice were immunized subcutaneously over two sites with a high dose of 10ug of recombinant eOD-GT8-60mer protein in 50uL co-formulated with 50uL Sigma adjuvant system (SigmaAldrich); the protein dose was 2.7-times higher than a prior study (Tokatlian et al., 2019).

All animal experiments in Chapter 6 were carried out in accordance with animal protocols 201214, 201115, and 201221 approved by the Wistar Institute Institutional Animal Care and Use Committee (IACUC). For DNA-based immunization, 6 to 8 week old female C57BL/6 or BALB/c mice (Jackson Laboratory) were immunized with DNA vaccines via intramuscular injections into the tibialis anterior muscles, followed by intramuscular electroporation with the CELLECTRA 3P device (Inovio Pharmaceuticals). For DNA immunizations using DLnano_LS_GT8 and DLnano_LS_HA(NC99) as antigens, 25ug of plasmid DNA was used, a standard DNA dose that has been utilized in prior study (Duperret et al., 2018b). For DNA immunizations involving DLnano_LS_Trp2188, DLnano_LS_Gp10025, DLmono_Trp2188, and DLmono_Gp10025, 10ug of individual plasmid DNA was used either alone or in combination. For vaccinations involving recombinant protein, 6 to 8 week old female BALB/c or C57BL/6 mice were immunized subcutaneously with a relative high dose of 10ug of recombinant eOD-GT8-60mer protein or HA(NC99)-60mer, and 4ug dose for LS_Trp2188-60mer and LS_Gp10025-60mer in 50uL PBS co-formulated with 50uL Sigma Adjuvant System (SigmaAldrich); a high protein dose was used in this study as compared to prior studies (Tokatlian et al., 2019).
For vaccinations in mechanistic study in Chapter 6, C57BL/6 mice received intramuscular injections of 80μg of DLnano_LS_GT8 co-formulated with 12U Hyaluronidase (SigmaAldrich) followed by intramuscular electroporation or 20μg of protein eOD-GT8-60mer co-formulated 1:1 with Sigma Adjuvant System. For in vivo macrophage depletion, 6 to 8-week-old female C57BL/6 mice received one or three intravenous injection of 750μg clodronate disodium formulated in clodosome (150μL injection, Encapsula Nanoscience) via retro-orbital injections; control mice each received 150μL of encapsosome (Encapsula Nanoscience) IV at the same timepoints.

For tumor challenge, B16-F10 (ATCC) were maintained in 10% FBS/DMEM under low passage (less than 10) and B16-F10-Luc2 cells (ATCC) were maintained in 10% FBS/DMEM enriched with 10μg/mL blasticidin (Gibco) with routine testing for mycoplasma contaminations. Cells were trypsinized and strained through 70μm strainer to generate single cell suspensions. They were then administered sub-cutaneously to mice (10^5 cells to each mouse in 100μL PBS). Tumor size was measured every two days with a digital caliper, and tumor volume was determined with the formula V=0.5W^2L (V=tumor volume, W= tumor width, L=tumor length). Mice with tumor volume greater than 2000mm^3 or with any dimension exceeding 2cm were euthanized for humane purposes. For recombinant anti-PD1 (Bio X Cell) administration in melanoma therapeutic treatment model, 200μg of antibody was injected intraperitoneally in 100μL PBS to each mouse weekly.

2.6.2 Guinea pig experiments

Female Hartley guinea pigs (8–10 weeks old) purchased from Charles River Laboratories (Wilmington, MA) were group housed and handled at BTS Research (San Diego, CA) with ad libitum access to food and water according to the standards of the Institutional Animal Care and Use Committee (IACUC). Following acclimation, each guinea pig was given a single immunization of 50μg of DLnano_LS_GT8 or DLmono_GT8
over 2 sites on the flank followed by intradermal EP with CELLECTRA 3P device. The animals were then bled at the indicated timepoints for humoral analyses.

2.6.3 NHP experiments

Two groups of cynomolgus macaques (N=4) (Primgen) were treated with plasmid dMAb constructs encoding either PGDM1400 or PGDM1400 and PGT121, with animals receiving a total of 6mg plasmid DNA. DNA was co-formulated with hyaluronidase (Hylenex – 135 U/ml). Multi-depth IM injection was performed followed by electroporation using the Elgen1000 Twinjector (Inovio Pharmaceuticals). Macaques were serially bled to obtain serum over 60 days.

2.7 ELISA

2.7.1 eCD4-Ig ELISA

For ELISA-based quantification of ReCD4-Ig, MaxiSor p plates (ThermoFisher Cat# 44-2404-21) were coated with 1ug/mL of JR-FL gp140 (Immune Technology Cat# IT-001-0024ΔTmp) overnight at 4°C. Plates were washed 4 times with Phosphate Buffered Saline + 0.1% Tween 20 (BioRad Cat# 1706531) (PBS-T) and blocked with 10% FBS in PBS for 1 hour at room temperature. Plates were subsequently washed and incubated with serum samples diluted in PBS-T for one hour at room temperature. Plates were washed again and incubated with secondary goat anti-human Fc HRP (Jackson ImmunoResearch Labs Cat# 109-035-008) at 1:5000 dilution for 1 hour. The plates were subsequently developed with SigmaFast OPD (SigmaAldrich Cat# P9187) for 10 minutes before OD450 measurements were performed with Biotek Synergy2 plate reader.

To detect sulfation of ReCD4-Ig in transfection supernatants or sera, MaxiSorp plate were coated at 4°C overnight with 5ug/mL JR-FL gp140. Plates were washed and blocked with 10% FBS/PBS for 3 hours at room temperature. Plates were washed, and
samples diluted in PBS-T were added for 1-hour incubation at room temperature. Plates were washed again and incubated with 1:250 dilution of mouse anti-sulfotyrosine antibody (Millipore Cat# 05-1100) for 1 hour at room temperature. Finally, the plates were washed and incubated with 1:5000 dilution of anti-mouse IgG2a HRP secondary antibody (Bethyl Cat# A90-107P) for 1 hour at room temperature. The plates were developed with SigmaFast OPD for 10 minutes and OD450 signals were measured.

2.7.2.1 HIV dMAb quantification ELISA- mouse

DNA encoded monoclonal antibody levels were quantified as previously described (Patel et al., 2018a). Briefly, high binding polystyrene 96 well plates (ThermoFisher) were coated with unconjugated purified goat anti-human IgG-Fc (1µg/ml) overnight in PBS. After blocking with 10% newborn calf serum (NCS) plates were washed with PBS containing 0.05% Tween-20. Mouse serum and standards were serially diluted and incubated for one hour at room temperature. Purified human IgG-Kappa (P80-111, Bethyl Laboratories) and human IgG-Lambda (P80-116, Bethyl Laboratories) were used as standards. After washing, plates were incubated with 1:20,000 dilution of either goat anti-human kappa (A80-115P, Bethyl Laboratories) or goat anti-human lambda (A80-116P, Bethyl Laboratories) light chain secondary antibodies conjugated to horseradish peroxidase (HRP) for one hour at room temperature. For quantification of total antibody levels in the mice dosed with four dMAb constructs, a secondary goat anti-human IgG H+L conjugated to horseradish peroxidase (A80-119P, Bethyl Laboratories) was used. After washing, plates were developed with o-phenylenediamine dihydrochloride (OPD) substrate (SIGMAFAST OPD, Sigma Aldrich). Plates were stopped with 2N H₂SO₄. Plates were read on a BioTek Synergy 2 plate reader (BioTek) at 450nm and 570nm wavelengths. Quantification of serum dMAb levels were determined by interpolating the unknown OD values to the standard curve.
2.7.2.2 dMAb quantification ELISA- Non-human primate

Quantification for human IgG dMAb in NHP sera was determined using the human therapeutic IgG1 ELISA kit from Cayman Chemicals following the manufacturer’s protocol. Serum was serially diluted to obtain two OD values within the linear range of the standard curve. Quantification measurements were repeated twice for Days 7-21.

2.7.2.3 dMAb binding to trimer ELISA

Binding curves of HIV serum-expressed dMAbs compared to recombinant proteins were obtained by coating 96 well half-area high binding polystyrene plates (ThermoFisher) with 1µg/ml of rabbit anti-His antibody (25B6E11, Genscript) in PBS at 4oC. Plates were blocked with 5% skim milk in PBS with 1% newborn calf serum (NCS) (Atlas Biologicals) and 0.2% Tween-20 for one hour at room temperature. His-BG505 MD39 trimer protein was then added at 1µg/ml in PBS with 1% NBS and 0.2% Tween-20 for two hours at room temperature. Serum was normalized based on the quantification concentration and serially diluted 2-fold from there. A similar amount of purified recombinant monoclonal antibody was added to match the serum concentration. Plates were incubated for one hour at 37oC. After washing, a 1:20,000 dilution of secondary goat anti-human kappa (A80-115P, Bethyl Laboratories) or goat anti-human lambda (A80-116P, Bethyl Laboratories) light chain secondary antibodies conjugated to horseradish peroxidase were added and incubated for one hour at room temperature. Plates were then developed with 1-step ultra-3,3',5,5'-tetramethylbenzidine (TMB) substrate (ThermoFisher) and read on a BioTek Synergy 2 plate reader (BioTek) at 450nm and 570nm wavelengths.

2.7.2.4 Anti-antibody detection ELISA

To determine the development of anti-drug antibody development, 96 well half-area high binding polystyrene plates (ThermoFisher) were coated with 1µg/ml of purified PGT121 or PGDM1400 (produced in-house) overnight in PBS at 4oC. Plates were then
blocked with 5% skim milk in PBS with 1% newborn calf serum (NCS) (Atlas Biologicals) and 0.2% Tween for one hour at room temperature. NHP sera was diluted 1:100 and added to plates for each time point and incubated for one hour at 37°C. After washing, a 1:20,000 dilution of secondary goat anti-NHP H+L min human secondary antibody conjugated to HRP (A140-202P, Bethyl Laboratories) was then added and incubated for 1hr at room temperature. Plates were then developed with 1-step ultra-TMB substrate (ThermoFisher) and read on BioTek Synergy 2 plate reader (BioTek) at 450nm and 570nm wavelengths.

2.7.3.1 GT8-binding ELISA

Corning 96-well half area plates were coated at room temperature for 6 hours with 1µg/mL MonoRab anti-His antibody (GenScript), followed by overnight blocking with solution containing 1x PBS, 5% skim milk, 10% goat serum, 1% BSA, 1% FBS, and 0.2% Tween-20. The plates were then incubated with 2µg/mL of his-tagged GT8-monomer at room temperature for 2 hours, followed by addition of mice sera serially diluted with PBS with 1% FBS and 0.1% Tween and incubation at 37°C for 2 hours. The plates were then incubated at room temperature for 1 hour with Peroxidase AffiniPure Goat Anti-Mouse IgG, Fcγ fragment specific at 1:5,000 dilution (Jackson ImmunoResearch) or AffiniPure Goat Anti-Mouse IgM, μ chain specific, (Jackson ImmunoResearch) at 1:5000 dilution followed by addition of TMB substrates (ThermoFisher) and then quenched with 1M H₂SO₄. Absorbance at 450nm and 570nm were recorded with BioTEK plate reader. Endpoint titer is defined as the highest dilution at which the OD of the post-immune sera exceeds the cut-off (mean OD of naïve animals plus standard deviations of the OD in the naïve sera multiplied with standard deviation multiplier f at the 99% confidence level).
2.7.3.2 VRC01 competition ELISA

The plates were coated, and blocked, followed by addition with GT8-his as described in the last section. Serially diluted mice sera were then incubated with the plates at 37°C for 1 hour, followed by addition of purified VRC01 antibody (NIH AIDS Reagent) for an additional 1 hour at room temperature. The plates were then incubated with anti-human Fc (cross-adsorbed against rabbits and mice) (Jackson Immunoresearch) at 1:10,000 dilution for 1 hour, followed by addition of TMB substrate for detection. Absorbance at 450nm and 570nm were recorded with BioTEK plate reader.

2.7.3.3 MBL binding ELISA

The plates were coated with 5ug/mL recombinant mouse MBL protein (R&D system) in 0.1M CaCl$_2$ at room temperature for 6 hours, followed by blocking with 1% BSA in 0.1M CaCl$_2$ in PBS overnight at 4°C. Transfection supernatant or muscle homogenates containing DLmono_GT8 or DLnano_LS_GT8 were then added to the plates for 2 hour incubation at 37°C, followed by Week 5 sera of BALB/c mice previously immunized twice with 25ug DLnano_LS_GT8. The plates were next incubated with anti-mouse IgG H+L (cross-adsorbed against human) HRP (Jackson Immunoresearch) at 1:10,000 dilution, followed by addition of TMB substrates. Absorbance at 450nm and 570nm were recorded with BioTEK plate reader.

2.7.3.4 VRC01 binding ELISA

ELISA format as described in the MBL binding ELISA section except that the recombinant MBL used in the coating step is replaced by 5ug/mL of VRC01 (NIH AIDS Reagent). Absorbance at 450nm and 570nm were recorded with BioTEK plate reader.
2.7.3.5 Antigenic profile characterization of designed GT8-nano-vaccines

Corning half-area 96-well plates were coated with 2ug/mL of GT8-monomer, or 3BVE_GT8-24mer, eOD-GT8-60mer, CD4Mut_LS_GT8-60mer and PfV_GT8-180mer at 4C overnight. The plates were then blocked with the buffer as described in the Section 2.7.3.1 for 2 hours at room temperature, followed by incubation with serially diluted VRC01 at room temperature for 2 hours. The plates were next incubated with anti-human Fc (cross-adsorbed against rabbits and mice) (Jackson Immunoresearch) at 1:10,000 dilution for 1 hour, followed by addition of TMB substrate for detection. Absorbance at 450nm and 570nm were recorded with BioTEK plate reader.

2.7.3.6 HA-binding ELISA

Corning 96-well half area plates were coated at 4C overnight with 2ug/mL of recombinant HA(ΔTM)(H1N1/A/New Caledonia/20/1999) or HA(ΔTM)(A/California/04/2009)(H1N1) (Immune Technology), and blocked at room temperature for 2 hour with the buffer as described in the GT8-binding ELISA section. The plates were subsequently incubated with serially diluted mouse sera in PBS with 1% FBS and 0.1% Tween at 37C for 2 hours, followed by 1-hour incubation with anti-mouse IgG H+L HRP (Bethyl) at 1:20,000 dilution at room temperature and development with the use of TMB substrate. Absorbance at 450nm and 570nm were recorded with BioTEK plate reader.

2.8 Western blot

For detection of ReCD4-Ig, 10uL of transfection supernatant was loaded onto precast 4–12% Bis-Tris gels under non-reducing condition and transferred to an Immobilon-FL PVDF membrane (EMD Millipore Cat# IPFL10100) with wet transfer. ReCD4-Ig was identified with IRDye 800CW goat anti-human IgG (LI-COR Biosciences, Lincoln, NE) (which cross-reacts with Rhesus IgG2 Fc) at 1: 10,000 dilution. For detection of sulfated tyrosine in ReCD4-Ig (Fig 3.1e), the membrane was incubated overnight with 1:1000
mouse anti-sulfotyrosine (1C-A2) at 4°C and developed with IRDye 680RD goat anti-
mouse IgG (LI-COR Biosciences Cat# 926-32232, RRID:AB_10806644). As anti-mouse
and anti-human antibodies are conjugated to dyes with different colors, it is possible to
visualize ReCD4-Ig and sulfotyrosine bands simultaneously in a single membrane. For
detection of sulfation enzymes expression in vivo, mice were sacrificed at indicated time
points after DNA injections/ IM-EP. TA muscles were harvested and homogenized in T-
PER extraction buffer (ThermoFisher Cat# 78510) and protease inhibitor. Fifty micrograms
of muscle homogenates were loaded onto 4-12% Bis-Tris gel under reducing condition
and transferred to a PVDF membrane with wet transfer. The membrane was incubated
overnight with polyclonal rabbit anti-TPST2 antibodies (Abcam Cat# ab87407), and
monoclonal mouse anti-GAPDH antibody (Cell Signaling Cat# 97166S) at 4°C. The
membrane was subsequently developed with IRDye 680RD goat anti-mouse IgG (LI-COR
Biosciences Cat# 925-68070) and IRDye 800CW goat anti-rabbit IgG. All membranes
were scanned with LI-COR Odyssey CLx (LI-COR Biosciences Cat# 926-32211).

For pseudo-native and SDS PAGE in Chapter 5, tibialis anterior muscles of
immunized animals were harvested and homogenized in T-PER extraction buffer
(ThermoFisher) and protease inhibitor (Roche). Muscle homogenates were subsequently
concentrated 20x with Amicon Ultra 0.5mL Centrifugation kits with 3kDA cut-offs (Milipore
Sigma) and protein concentrations were quantified with BCA assays (ThermoFisher). For
electrophoresis, 8uL supernatants of Exp293F cells transfected with pVAX,
DLmono_GT8, eOD-GT8-60mer or 50ug muscle homogenates from mice immunized with
the 80ug aforementioned constructs co-formulated with 12U hyaluronidase were loaded
onto 4-12% SDS Bis-Tris Gel (SDS-PAGE) or 3-8% Tris-Acetate Gel (pseudo-Native
PAGE) for electrophoresis. For SDS-PAGE, all samples were reduced with heating of the
samples in the presence of a reducing agent and LDS sample buffer (ThermoFisher) at
70C for 10min. For Pseudo-Native PAGE, samples were only incubated with the LDS buffer at room temperature and loaded directly onto the 3-8% TA gel without boiling. Proteins were subsequently transferred to PVDF membrane from the gels, and stained with 3ug/mL of VRC01 and 1ug/mL anti-human GAPDH (for SDS-PAGE only, Clone D4C6R, Cell Signaling) in Odyssey Blocking Buffer/ PBS/ 0.1% Tween (LI-COR Biosciences) overnight at 4C, and 1:10,000 IRDye 800CW goat anti-human IgG (LI-COR Biosciences) in Odyssey Blocking Buffer/ 0.1% Tween/ 0.1% SDS at room temperature for 1 hour, and then scanned with LI-COR Odyssey CLx.

2.9 Fluorescence microscopy

Eight-well chamber slides (Nunc Cat# 154534) were pre-coated with poly-L-lysine solution (SigmaAldrich Cat# P8920) before HEK293T cells were seeded at a density of 2×10⁵ per well overnight. The cells were then transfected with 1.0ug of p-reCD4-Ig and 0.05ug of p-TPST2, p-IgE-TPST2 or p-ΔTM-TPST2 with GeneJammer. Forty-eight hours after transfection, the cells were fixed and permeabilized with 4% formaldehyde in PBS and 0.5% Triton-X-100 (Image-It-Fixation/Permeation Kit, ThermoFisher Cat# R37602) and blocked with 3% BSA in PBS at room temperature for 1 hour. The cells were then stained overnight at 4°C with 1:200 dilution of anti-Golgin 97 antibody (LI-COR Biosciences Cat# 926-32211) in 1% BSA/PBS-T, and 1:200 dilution of polyclonal rabbit anti-TPST2 antibody (Abcam Cat# ab87407, RRID:AB_10672926). The cells were then washed with PBS-T and stained with 1:500 dilution of Goat anti-Rabbit Alexa Fluor 594 (Thermo Fisher Scientific Cat# A-11037), and goat anti-mouse Alexa Fluor 488 (Thermo Fisher Scientific Cat# A32723). For nuclear staining, the cells were incubated with 0.5ug/mL of DAPI (SigmaAldrich Cat # D9542) in PBS-T and mounted with cover slips using Prolonged Diamond AntiFade Mountant (ThermoFisher Cat# P36970). Z-stack images were then acquired with Leica TCS SP5 II Scanning Confocal Microscope with a
64× objective. Maximal projections of the Z-stacks, deconvolution, and regions of interest analyses were performed with Leica LASX software to obtain Pearson correlation coefficients.

For lymph node staining in Chapter 5, 7 days after BALB/c mice were immunized with 80ug DNA co-formulated with 12U Hyaluronidase (Sigma) encoding GT8-monomer or DLnano_LS_GT8, tibialis anterior muscles of the mice were injected with 5ug of anti-mouse CD35 BV421 (BD-Bioscience) for in situ labelling of follicular dendritic cells 16 hours prior to harvest. Ipsilateral iliac lymph nodes from the mice were harvested the next day and preserved in O.C.T medium (Fisher) for cryo-sectioning. The sections were fixed with 4% paraformaldehyde, then blocked in 3% BSA/ PBS for 1 hour at room temperature, followed by overnight staining with 6ug/mL VRC01. The sections were then washed and stained with anti-human Alexa Fluor 488 antibody and imaged with Leica SP5 confocal microscopes.

For muscle staining in Chapter 5, four days after BALB/c mice were immunized with 80ug DNA encoding GT8-monomer, DLnano_LS_GT8, DLnano_3BVE_GT8 or DLnano_PfV_GT8 co-formulated with 12U Hyaluronidase in the tibialis anterior muscles of the mice were harvested the and preserved in 4% PFA/PBS for 2 hours at room temperature and then stored overnight in 70% EtOH/H₂O at 4C. The tissues were then serially dehydrated and blocked in 3% BSA/ PBS for 1 hour at room temperature, followed by overnight staining with 6ug/mL VRC01. The sections were then washed, and stained with anti-human Alexa Fluor 488 antibody, counterstained with 0.5ug/mL DAPI and imaged with Leica SP5 confocal microscopes.
2.10 Immunohistochemistry

For immunohistochemistry staining of muscle sections, BALB/C mice were immunized with 80ug DLmono_GT8 or DLnano_LS_GT8 co-formulated with 12U hyaluronidase (Sigma). Transfected muscles were harvested seven days post immunization, cryo-sectioned, fixed, permeabilized, and blocked as described in the Immunofluorescence section. The muscle sections were then stained with goat anti-mouse MBL at 1:200 dilution in 1% BSA/PBS (R&D system) overnight, and then with secondary Rabbit anti-goat (H+L) HRP conjugated at 1:500 dilution (BioRad) and DAB substrates for development.

2.11 TUNEL assay

TUNEL assay was performed on fixed muscle specimen according to manufacturer’s protocol (Abcam). Briefly, four days post treatments with DNA or protein vaccine, tibialis anterior muscles were harvested and fixed in 4% paraformaldehyde/ PBS for 3 hours at 4C. The tissues were then dehydrated and paraffin-embedded, the sample sections were then rehydrated by serial transfer of the tissues from 100% Ethanol to 70% Ethanol/ water. The specimen was then permeabilized by treatment with Proteinase K at room temperature for 20 minutes. Endogenous peroxidase was then quenched by treatment of tissue sections with 3% H$_2$O$_2$ in methanol. The sections were then incubated with TdT enzyme in the equilibration buffer at room temperature for 1.5 hours in a humidified chamber, and the reactions were stopped with the stop solution provided in the kit. The sections were then blocked with provided blocking buffer at room temperature for 10 minutes and labelled with provided detection conjugate diluted 1:25 in blocking buffer at room temperature for 30 minutes. The sections were then developed with DAB substrate at room temperature for 15 minutes and then counter stained with methyl green before they were imaged with Nikon Eclipse 80i under 20x and 60x magnification.
2.12 Electron microscopy

2.12.1 Transmission EM of muscles

Tibialis anterior muscles from BALB/c mice immunized with 80μg DLmono_GT8 or DLnano_LS_GT8 co-formulated with 12U hyaluronidase were collected seven d.p.i. The muscles were then fixed in 2.5% glutaraldehyde, serially dehydrated in acetone/ethanol solvents, and then embedded in epoxy and LR white resin. The resin was then sectioned to a thickness of 70nm and deposited onto a metal grid, blocked overnight in 3% BSA/PBS, followed by staining with 60μg/mL VRC01 (diluted in 3% BSA/PBS) overnight, and with 1:200 anti-human 6nm gold nanoparticles (Jackson Immunoresearch) for 1 hour. The sections were then washed with 0.1% Tween in PBS, and water, followed by post-staining fixation with 2.5% glutaraldehyde in PBS for 5 minutes at room temperature followed by staining with 2% Uranyl acetate for 1 hour. The grids were subsequently imaged with JEOL JEM 1010 transmission electron microscope. For quantitative analyses, total number of gold-labeled clusters, and order of each cluster were manually counted. Frequency of a cluster of a particular order in a field of view was normalized relative to the total number of clusters observed.

2.12.2 Negative Stain EM of purified nanoparticles

The nanoparticles were produced in Expi293 cells, purified using Agarose bound lectin beads (Agarose Galanthus Nivalis Lectin, Vector Laboratories) followed by size exclusion chromatography (GE Healthcare) using the Superose 6 Increase 10/300 GL column. The proteins were further dialyzed into Tris-buffered saline (TBS). A total of 3 μL of purified proteins was adsorbed onto glow discharged carbon-coated Cu400 EM grids. The grids were then stained with 3 μL of 2% uranyl acetate, blotted, and stained again with 3 μL of the stain followed by a final blot. Image collection and data processing was
performed on a FEI Tecnai T12 microscope equipped with a Oneview Gatan camera at 90,450X magnification at the camera and a pixel size of 1.66 Å.

2.13 ELISpot Assay

Spleens from immunized mice were collected 5 weeks post the first immunization (2 weeks post the second) and homogenized into single cell suspension with a tissue stomacher in 10% FBS/ 1% Penicillin- streptomycin in RPMI 1640. Red blood cells were subsequently lysed with ACK lysing buffer (ThermoFisher) and percentage of viable cells were determined with Trypan Blue exclusion. 200,000 cells were then plated in each well in the mouse IFNγ ELISpot plates (MabTech), followed by addition of peptide pools that span both the lumazine synthase, GT8 or HA domains at 5ug/mL of final concentration for each peptide (GenScript). The cells were then stimulated at 37C for 16-18 hours, followed by development according to the manufacturer’s instructions. Spots for each well were then imaged and counted with ImmunoSpot Macro Analyzer.

2.14 Intracellular cytokine staining

Single cell suspension from spleens of immunized animals were prepared as described in the previous section and stimulated with 5ug/mL of peptides spanning both the lumazine synthase, GT8 or HA domains(GenScript) for 5 hours at 37C in the presence of 1:500 protein transport inhibitor (ThermoFisher) and anti-mouse CD107a-FITC(ThermoFisher). The cells were then incubated with live/dead for 10 min at room temperature, surface stains (anti-mouse CD4 BV510, anti-mouse CD8 APC-Cy7, anti-mouse CD62L BV711 and anti-mouse CD44 AF700) (BD-Biosciences) at room temperature for 30minutes. The cells were then fixed and permeabilized according to manufacturer’s instructions for BD Cytoperm Cytofix kit and stained with intracellular stains anti-mouse IL-2 PE-Cy7, anti-mouse IFN-γ APC, anti-mouse CD3e PE-Cy5 and
anti-mouse TNFα BV605 (BioLegend) at 4C for 1 hour. The cells were subsequently analyzed with LSR II 18-color flow cytometer.

2.15 Determination of the antigen-specific B-cells in spleen

Recombinant 3BVE-GT8 was labelled with FITC with the lightning link kits according to manufacturer’s instructions (Expedon). Spleens were harvested five weeks post the second immunization of 25ug of DLnaono_LS_GT8, DLmono_GT8 or from Naïve mice. Single cells were then labelled with Live/Dead dye ultraviolet reactive (ThermoFisher) at room temperature for 10min and incubated with mouse Fc-Block (Clone 93, ThermoFisher) at 1:200 dilution. Avi-Tagged GT8 was biotinylated and tetramerized with an excess of APC-streptavidin (ThermoFisher) as previously described (Jardine et al., 2015). The cells were washed with PBS and incubated with 1:200 A488-3BVE-GT8 and 1:200 APC-GT8-tetramer at 4C for 30min. Without being washed, the cells were incubated with 1:200 anti-mIgD-APC/Cy7 (BioLegend), anti-mIgM-BV711 (Fisher Scientific), anti-mCD19-PECy7 (Biolegend), anti-mIgG-BV510 (Biolegend) in 1% FBS/PBS solution. The cells were then resuspended in 1x BDFix buffer and analysed with LSR II 18-color flow cytometer.

2.16 In vivo imaging

For B16-F10-Luc2 challenge study, tumor challenged mice received 150mg/kg administration of VivoGlo™ Lucifier (Promega) at each timepoint formulated in sterile PBS, and then imaged with IVIS Spectrum CT for Bioluminescence with the auto-exposure settings (or for 60s, whichever is shorter) 10 minutes post injection.

2.17 Ex vivo neutralization assay

Synthesis of HIV Env pseudotyped viruses and TZM-bl assays were performed as previously described (Sarzotti-Kelsoe et al., 2014). Briefly, HEK293 T cells were
transfected with 4ug of plasmid encoding HIV envelope and 8ug of plasmid encoding HIV backbone pSG3Δenv (NIH-ARP Cat# 11051) with GeneJammer. Forty-eight hours after transfection, the supernatants were filtered with Steriflip (MiliporeSigma Cat# SCGP00525) and stored at -80°C. Pseudoviruses were titrated with a TZM-bl luciferase reporter assay using Britelight Plus (PerkinElmer Cat# 6066769) to determine a titer that corresponds to at least 150,000 RLU. Mice sera were heat inactivated at 56°C for 10 minutes for the TZM-bl neutralization assays to determine serum concentration/titer that would result in 50% virus neutralization (IC₅₀).

2.18 HAI assay

Mice sera were treated with receptor-destroying enzyme (RDE, 1:3 ratio) at 37°C overnight for 18–20 h followed by complement and enzyme inactivation at 56°C for 45 min. RDE-treated sera were subsequently cross-adsorbed with 10% rooster red blood cells (Lampire Biologicals) in PBS at 4C for 1 hour. The cross-adsorbed sera were then serially diluted with PBS in a 96-well V-bottom microtiter plates (Corning). Four hemagglutinating doses (HAD) of A/Solomon Islands/03/06 virus, A/New Caledonia/20/99, or A/California/07/2009 (BEI) were added to each well and the serum–virus mixture was incubated at room temperature for 1 hour and then incubated with 50 µl 0.5% v/v rooster red blood cells in 0.9% saline for 30 min at room temperature. The HAI antibody titer was scored with the dot method, and the reciprocal of the highest dilution that did not exhibit agglutination of the rooster red blood cells was recorded.

2.19 Infectious molecular clones (IMC)

The HIV-1 reporter virus used were replication-competent IMC designed to encode the env genes of DU151 (subtype C; GeneBank No. DQ411851) in cis within a Nef deficient isogenic backbone that expresses the Renilla luciferase reporter gene. All the IMCs were built using the original NL-LucR.T2A-ENV.ecto backbone as previously
described (Adachi et al., 1986). Reporter virus stocks were generated by transfection of 293T cells with proviral IMC plasmid DNA, and virus titer was determined on TZM-bl cells for quality control.

Infection of CEM.NKRCCR5 cell line with HIV-1 IMCs: CEM.NKRCCR5 (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) cells were infected with HIV-1 IMCs as previously described (Pollara et al., 2011). Briefly, IMCs were titrated in order to achieve maximum expression within 48-72 hours post-infection as determined by detection of Luciferase activity and intracellular p24 expression. IMC infections were performed by incubation of the optimal dilution of virus with CEM.NKRCCR5 cells for 0.5 hour at 37°C and 5% CO₂ in presence of DEAE-dextran (7.5 μg/ml). The cells were subsequently resuspended at 0.5x10⁶/ml and cultured for 48-72 hours in complete medium containing 7.5μg/ml DEAE-dextran. For each ADCC assay, the frequency infected target cells were monitored by intracellular p24 staining. Assays performed using infected target cells were considered reliable if cell viability was ≥60% and the percentage of viable p24+ target cells on assay day was ≥20%.

2.20 Luciferase ADCC Assay

ADCC activity was determined by a luciferase (Luc)-based assay as previously described (Pollara et al., 2014). Briefly, CEM.NKRCCR5 cells were used as targets after infection with the HIV-1 IMCs. PBMC obtained from a HIV-seronegative donor with the heterozygous 158F/V and 131H/R genotypes for FcR3A and FcR2A, respectively, were used as a source of effector cells, and were used at an effector to target ratio of 30:1. Sera from NHPs inoculated with dMAb plasmids for PDGM1400 and PGT121 were initially diluted to reach an initial concentration ranging from 4 to 6μg/ml of dMAb and tested across a range of concentrations using 5-fold serial dilutions. The reference mAbs were tested across a range of concentrations using 5-fold serial dilutions starting at 50 μg/ml.
The effector cells, target cells, and Ab dilutions were plated in opaque 96-well half area plates and were incubated for 6 hours at 37°C in 5% CO₂. The final read-out was the luminescence intensity (relative light units, RLU) generated by the presence of residual intact target cells that have not been lysed by the effector population in the presence of ADCC-mediating mAb (ViviRen substrate, Promega). The percent of specific killing was calculated using the formula: percent specific killing = \[\frac{(\text{number of RLU of target and effector well} - \text{number of RLU of test well})}{\text{number of RLU of target and effector well}}\] ×100.

In this analysis, the RLU of the target plus effector wells represents spontaneous lysis in absence of any source of Ab. The ADCC detectable in NHP serum is reported after subtracting the activity observed in the serum before dMAb injection (baseline subtracted activity). Results are considered positive if percent specific killing is greater than 15%.

The RSV-specific mAb Palivizumab was used as a negative control and a combination of C1/C2 A32, CD4bs CH44, glycosylation site 2G12, and gp41 7B2 mAbs (mAb mix) was used as positive control.

2.21 Lethal H1/A/California/07/09 influenza challenge

6-8 week old female BALB/c mice (Jackson Laboratory) were immunized with 1µg of pVAX vector, DLmono_HA_CA09 or DLnano_3BVE_HA_CA09 twice three weeks apart. The mice were subsequently transferred to BioQual Inc for challenge experiment. 35 days post the second immunization, the mice were intranasally inoculated with 10LD₅₀ H1/A/California/07/09 in PBS. Weights of the mice were pre-recorded prior to the challenge and daily after the challenge until 7 days post inoculation, at which lungs from the mice were harvested and snap-frozen for viral load assay by RT-qPCR and histopathology by H&E staining. At any point, mice exhibiting more than 20% of weight loss as compared to baseline were euthanized (humane endpoint).
2.22 RT-qPCR assay for viral load determination

The amounts of RNA copies per gram lung tissue was determined using a real-time quantitative PCR (qPCR) assay. This assay utilized primers and a probe specifically designed to amplify and bind to a conserved region of the NP gene of influenza virus. The signal was compared to a known standard curve and calculated to give copies per gram tissue. Viral RNA was extracted from lung homogenates using MiniElute Virus Spin Kit (Qiagen). TAQMAN RT-PCR kit (Applied Biosystems, Inc., Carlsbad, CA) was used for amplification of viral RNA in the presence of 600nM primers (CAL-1-U: ATGGCGTCTCAAGGCACCAA and CAL-1-D: GCACATTGGATGTAATCTC) and 140nM probe (CAL-1-P: 6FAM-CAGAGCATCTGTCGGAAGAATGATTG-TAMRA) with the following Thermocycler setting: 48°C for 30 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, and 1 minute at 60°C.

2.23 Statistics

Power analysis was performed with R based on our preliminary data to determine the smallest sample size that would allow us to achieve a power of 0.9 with a pre-set α-value of 0.05. One-way ANOVA analysis and pair-wise T-tests (with Holm-Bonferroni Adjustments in the case of multiple comparisons) or Two-tailed Mann Whitney Rank Tests were performed with GraphPad Prism 8.0. IC_{50} values were computed with a non-linear regression model of percentage neutralization versus log (reciprocal serum dilution) using Prism 8.0. P-values less than 0.05 were considered as statistically significant.
Chapter 3 Co-delivery of Synthetic DNA encoding a complex anti-HIV-1 biologic with a DNA-encoding enzyme promotes \textit{in vivo} expression and post-translational modification

3.1. Abstract

Despite vigorous and ongoing efforts, active immunizations have yet to induce broadly neutralizing antibodies (bNAbs) against HIV-1. An alternative approach is to achieve prophylaxis with long-term expression of potent biologic HIV-1 inhibitors with Adeno-associated Virus (AAV), which could however be limited by hosts’ humoral and cellular responses. An approach that facilitates in vivo production of these complex molecules independent of viral-vectored delivery will be a major advantage. We used synthetic DNA and electroporation (DNA/EP) to deliver an anti-HIV-1 immunoadhesin eCD4-Ig in vivo. In addition, we engineered a TPST2 enzyme variant (IgE-TPST2), characterized its intracellular trafficking patterns and determined its ability to post-translationally sulfate eCD4-Ig in vivo. With a single round of DNA injection, a peak expression level of 80-100ug/mL was observed in mice 14 days post injection (d.p.i). The engineered IgE-TPST2 enzyme trafficked efficiently to the Trans-Golgi Network (TGN). Co-administrating low dose of plasmid IgE-TPST2 with plasmid eCD4-Ig enhanced the potency of eCD4-Ig by three-fold in the ex vivo neutralization assay against the global panel of HIV-1 pseudoviruses. This work provides a proof-of-concept for delivering anti-HIV-1 immunoadhesins by advanced nucleic acid technology and modulating protein functions in vivo with targeted enzyme-mediated post-translational modifications.

3.2 Introduction

There are currently 37 million people living with HIV/AIDS worldwide, and two million people are newly infected each year (Tetteh et al., 2017). 15-50% of patients chronically infected with HIV have developed antibodies that are considered broadly neutralizing (Hraber et al., 2014). However, to date, active vaccination with HIV envelope immunogens have failed to elicit bNAbs in NHPs and humans (Sok et al., 2017). In contrast, passive transfer of bNAbs have protected NHPs from SHIV challenges (Julg et
al., 2017a; Moldt et al., 2012; Shingai et al., 2014). Additionally, bNAb 10-1074 could transiently suppress viremia in HIV viremic patients (Caskey et al., 2017), while bNAb 3BNC117 can delay viral rebound in HIV patients on analytic interruptions of ART (ATI) (Scheid et al., 2016). Viral rebound in these patients, typically occurring seven to ten weeks after ATI, is potentially driven by the emergence of HIV viruses resistant to the bNAb.

Recently, AAV-delivery of a potent immunoadhesin construct eCD4-Ig demonstrated protection of Rhesus Macaques (RhM) from repeated challenges of SHIV-AD8 and SIV-Mac239 (Gardner et al., 2015). eCD4-Ig is a fusion protein consisting of (from N to C-terminus) extracellular D1-D2 domains of CD4, IgG-Fc, and a 15-amino acid CCR5-mimetic peptide. As eCD4-Ig targets the conserved receptor and co-receptor binding sites on HIV envelope, it has demonstrated extreme breadth and potency, neutralizing all isolates tested with IC\textsubscript{50}<5ug/mL (Gardner et al., 2015). In addition, mutations that allow HIV-1 to escape from eCD4-Ig potentially come at a fitness cost to the virus by lowering the affinity of Env to CD4 and CCR5. To further enhance the potency of eCD4-Ig, AAV-encoded TPST2 was co-administered with AAV-encoded eCD4-Ig because TPST2 can specifically sulfate tyrosine residues in the CCR5 mimetic peptide of eCD4-Ig (Gardner et al., 2015).

While the utility of AAV gene delivery is well-established (Naso et al., 2017), its successful use in targeting non-immuno-privileged tissues (livers or skeletal muscles) is frequently hindered by pre-existing neutralizing antibodies against the capsid (Fitzpatrick et al., 2018), which are extremely prevalent in the human population. For example, neutralizing antibodies to AAV2 are found in 30%-60% of human sera analyzed across different studies (Li et al., 2012; Mimuro et al., 2014). In addition, delivery by AAV induces seroconversion, which precluded the possibility of redosing the biologics with the same
vector (Tse et al., 2017). Even after successful transduction of target tissues, cytotoxic T-
lymphocytic response against the viral capsids may result in premature loss of the 
transgenes (Manno et al., 2006).

Advances in electroporation technologies (EP) have increased in vivo DNA plasmid driven expression by 100 fold or more, (Sardesai and Weiner, 2011) which has recently been translated with success in the vaccine space in the clinic (Tebas et al., 2017; Trimble et al., 2015). As plasmid delivery is serologically independent, DNA/EP represents a more reproducible approach for gene deliveries (Almqvist et al., 2011). Our group has recently demonstrated the ability of DNA/EP to deliver high levels of plasmid-encoded monoclonal antibodies (DMAbs) against pseudomonas and influenza that can protect mice from lethal challenges (Elliott et al., 2017; Patel et al., 2017). Here, we built on this technology and demonstrated that DNA/EP could express a non-native, highly structured molecule, eCD4-Ig, in vivo. We also showed, for the first time, DNA/EP-mediated in vivo delivery of enzyme IgE-TPST2 that can colocalize with and post-translationally sulfate eCD4-Ig to improve its functionality.

3.3. Results

3.3.1 Transfection of HEK293T cells enables expression and secretion of ReCD4-Ig in vitro

Since AAV-delivered Rhesus eCD4-Ig (ReCD4-Ig) protected RhMs against SHIV challenges (Gardner et al., 2015), we had focused our current work on ReCD4-Ig for downstream comparisons. An optimized transgene encoding ReCD4-Ig with an N-terminal IgG kappa-leader sequence was designed and synthesized de novo and cloned into a modified pVAX-1 plasmid backbone. Expression of this engineered p-ReCD4-Ig DNA construct was studied in vitro by transfecting HEK293T cells followed by ELISA quantification. In both the transfection supernatant and cell lysate, robust expression of
ReCD4-Ig was detected (Fig 3.1a). Western blot of the transfection supernatant with anti-
human IgG confirmed secretion of ReCD4-Ig (Fig 3.1b).

3.3.2 Co-transfection of p-ReCD4-Ig and p-TPST2 variants allows in vitro sulfation of 
ReCD4-Ig

Previous studies have demonstrated that tyrosine sulfation of CCR5 mimetic 
peptide by trans-Golgi resident enzymes, TPST2, enhances potency of ReCD4-Ig 
(Gardner et al., 2015; Seibert et al., 2002). As ReCD4-Ig is targeted to the secretory 
pathway early in the translation process by the IgG leader sequence, tyrosine sulfation of 
ReCD4-Ig could only occur if the TPST2 variant is also expressed in the secretory 
compartment.

To highlight our ability to target TPST2 to the right subcellular compartment, we 
co-transfected HEK293T cells with p-ReCD4-Ig and plasmids encoding TPST2 enzyme 
variants. The p-IgE-TPST2, a construct with an IgE leader sequence upstream of TPST2, 
was predicted to sulfate ReCD4-Ig since the IgE leader sequence facilitates trafficking of 
TPST2 into the endoplasmic reticulum. A second TPST2 construct with a deletion in the 
transmembrane (TM) motif, p-ΔTM-TPST2, was not expected to sulfate ReCD4-Ig since 
the TM deletion removes the signal anchor sequence required for Trans-Golgi Network 
(TGN) targeting of TPST2. Finally, a control plasmid, p-HS3SA, was tested. HS3SA is a 
Golgi-resident enzyme which can transfer sulfate groups to heparin sulfate and has a 
similar catalytic site as compared to TPST2 (Teramoto et al., 2013). Varying doses of 
DNA-encoded enzymes (1:5000 to 1:20, enzyme: ReCD4-Ig) were used to determine the 
minimal dose required to maximize sulfation of ReCD4-Ig. Using an anti-sulfotyrosine 
binding ELISA on cell supernatant, higher sulfation was observed for both TPST2 and IgE-
TPST2 groups, even at the lowest enzyme dose of 1:5000, as compared to the baseline 
ReCD4-Ig only group (Fig 3.1c). Furthermore, for both TPST2 and IgE-TPST2, sulfation
signals were saturated at a remarkable 1:1000 enzyme dose, and higher dose of DNA did not increase sulfation. In comparison, RecCD4-Ig sulfation for both the ΔTM-TPST2 and HS3SA groups were not higher than the baseline. The lack of sulfation with the HS3SA group indicates remarkable specificity of sulfotransferases. To further confirm enzyme-mediated sulfation of ReCD4-Ig, the supernatants were analyzed with Western blots (Fig 3.1d). Again, stronger sulfotyrosine bands were observed for the 1:1000 TPST2 and IgE-TPST2 groups than for the ReCD4-Ig only, ΔTM, and HS3SA groups. Taken together, these results suggest that DNA-encoded TPST2, and IgE-TPST2 can mediate in vitro sulfation of ReCD4-Ig at a remarkably low dose.

3.3.3 Incorporation of the N-terminal IgE leader sequence enhances targeting of TPST2 to TGN

We used fluorescence microscopy to determine whether IgE leader sequence can improve trafficking of DNA-encoded IgE-TPST2 to the cellular secretory compartment. HEK293T cells were transfected with either p-ReCD4-Ig only, or p-ReCD4-Ig in combination with p-TPST2, p-IgE-TPST2, or p-ΔTM-TPST2. 48 hours after transfections, cells were harvested and stained with DAPI (blue), anti-TPST2 (red), and anti-Golgin 97 (green). Golgin-97 is a trans-Golgi resident protein commonly used to determine the expression of a protein of interest in TGN (Lu et al., 2004). Confocal microscopy images of harvested cells illustrate robust expression of TPST2, IgE-TPST2 and ΔTM-TPST2 upon transfection (Fig 3.2a). More importantly, IgE-TPST2 appears to co-localize with Golgin 97 to a greater extent than TPST2, whereas ΔTM-TPST2 does not co-localize with Golgin 97. To quantify the extent of co-localization between Golgin 97 and TPST2 variants, we analyzed the Pearson correlation coefficients (PCC) between red and green channels for 16 regions of interests for each group (Fig 3.2b). The global one-way ANOVA analysis yields a p-value<0.0001 and post-hoc pairwise T-test shows PCC for the IgE-
TPST2 group (PCC=0.542) is significantly higher than that for the TPST2 group (PCC=0.275, p<0.0001). To ensure co-transfection with ReCD4-Ig did not change enzyme localization, we also co-transfected HEK293T cells with plasmid enzymes and pVAX1 backbone, and observed similar colocalization patterns (Supplemental Fig 3.1). Taken together, the results support that while both TPST2 and IgE-TPST2 can traffic to TGN, IgE-TPST2 can be targeted to the secretory compartment more efficiently than TPST2, potentially because the N-terminal IgE leader sequence is recognized by signal recognition particle (SRP) more efficiently than the internal signal anchorage sequence of TPST2.

3.3.4 DNA/EP mediates in vivo expression of ReCD4-Ig and sulfation enzymes

We next determined whether we can express ReCD4-Ig and the sulfation enzyme TPST2 in vivo with DNA/EP. We observed strong TPST2 expression in the injected as compared to the contralateral legs of mice for at least 56 days (Fig 3.3a). DNA/EP also mediated robust in vivo expression of IgE-TPST2 (Supplemental Fig 3.2). Since ReCD4-Ig sequence is RhM based, strong anti-drug antibodies could develop in immune competent mice and influence the expression profile of ReCD4-Ig. Thus, immunodeficient B6.Cg-Foxn1nu/J (nude) mice were used initially. We observed a high level of expression of ReCD4-Ig, which peaked at an average of 35ug/mL 14 days post injection (d.p.i) (Fig 3.3b). Remarkably, expression was detected as early as 3 d.p.i (average 5.7ug/ml) and lasted for at least 150 days (average 3.1ug/ml). In addition, ReCD4-Ig expression profile in transiently immune-depleted BALB/c mice demonstrated a similar pattern.

3.3.5 Low dose of p-IgE-TPST2 leads to in vivo sulfation of ReCD4-Ig without decreasing its expression

We next tested the ability of DNA-encoded IgE-TPST2 to sulfate ReCD4-Ig in vivo in transiently immune-depleted BALB/c mice.Remarkably, we observed that 1:1000 dose
of IgE-TPST2 can saturate the sulfation OD450 signals detected in the mice sera 7 d.p.i, as compared to 1:20 IgE-TPST2 dose group (Fig 3.3c). Additionally, sulfation of ReCD4-Ig was significantly higher, even at a lower 1:5000 dose of the enzyme, as compared to the baseline ReCD4-Ig only group. Previous studies describe that co-transfection of a high dose of TPST2 with its target proteins in vitro could lead to decreased secretion of the target proteins (Chen et al., 2016). We observed a similar phenomenon both in vitro and in vivo (Fig 3.3d and Supplemental Fig 3.3a). To study if we could find an effective dose that would not limit ReCD4-Ig expression, we titrated the amount of IgE-TPST2 co-administered in vivo. A high dose (1:20) of IgE-TPST2 co-transfected with ReCD4-Ig resulted in 67% and 70% decreases in the expression of ReCD4-Ig in transfection supernatants and mice sera, respectively, as compared to ReCD4-Ig only groups. Additionally, suppression of ReCD4-Ig secretion is not directly driven by IgE-TPST2-mediated sulfation since co-administration of p-ReCD4-Ig and 1:20 p-HS3SA also reduces ReCD4-Ig expression by 52% (Supplemental Fig 3.3b). Importantly, at the minimal 1:1000 dose of p-IgE-TPST2 required for optimal sulfation of ReCD4-Ig, we did not observe a difference in ReCD4-Ig expression 7 d.p.i between ReCD4-Ig only and ReCD4-Ig + 1:1000 IgE-TPST2 groups (Fig 3.3d). Such pattern was again observed when the mice injected with either pReCD4-Ig alone or pReCD4-Ig + 1:1000 p-IgE-TPST2 were followed over time for ReCD4-Ig expression (Fig 3.3e). In addition, using binding ELISA to assess the durability of sulfation of ReCD4-Ig by IgE-TPST2 in vivo, we observed peak levels of sulfation in the co-treated mice between 7 and 14 d.p.i (Fig 3.3f). Sulfation of ReCD4-Ig in these co-treated mice declines over time but remains significantly higher than the mice which did not receive plasmid enzyme co-treatment for at least three weeks. Taken together, these results illustrate that plasmid encoded enzymes delivered by electroporation can enable in vivo sulfation of ReCD4-Ig at a remarkably low dose without decreasing the expression of ReCD4-Ig.
3.3.6 *In vivo* sulfation increases the potency of ReCD4-Ig

We next determined if *in vivo* sulfation of ReCD4-Ig can enhance its potency. Similar levels of ReCD4-Ig expression (40ug/mL) was again observed for both p-ReCD4-Ig only and p-ReCD4-Ig + 1:1000 p-IgE-TPST2 groups 7 d.p.i in immune-depleted BALB/c mice (Fig 3.4a). We first tested the ability of *in vivo* produced ReCD4-Ig to neutralize one of the pseudoviruses from the global panel (25710, Tier 2, clade C) using the standard TZM-bl assay (deCamp et al., 2014). Sulfation mediated by IgE-TPST2 significantly enhanced the ability of ReCD4-Ig to neutralize this isolate, as evidenced by a right-ward shift in the neutralization curve (Fig 3.4b). We then evaluated the effects of ReCD4-Ig sulfation with a panel of 13 pseudoviruses comprising of the global panel and the tier 3 isolate SIV<sub>mac239</sub> (deCamp et al., 2014). We observed that ReCD4-Ig neutralized all 13 viruses in the panel with an IC<sub>50</sub> less than 5ug/mL and a mean IC<sub>50</sub> of 0.83ug/mL (Fig 3.4c and Fig 3.4d). Naïve mice sera, in comparison, did not neutralize any of the pesudovirus at a titer of 1:20. Our results validated the remarkable breadth of eCD4-Ig as described in previous studies (Gardner et al., 2015). In addition, sulfation of ReCD4-Ig enhances its potency in neutralizing 8/12 pseudoviruses in the global panel (CE1176, 25710, X2278, TRO, BJOX, X1632, CH119, CNE55) and Mac239 (Fig 3.4c-d and Supplemental Fig 3.4). Sulfation exhibits the most drastic effect on the ability of ReCD4-Ig to neutralize CE1176, with a 10-fold drop in IC<sub>50</sub> (0.57 ± 0.27 ug/mL to 0.05 ± 0.02 ug/mL). Overall, IgE-TPST2 mediated sulfation led to a decrease in the geometric mean of IC<sub>50</sub> against the viral panel from 0.83ug/mL to 0.27ug/mL. Of note, the IC<sub>50</sub> of sulfated ReCD4-Ig in neutralization of Mac239 is 0.16 ± 0.06 ug/mL, similar to the IC<sub>50</sub> reported for AAV delivered ReCD4-Ig (Gardner et al., 2015). Taken together, our results validated *in vivo* sulfation of ReCD4-Ig by plasmid-encoded IgE-TPST2 from a functional standpoint and demonstrated the ability of DNA-encoded enzymes to modulate biological functions of a target protein through post-translational modification (PTM).
3.4. Discussion

Sulfation plays an important role in molecular interactions between CCR5, gp120, and CD4 to support HIV infection. Sulfated tyrosine residues on the N-terminus of CCR5 and CD4i antibody (412d) mediate their intermolecular interactions with the V3 loop of Env in the CD4-bound post-fusion state (Farzan et al., 1999). As such, sulfated CD4i antibodies neutralize primary HIV-1 isolates more potently than non-sulfated prototypical CD4i antibodies 17b and 48d (Choe et al., 2003). In addition, the CDRH3s of many V2-apex bNAbs (PGDM1400, PG9) are also tyrosine sulfated (Moore et al., 2017). By using DNA/EP to express both ReCD4Ig and IgE-TPST2 in vivo, we have significantly increased potency of the immuneadhesin through post-translational sulfation, validating and extending prior findings (Gardner et al., 2015).

Importantly, this is the first report utilizing DNA to encode an enzyme that can carry out PTM of a target protein in vivo. Through modulating protein function in vivo, DNA/EP provides a platform with diverse applications. For example, by modifying the glycosylation pattern in the Fc portion of immunoglobulin, we can fine-tune its effector functions. Afucosylation of IgG1 Fc with endoglycosidase/ fucosidase, for instance, can potentially enhance antibody-dependent cell-mediated cytotoxicity (ADCC) of the modified antibody. Terminal sialylation, in the context of core fucosylation, exhibits an opposite effect (Arnold et al., 2007; Li et al., 2017). In the area of vaccine design, PTMs of an antigen can create new epitopes for recognition by the immune system. For instance, both germline encoded and somatically mutated antibodies in the CAP256.VRC26 lineage recognize sialic-acid bearing glycans at N160, or N156 positions of the HIV envelope (Andrabi et al., 2017). Co-administration of DNA-encoded HIV Env antigens and sialytransferases could likely elicit such glycan-dependent neutralizing antibodies.
Our results illustrate that a remarkably low dose of 1:1000 p-IgE-TPST2 is required for *in vivo* sulfation of ReCD4-Ig. We expected this finding since a single molecule of the enzyme should be able to turn over multiple copies of target proteins. Specifically, since TPST2 has a turnover number ($k_{\text{cat}}$) of $5.1 \times 10^{-3} \text{s}^{-1}$ (for a mono-sulfated CCR8 peptide) and half-life of a Golgi-resident enzyme is about 20 hours, a single copy of TPST2 enzyme should be able to turnover at least hundreds of copies of ReCD4-Ig (Danan et al., 2010; Strous, 1986). Of note, the dose required to sulfate ReCD4-Ig is much lower for DNA-encoded IgE-TPST2 (1:1000) than AAV-encoded TPST2 (1:4). This demonstrates the high efficiency of DNA/EP mediated enzyme delivery and that muscle cells have received separate copies of both p-IgE-TPST2 and p-ReCD4-Ig simultaneously. This is likely due to directionally pulsed electric fields that can create transient pores in the plasma membrane, and move polyanionic plasmid DNA directly into the cells to improve transfection efficiency by 100-1000 folds (Sardesai and Weiner, 2011). In comparison, uptake of AAV-encoded genetic materials (ReCD4-Ig and TPST2) into cells requires clathrin-dependent endocytosis or micropinocytosis (Stoneham et al., 2012; Weinberg et al., 2014), and transduction of muscles cells by both AAV-TPST2 and AAV-eCD4-Ig can occur in a stochastic fashion.

The results also support an approach to target an enzyme to a specific subcellular compartment to maximize its functions. While the efficiency of IgE-TPST2 mediated sulfation appears similar to that of TPST2-mediated sulfation (Fig 3.1d), selective targeting of the IgE-TPST2 can potentially reduce cytosolic expression of the enzyme and off-target effects. This approach can be further extended to target proteins to other subcellular compartments for therapeutic and investigational purposes. Specifically, an N-terminal sequence consisting of 10-70 amino acids that forms amphipathic helices could target proteins to the mitochondria; a dileucine motif DXXLL, or a tyrosine-based motif YXXØ, in
the cytoplasmic tail of a transmembrane protein could target it to the lysosome; whereas a unit of 5 basic positively charged amino acids could target a protein to the nucleus (Braulke and Bonifacino, 2009; Regev-Rudzki et al., 2008).

Finally, we have demonstrated the ability of DNA/EP to enable robust and long-term in vivo expression of immunoadhesins like ReCD4-Ig. With a single round of injection, a peak expression level of 80-100ug/mL in mice was observed, with levels that remains above 3ug/mL for 150 days. The decline in ReCD4-Ig expression between 28 and 42 d.p.i (Fig 3b) and in ReCD4-Ig sulfation between 14 and 28 d.p.i (Fig 3f) is under further investigation. Approaches such as modifications to the plasmid backbone to reduce in vivo promoter silencing (Chen et al., 2008) and protein engineering to increase enzyme half-lives are worthy of future investigations. The in vivo folding of ReCD4-Ig into its proper conformational state is confirmed by the observation that DNA-encoded ReCD4-Ig demonstrates excellent potency and breadth in neutralizing all isolates from the global panel with an IC\textsubscript{50} less than 5ug/mL and a mean IC\textsubscript{50} of 0.27ug/mL. Recently, our preliminary studies demonstrate microgram per milliliter levels of expression of other monoclonal antibodies can be achieved in NHPs with Cellectra EP delivered DNA, supporting the potential translation of this approach (work in progress). This study illustrates the utility of DNA/EP for in vivo expression and subcellular targeting of an enzyme and provides a strategy for modifying protein functions in vivo through PTM. Further study to determine whether the DNA/EP can be a tool to deliver and modify immunoadhesins and other important biologics to prevent and control HIV-1 infection and other diseases is likely important.

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**Figure 3.1** In vitro expression and sulfation of ReCD4-Ig.

Error bars represent standard deviations.

(A) Expression of ReCD4-Ig in transfection lysate and supernatant of HEK293T cells (n=4).

(B) Western blot of supernatants of HEK293T cells transfected with either p-ReCD4-Ig or pVAX-1.

(C) Binding ELISA to detect tyrosine sulfation of ReCD4-Ig in transfection supernatant. HEK293 T cells were transfected with p-ReCD4-Ig or p-ReCD4-Ig with varying doses of plasmid enzyme constructs at (normalized to p-ReCD4-Ig, n=3).

(D) Western blot of supernatants of HEK293T cells transfected with either p-ReCD4-Ig alone or p-ReCD4-Ig and 1:1000 plasmid enzymes. The lower and upper panels show the total amount of ReCD4-Ig and sulfotyrosine respectively.
Figure 3.2 Subcellular targeting of IgE-TPST2.

(A) Confocal microscopy to determine colocalization between TPST2 variant (red) and Golgin 97 (green). Nuclei are stained with DAPI (blue).
(B) Quantification of colocalization between TPST2 variants and Golgin 97 with PCC and regions of interest analyses (n=16). P-values from posthoc pairwise T-tests are indicated: **, p<0.005, ****, p<0.0005.
Figure 3. In vivo expression and sulfation of ReCD4-Ig.

Each dot represents an individual mouse; error bars represent standard deviation. P-values were computed with pairwise T-tests: *, p<0.05, **, p<0.005, ***, p<0.0005, ****, p<0.0005.

(A) Western blot of muscle homogenates 7-56 d.p.i demonstrates expression of TPST2 (43kDa) in the injected legs as compared to the contralateral legs. GAPDH (37kDa) serves as loading controls.
(B) Serum expression of ReCD4-Ig in B6.Cg-Foxn1nu/J and transiently immune-depleted BALB/c from a single round of DNA injection (160μg). N=5 for each group.

(C) Binding ELISA to determine ReCD4-Ig tyrosine sulfation in sera of BALB/c mice injected with p-ReCD4-Ig alone or p-ReCD4-Ig with varying doses of p-IgE-TPST2.

(D) Serum expression level of ReCD4-Ig in mice cotreated with 320μg p-ReCD4-Ig and varying plasmid IgE-TPST2 doses 7 d.p.i.

(E) Serum expression level of ReCD4-Ig at different timepoints in BALB/c treated with either 320μg of p-ReCD4-Ig or 320μg of p-ReCD4-Ig and 0.32μg of p-IgE-TPST2. Each line represents an individual mouse.

(F) Average sulfation signals of ReCD4-Ig in the sera of BALB/c treated with 320μg of p-ReCD4- and 0.32μg of p-IgE-TPST2. N=5 for the group. Dotted line represents average sulfation signals of ReCD4-Ig in the sera of BALB/c treated with 320μg of p-ReCD4 alone 7 d.p.i.
**Figure 3. 4** Functional characterization of IgE-TPST2 mediated sulfation on ReCD4-Ig.

Each dot represents an individual mouse; error bars represent standard deviation. P-values were computed with pairwise T-tests.

(A) Serum concentrations of ReCD4-Ig at the time of terminal bleed (7 d.p.i) in immune-depleted BALB/c mice injected with 320ug of p-ReCD4-Ig alone or p-ReCD4-Ig + 1:1000 p-IgE-TPST2.

(B) Neutralization of 25710 pseudovirus versus serum concentration of ReCD4-Ig.

(C) Comparison of ReCD4-Ig IC\(_{50}\) with or without IgE-TPST2 treatment.

(D) IC\(_{50}\) values of ReCD4-Ig in sera of mice with and without IgE-TPST2 treatment. Geometric mean of IC\(_{50}\) across the panel (except for MLV) is also given.
**Supplemental Figure 3.** Fluorescence microscopy images of HEK293T cells transfected with pVAX backbone plasmid alone, or pVAX with plasmid encoded.

Golgin 97 (green), TPST2 (red) and nuclei (blue) are stained as described in Fig 2(a). Overlay of the three channels show enhanced trafficking of IgE-TPST2 to TGN as compared to TPST2 and ΔTM-TPST2.
Supplemental Figure 3. 2 Western blot of muscle homogenates 7 d.p.i demonstrates expression of IgE-TPST2 (43kDa) in the injected legs as compared to the contralateral legs.

GAPDH (37kDa) serves as loading controls.
Supplemental Figure 3. 3 In vitro cotransfection and in vivo co-delivery of a Golgi-resident enzyme decrease ReCD4-Ig expression.

Pairwise T-tests were used to compare level between each group: *, p<0.05, **, p<0.005.

(A) Quantification of ReCD4-Ig in supernatants of HEK293T cells transfected with p-ReCD4-Ig and varying doses of p-IgE-TPST2.

(B) Serum concentrations of ReCD4-Ig 7 d.p.i in transiently immune-depleted BALB/c mice injected with 320ug of p-reCD4-Ig alone, or 320ug of p-reCD4-Ig with 16ug of p-TPST1/ p-HS3SA.
Supplemental Figure 3. 4 IC$_{50}$ values of ReCD4-Ig with or without sulfation in ex vivo neutralization assay.

Each dot represents IC$_{50}$ value computed from a single mouse, and p-value for each virus is computed with pairwise T-test with Holm adjustment for multiple comparisons. The panels are separated into (A) viruses in which sulfated ReCD4-Ig has significantly enhanced potency (virus X2278 is borderline significant) and (B) viruses in which the potency of sulfated ReCD4-Ig is not significantly higher.
CHAPTER 4 Harnessing synthetic DNA for in vivo expression of multiple different bNAbs against HIV-1


*MCW and ZX contribute equally to the work
4.1 Abstract

Interventions to prevent HIV-1 infection and alternative tools in HIV cure therapy remain pressing goals. Recently, numerous broadly neutralizing HIV-1 monoclonal antibodies (bNAbs) have been developed which possess the characteristics necessary for potential prophylactic or therapeutic approaches. However, formulation complexities especially for multi-antibody deliveries, long infusion times, and production issues could limit the use of these bNAbs when deployed globally impacting their potential application. Here, we describe an approach utilizing synthetic DNA-encoded monoclonal antibodies (dMAbs) for direct in vivo production of prespecified neutralizing activity. We designed 16 different bNAbs as dMAbs cassettes and studied their activity in small and large animals. Sera from animals administered dMAbs neutralized multiple HIV-1 isolates with similar activity to their parental recombinant MAbs. Delivery of multiple dMAbs to a single animal led to increased neutralization breadth. Two dMAbs, PGDM1400 and PGT121, were advanced into non-human primates for study. High peak circulating levels (between 6-34µg/ml) of these dMAbs were measured and the sera of all animals displayed broad neutralizing activity. The dMAb approach provides an important local delivery platform for the in vivo generation of HIV-1 bNAbs and for other infectious disease antibodies.

4.2 Introduction

In just over four decades since its global emergence, the AIDS epidemic has taken millions of lives. While there have been exceptional advances in antiretroviral therapies, there remains a need for preventive treatments and interventions to eliminate HIV-1 infection (Maartens et al., 2014). In recent years, multiple monoclonal antibodies with potent neutralization capacity have been isolated from HIV-1 infected persons (McCoy and Burton, 2017; Pegu et al., 2017). A few of these broadly neutralizing antibodies (bNAbs) have demonstrated efficacy in preventing infection after a single dose of
intravenous recombinant protein in non-human primates (Gautam et al., 2018). Such observations have generated enthusiasm in the field and progressed HIV-1 bNAbs into the clinic for studies of prevention (NCT02256631, NCT02568215, NCT02716675) as well as for HIV treatment towards cure strategies (Bar et al., 2016; Bar-On et al., 2018; Cohen and Caskey, 2018; Mendoza et al., 2018; Scheid et al., 2016). Recently, clinical trials have explored the capability of these antibodies to lower viral loads or prevent rebound after analytical treatment interruption (ATI) (Bar et al., 2016; Scheid et al., 2016). Most notably, a study by Mendoza et al. demonstrated that a combination of two bNAbs, 3BNC117 and 10-1074, prevented viral rebound for a median of 21 weeks in a subset of individuals compared to 2.3 weeks in historical controls (Mendoza et al., 2018).

The widespread use of passive delivery of recombinant antibodies is impacted due to infusion time, formulations issues, product temperature stability, re-dosing requirements and significant manufacturing costs (Awi and Teow, 2018). Viral vector delivery with adeno-associated virus (AAV) has been previously evaluated as a delivery platform for HIV-1 bNAbs, with high-level and long-term expression of the transgene antibody (Balazs et al., 2011; Johnson et al., 2009; Saunders et al., 2015). However, AAV delivery can be limited in populations by pre-existing neutralizing antibodies to the vector, safety concerns of permanent gene marking of the patient, temperature stability, manufacturing cost, as well as vector seroconversion potentially preventing re-administration ultimately resulting in reduced antibody levels in many subjects (Fuchs and Desrosiers, 2016). Recent clinical results of recombinant AAV-1 delivered PG9 demonstrated limited detection of circulating PG9 in healthy males who were delivered a range of vector doses (4x10^{12} – 1.2x10^{14} vector genomes) (Priddy et al., 2019). In this study, we explored the use of synthetic DNA encoded monoclonal antibodies (dMAb) as a possible alternative, serology-independent, approach to passive transfer and AAV delivery. Upon injection and electroporation of
optimized plasmid DNA with transgenes encoding antibody, locally transfected cells become the in vivo bio-factory for antibody production. We have previously demonstrated this dMAb technology was able to induce protective levels of antibody in mice against important infectious disease targets, including influenza A and B viruses, Pseudomonas aeruginosa, Zaire Ebolavirus, dengue virus, Zika virus and chikungunya virus (Elliott et al., 2017; Esquivel et al., 2019; Flingai et al., 2015; Muthumani et al., 2016; Patel et al., 2017; Patel et al., 2018a).

Here, we studied the activity of a panel of 16 engineered dMAbs encoding HIV-1 specific monoclonal antibodies that exhibit broad neutralizing activity. Following dMAb administration, we observed rapid expression and sustained blood levels for months in small animals. These in vivo produced dMAbs were functionally active and neutralized to varying degrees the 12 global panel viruses in an envelope-pseudotyped virus neutralization assay (deCamp et al., 2014). To decrease the possibility of viral escape, we next explored administration of multiple (up to four) dMAbs into a single animal and demonstrated expansion of serum neutralizing breadth. Based on in vivo dMAb levels and neutralizing potency, we advanced two dMAbs, PGT121 and PGDM1400, for a pilot non-human primate (NHP) study. In this study, NHP were delivered a single dMAb (PGDM1400) or a combination of the two (PGT121 and PGDM1400). Strong expression was observed in both groups with a range from 6-34.3 µg/ml at peak levels. All animals expressed dMAbs and the serum demonstrated strong tier-2 neutralization breadth. Additionally, the levels of dMAb observed in this study were, on average, more than ten times higher compared to an initial NHP dMAbs study (Esquivel et al., 2019). This provides evidence for further development of this platform, which represents an alternative modality for in vivo antibody production against HIV-1 and other biological targets.
4.3 Results

4.3.1 Robust expression of optimized HIV-1 dMAbs expressed in vitro and in vivo

dMAbs utilize optimizations that were developed to increase plasmid uptake and expression in context of delivery by adaptive electroporation (Sardesai and Weiner, 2011). These improvements resulted in significant enhancement of in vivo expression launched from plasmids. Here we adapted multiple HIV-1 specific bNAbs and tested their expression and in vivo levels. We compared 16 different bNAbs targeting five different regions of the HIV-1 envelope: CD4 binding site (VRC01, N6, 12A21, 3BNC117, IOMA, NIH45-46); high mannose glycan patch (PGT121, PGT128, 10-1074, PGT130); apex (PGT145, PGDM1400, PG9); gp120-gp41 interface (PGT151, 35O22) and gp41 fusion domain (VRC34.01) (Gristick et al., 2016; Huang et al., 2016; McCoy and Burton, 2017; Scheid et al., 2011; Walker et al., 2011). Broadly neutralizing antibodies encoded as dMAbs were selected based on target epitope, neutralization capacity and other characteristics such as length of heavy chain complementarity-determining regions 3 (CDR3) and percent of somatic hypermutation to obtain a range of antibody characteristics (Table S1). The heavy chain length of the CDR3 region ranged from 12 (3BNC117) to 35 (PGDM1400) with an average length across all dMAbs of 22. The percent of amino acid somatic mutations from germline in the heavy chain ranged from 18% (IOMA) to 42% (VRC01) with an average of 28%. Plasmids encoding the heavy chain or the light chain of each of the broadly neutralizing antibodies were RNA and codon optimized, synthesized and cloned into the modified pVax1 backbone. All dMAbs were of the human IgG1 isotype. Expression levels in vitro of all dMAbs were confirmed using transient transfection of HEK 293T cells (Figure S4.1).

Next we proceeded to assess in vivo expression in transiently immunodepleted mice to prevent the development of anti-drug antibodies responses against the human
IgG1. Mice were injected with plasmid dMAb constructs followed by intramuscular electroporation (IM-EP) using the CELLECTRA® 3P device. We observed dMAb expression in sera two days post injection with peak levels around Day 21 (Figure S4.2). dMAb was continuously detected in the sera for over 300 days. Peak levels of dMAbs varied from below 1µg/ml to greater than 80µg/ml (Figure 4.1A). The majority of dMAbs exhibited peak levels between 10-30µg/ml. There was minimal variability among mice receiving a given dMAb, supporting a model where the intrinsic properties of each antibody sequence influence the overall levels observed in vivo. We did not observe any correlation in dMAb levels for different families, heavy chain CDR3 length or percent somatic hypermutation rate of the heavy chain.

4.3.2 Functionality of in vivo produced dMAbs is comparable to recombinant MAb counterparts

To further characterize in vivo produced dMAbs, we investigated their ability to bind to trimer as compared to recombinantly produced monoclonal antibodies. We observed similar strength of binding to trimer as compared to recombinant protein monoclonal antibody for all tested dMAbs (Figure 4.1B and Figure S4.3A). In agreement with our other dMAb studies, this suggests proper folding of the dMAb in vivo and supports retention of their antigen specificity (Elliott et al., 2017; Flingai et al., 2015; Muthumani et al., 2016; Patel et al., 2017; Patel et al., 2018a).

We next examined dMAb functionality in the context of neutralization using HIV-1 envelope pseudotyped viruses representing the global diversity of HIV-1 glycoprotein (deCamp et al., 2014). We observed strong neutralization titers for all the studied dMAbs (Figure 4.1C-D). Importantly, there was low variability in neutralization titers among mice given a specific dMAb based on each specific group and serum neutralization titers (IC$_{50}$) were similar to titers reported in the literature (Figure 4.1C and Figure S4.3B). The
neutralization data further confirm dMAbs were assembled, formed and properly folded in vivo and then exhibit similar potency to the recombinant protein monoclonal antibodies.

4.3.3 Modifications improved production of low expressing N6 dMAb

N6 is an extremely potent and broad neutralizing anti-HIV-1 antibody (Huang et al., 2016), however its in vivo levels were among the three lowest (Figure 4.1A). Based on these previous studies, we observed that single amino acid modification can significantly increase dMAb expression (Patel et al., 2018a). Thus, we sought to increase its expression by designing modifications to both the heavy chain (HC) and light chain (LC) of the original N6 amino acid sequence at the C- and N-terminus of the variable region. These modifications were selected to make the antibody more similar to the human parental germline antibody sequence (Figure S4.4A). Mice injected with HC unmodified + LC modified (LCmod) or HC modified + LC unmodified (HCmod) dMAb had 3.5-fold increases in levels over the unmodified original N6 (Figure S4.4B). When both modified plasmids were used to assemble modified N6 (N6mod), levels increased 9-fold over unmodified and a 2.5-fold over each modified chain. Modifications to the variable regions can change an antibody’s ability to bind to its target and impact its functionality. Binding to trimer was similar between serum from mice injected with N6mod dMAb and recombinant unmodified N6 (Figure S4.4C). Furthermore, recombinant antibodies expressing either N6 or N6mod were able to neutralize multiple viruses from the global panel to similar degrees as levels previously reported in the literature (Figure S4.4D) (Huang et al., 2016). Thus, these modifications appear to be important for increasing overall production in vivo, resulting in increased serum levels of antibody while maintaining the functionality of this antibody. While there was marked improvement in the in vivo production levels of N6mod, these levels remain on the lower end and additional rounds of optimization could further improve in vivo levels.
4.3.4 Delivery of multiple dMAbs to provide enhanced coverage of viral mutations

Due to the high error rate of HIV-1 reverse transcription and resultant high antigenic variability, viral immune escape from a single-antibody therapy is likely (Cohen and Caskey, 2018). Additionally, escape mutations to the mAb may already exist in populations as no single mAb targets all circulating HIV-1 strains (Cohen and Caskey, 2018). In order to overcome such issues, we evaluated co-delivery of multiple bNAbs against distinct HIV-1 envelope epitopes in the dMAb delivery platform. We selected two combinations which are currently in clinical trials, 3BNC117 + 10-1074 (Bar-On et al., 2018; Mendoza et al., 2018) and PGDM1400 + PGT121 (NCT0320591), as well as PGT121 + PGT145. PGT121 and PGT145 were chosen based on in vivo dMAb levels, target epitope and neutralization profile of the antibodies. Mice were dosed with either a single dMAb construct or with two dMAb constructs in separate distinct muscle sites. In vivo levels of each individual antibody were similar in the combination mice as compared to mice delivered only a single antibody (Figure 4.2A). Individually, bNAb targets between 7 and 11 viruses in the global panel with various gaps in neutralization capacity (Figure 4.1C). By expressing two dMAbs in a single mouse, we observed an increase in their overall breadth of neutralization, targeting never less than 11 different members of the global panel as compared to each of the individual dMAbs with the PGDM1400 + PGT121 (NCT0320591) combination now providing 100% viral coverage (Figure 4.2B) (Julg et al., 2017b).

We next sought to deliver and express four dMAbs in a single mouse using antibodies PGDM1400, PGT151, VRC01, and PGT121. Such deliveries of multiple antibodies are difficult in other methods. For this study the antibodies were selected based on their neutralization capacity, overall in vivo levels, and ability to target distinct epitopes on the HIV-1 envelope. In these studies, animals were injected with a single dMAb or with
all four. As we do not have anti-idiotype antibodies for these antibodies, we measured the total amount of the xenogeneic human antibody expressed in the mice (Figure 4.2C). The total serum hlgG1 dMAb levels in the mice administered with all four dMAb constructs were comparable to the sum of the levels of each dMAb construct administered individually (sum of mice injected the individual dMAbs: 26.01µg/ml vs combination dMAb mice: 25.10µg/ml). Once again, we observed increased neutralization breadth in the sera of mice that received all four dMAb constructs compared to neutralization breadth in the sera of mice that received each individual dMAb construct (Figure 4.2D). By delivering all four dMAb constructs at once, we observed neutralization IC\textsubscript{50} levels below 0.1µg/ml across the entire global panel.

4.3.5 HIV-1 dMAbs express in NHPs

Based on the promising studies in mice, we next explored dMAb delivery of HIV-1 specific dMAbs in a pilot NHP animal model which is more relevant for translation to humans. Two dMAbs were selected to move into NHPs, PGDM1400 and PGT121, based on high in vivo dMAb levels in mice (Figure 4.1). Two groups of four macaques were dosed with either 6mg of PGDM1400 dMAb plasmid construct (Group 1) or 3mg of PGDM1400 plus 3mg of PGT121 dMAb plasmid construct (Group 2). Expression of human IgG1 (hlgG1) was detected in NHP serum as early as 3 days post-injection and peaked at Day 14 (Figure 4.3A, Figure S4.5A and S4.6A). Total human IgG1 levels at peak were slightly higher for the group receiving PGDM1400 dMAb alone (Group 1) compared to the two dMAbs PGDM1400 and PGT121 (Group 2) (Figure 4.3B). The total hlgG1 detected in the serum from Group 1 ranged between 11.2 and 34.3µg/ml (mean 25.1µg/ml) and for Group 2 between 6.3 and 20.4µg/ml (mean 10.1µg/ml). The levels of human IgG1 dMAb in the sera declined after Day 14 to undetectable levels by Day 35, which is expected in this context where a xenogeneic human IgG was being expressed in an immune-competent
NHP host (Gardner et al., 2019b; Kuriakose et al., 2016; Martinez-Navio et al., 2016). Accordingly, the decrease in dMAb levels after Day 14 corresponded with the development of NHP anti-human IgG-dMAb antibodies in the sera (Figure S4.5B and S4.6B). Using envelope antigen and secondary antibodies specifically recognizing hlgG1 kappa (PGDM1400) versus hlgG1 lambda (PGT121) light chains, we were able to confirm expression of both PGDM1400 and PGT121 dMAbs in Group 2 NHP sera (Figure 4.3C, S4.5C and S4.6C).

We proceeded to determine the anti-viral activity of the sera harboring the anti-HIV-1 dMAbs. Pre-bleed sera (Day 0 (D0)) and sera from the peak dMAb level time point (Day 14 (D14)) were tested for neutralization against the global panel tier-2 viruses. NHP sera contained no neutralizing antibodies before dMAb administration (D0) and no non-specific neutralization was detected against mouse leukemia virus (MLV) on D0 and D14 (Table 4.1). Sera collected at peak dMAb levels were able to neutralize 11 out of 12 viruses (Group 1) and 12 out of 12 viruses (Group 2) (Figure 4.3D and Table 4.1). For several viruses, specifically 243F6, 25710, CE0217, CNE55, and CNE8, titers (IC₅₀) for both groups were less than 0.1µg/ml. These pseudotype neutralization titers, originally performed at The Wistar Institute, were then retested and confirmed at Duke University. Similar Group 1 and Group 2 NHP neutralization titers (IC₅₀) were obtained across the 9 HIV-1 pseudotype viruses re-evaluated (Figure S4.7A). Delivery of a second dMAb in Group 2 modestly improved neutralization for some isolates and added neutralization coverage for two additional viruses, 398F1 and TRO.11. We further explored the anti-viral activity by exploring the antibody-dependent cell-mediated cytotoxicity (ADCC) against a subtype C HIV-1 infectious molecular clone (IMC) DU151 infected cells, chosen based on the sensitivity to both mAbs. Though ADCC activity of the serum was usually only detected at concentration >4µg/mL, it was comparable to the recombinant protein monoclonal
antibody activity (Figure 4.3E). No ADCC activity was observed for the negative control recombinant Palivizumab antibody (Figure S4.7B). In summary, the NHP data support that HIV-1 dMAbs expressed at high levels, bind to envelope trimers, neutralize numerous tier-2 viruses and have effector functions and can complement each other in vivo.

4.4 Discussion

Recently, the use of protein monoclonal antibodies has become a first-line treatment for numerous cancers, and similarly plays a major role in autoimmune disease therapies (Singh et al., 2018). In general though, the adoption of MAb for infectious disease is very exciting but to date there have been limited approvals (Singh et al., 2018). Due to their exceptional breadth and potency, clinical trials are in progress to explore the ability of broadly neutralizing antibodies (bNAbs) against HIV-1 to both prevent and treat infection (Cohen and Caskey, 2018). Additional strategies for delivery are likely important especially for providing these strategies in the developing world (Sparrow et al., 2017). In this manuscript, we describe a recently developed DNA-encoded monoclonal antibody (dMAb) platform for delivering bNAbs and provide the first proof of concept for this delivery targeting HIV-1.

Through iterative studies, we describe dMAb delivery resulting in expression of multiple HIV-1 specific antibodies up to 80µg/ml in mice. To date, in vitro expression of bNAbs from transfected cell lines does not correlate or predict in vivo levels when delivered via the dMAb platform (Patel et al., 2018a). Interestingly, similar variations in dMAb levels across multiple different antibodies were observed with AAV-mediated gene delivery (Balazs et al., 2011; Gardner et al., 2019b; Welles et al., 2018). Even within the same class of antibodies which share the germline VH gene (IGHV1-2) usage, dMAb levels vary significantly between 1.3µg/ml (N6) and 52.2µg/ml (IOMA) (Figure 4.1A). Furthermore, we did not observe any correlation of in vivo levels and heavy chain CDR3
length, light chain usage or rate of somatic hypermutation. We demonstrate that modifications to the beginning and end of N6 variable regions of the heavy and light chains improved in vivo levels while maintaining the antibody’s activity (Figure S4.4). As we acquire more data on antibody sequences and dMAb expression, a better understanding of in vivo sequence liabilities will be obtained. As HIV-1 bNAbs are highly somatically mutated, and many of these mutations are required for maintaining the functionality of the antibody, balancing mutations made for increasing dMAb levels will need to be weighted with impacts on functionality. DNA encoded monoclonal antibodies are an important tool for these studies since DNA can be easily modified to encode different amino acids and the effects on both in vivo expression as well as functionality can be quickly and cost-effectively explored. Furthermore, the monoclonal antibody sequence rules which dictate the in vivo expression of dMAbs delivered to muscle tissue could translate to other platforms such as AAV where muscle expression is also being tested.

We demonstrated the ability to encode two HIV-1 specific bNabs in NHPs using the dMAb technology. We observed dMAb expression in NHP sera within three days of dMAb administration and levels that peaks around Day 14. We believe these peak levels (ranging from 6 to 34µg/ml) would be protective against multiple SHIV strains upon challenge based upon prior studies which utilized recombinant monoclonal antibody protein (Gautam et al., 2018; Julg et al., 2017a; Julg et al., 2017b; Moldt et al., 2012; Shingai et al., 2014). Specifically, Julg et al. demonstrated that NHPs delivered passive infusions of 2 and 0.4mg/kg of PGDM1400 one day before challenge with SHIV-325c were protected from infection (Julg et al., 2017b). The average level of PGDM1400 in the serum at time of challenge was 6.9 and 2.5 µg/ml for 2 and 0.4mg/kg infusions respectively. There was breakthrough infection in the group delivered 0.08mg/kg which corresponded to a serum level of 0.22 µg/ml at time of challenge. Additionally, PGT121 has
demonstrated protection against SHIV-SF162P3 and SHIV-AD8EO at levels of 15 and 22 µg/ml respectively with partial infection at serum levels of 1.8 µg/ml (Moldt et al., 2012; Shingai et al., 2014). In the context of treatment, NHPs chronically infected with SHIV-SF162P3 and delivered a single infusion of 10mg/kg of PGT121 were able to control viral loads to undetectable levels (Barouch et al., 2013). Rebound occurred in 3 out of 4 animals once mAb serum concentrations reached undetectable (<1µg/ml) with one animal having long-term virologic control. While we are unaware of a prior study for the use of PGDM1400 in pre-clinical treatment setting, the peak levels of this antibody in the NHPs might be relevant in therapeutic settings especially considering that the mean concentration of 3BNC117 and 10-1074 were between 1.9 and 14.8 µg/ml at the time of viral rebound in the study by Mendoza et al (Mendoza et al., 2018). However, additional testing of the ability of dMAbs, that are developed as species matched antibodies, to impact challenge outcomes or control of infection will be informative.

There was a decline in human dMAb levels the NHP study observed after Day 14 corresponds with the development of anti-drug antibodies (ADA) due to the expression of the xenogeneic protein. The development of ADA against a cross-species human IgG has also been observed in NHPs following AAV, recombinant protein (Kuriakose et al., 2016), as well as RNA (Pardi et al., 2017) and adenovirus delivery of antibodies in mice (Badamchi-Zadeh et al., 2018). In studies of AAV delivery of HIV-1/SIV bNAbs, induction of ADA was closely associated with the distance of the variable regions from germline (Martinez-Navio et al., 2016). Furthermore, AAV delivery of fully rhesus monoclonal antibodies against SIV led to a lower incidence of ADA responses in rhesus macaques (Welles et al., 2018). In mice, we eliminated host immune responses to human IgG1 dMAb via transient immunosuppression. Additionally, we have observed that using a species matched fully murine dMAb in mice can avoided significant ADA development and allowed
for dMAb detection in the serum of several months (Khoshnejad et al., 2019). However, we have not yet developed fully simian dMAbs and future studies will test this principle. Ultimately, human studies will be particularly informative. In this regard, recent monoclonal antibody delivery clinical trials include an AAV vector encoding HIV-1 bNAb VRC07 (NCT03374202), the first dMAb construct for expression of antibody against Zika virus (NCT03831503), and an mRNA platform for delivery of anti-chikungunya virus antibody (NCT03829384) will be particularly valuable to provide additional information on this important question.

Depending on the application of the monoclonal antibody, different effector functions and modifications in the antibody Fc domain may be required. Monoclonal antibodies used for HIV-1 prevention and treatment will likely benefit from longer in vivo half-lives. Amino acid mutations to the Fc, including YTE and LS, yield prolonged recombinant in vivo antibody half-lives in both preclinical NHP studies and in the clinic (Kontermann, 2009). We have previously demonstrated the feasibility of dMAbs to encode Fc modifications such as the LALA mutation to prevent antibody dependent enhancement (ADE) in dengue virus infection (Flingai et al., 2015). Furthermore, modifications to the Fc region of the antibody can increase ADCC activity which is important in HIV-1 protection and control of infection (Margolis et al., 2017). Inclusion of these modifications to increase activity of dMAb against infected cells is imperative for therapeutic cure approaches. Exploration of half-life extension and Fc activity modification for HIV-1 dMAbs is ongoing.

Our data describe a new important technology for in vivo HIV-1 antibody delivery. We demonstrated that dMAb constructs can be developed to encode multiple HIV-1 specific IgG1 and that the in vivo expressed dMAbs retain their functional activity in both small and large animals. The observation that in vivo produced dMAbs had similar HIV-1 envelope trimer binding and neutralization capacity as recombinantly produced
counterparts highlights the effective folding of the dMAb antibody in this system. Furthermore, the ability to deliver multiple antibodies, in this case four at one time, to limit viral escape and resistance may be important for HIV-1 prevention and treatment strategies. To our knowledge, this is the most comprehensive screening of multiple HIV-1 antibodies delivered by the same platform, demonstrating how inherent antibody characteristics influence in vivo production. Additionally, through numerous optimization efforts aiming at both the DNA synthetic design and the delivery technology, we were able to consistently reach greater than 5µg/ml of antibody in NHPs, with some NHPs reaching serum levels of more than 30µg/ml. These levels represent a significant improvement compared to the original NHP dMAb studies, which achieved on average a tenth the in vivo production observed here (Esquivel et al., 2019). These studies demonstrate the possibility of dMAbs as an approach to delivery of monoclonal antibody specificities in a simple to produce, temperature stable, and rapid delivery format. Further study of the dMAb platform for anti-HIV strategies appears important.

4.5 Acknowledgements

We would like to thank the Animal Facility staff at the Wistar Institute for providing house and care to the animals. The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Panel of Global HIV-1 Env Clones from Dr. David Montefiori; SV-A-MLV-env from Dr. Nathaniel Landau and Dr. Dan Littman; HIV-1 SG3 ΔEnv non-infectious molecular clone and TZM-bl from Drs. John C. Kappes and Xiaoyun Wu; and CEM.NKRCCR5 cells from Dr. Alexandra Trkola. This work was supported by an NIH IPCAVD Grant U19 AI109646-04, WW Smith Charitable Trust 6743101374, and Martin Delaney Collaboration HIV Cure Research Grant 2528109374 awarded to D.B.W.
**Figure 4.1** In vivo expression of dMAb-encoded HIV-1 bNabs in mice.
(A) Peak dMAb expression levels (Day 14) of HIV-1 broadly neutralizing antibodies in the sera of transiently immunodepleted mice. Groups of mice (n=5) were administered dMAb constructs expressing one of sixteen different bNAbs.

(B) Binding curves for four dMAbs against HIV-1 trimer BG505_MD39. Serum dMAb levels were normalized for expression (colored lines, n=5 mice) and compared to the similar purified recombinant protein (black line) over various concentrations.

(C) Individual mouse IC₅₀ (n=5) for four dMAbs across the 12 viruses global panel (blue circles) vs values reported in the literature (red squares). Literature values gathered from Los Alamos CatNaber.

(D) Mean (n=5) IC₅₀ pseudotype neutralization of Day 14 mouse sera against the 12 viruses of the global panel, and MLV control. Value of 45 corresponds to no neutralization at a 1:45 dilution the lowest dilution tested of the mouse serum. All other values are in µg/ml. Horizontal bars indicate mean; error bars represent standard error of the mean. Expression levels are representative of two experimental replicates, binding and neutralization testing performed once.
Figure 4. Delivery of multiple dMAb constructs in a single mouse maintains individual dMAb expression levels and increases serum neutralization breadth.

(A) Groups of mice (n=5) were administered a single dMAb (PG121, PGT145, PGDM1400, 3BNC117 or 10-1074), or a combination of two dMAbs (PGT121 + PGT145, PGDM1400 + PGT121, 3BNC117+10-1074). Peak serum expression levels of human IgG were quantified by ELISA.

(B) Mean (n=5) IC₅₀ pseudotype neutralization against the 12 viruses of the global panel, and MLV control, of sera collected at Day 14 from mice administered a single or two dMAbs. Value of 45 corresponds to no neutralization at a 1:45 dilution the lowest dilution tested of the mouse serum. All other values are in µg/ml.

(C) Total human IgG serum expression levels following administration of individual dMAbs (PGDM1400, PGT151, VRC01, and PGT121) and co-administration of all four dMAbs (combo) in mice (n=5).

(D) Mean (n=5) IC₅₀ pseudotype neutralization against the 12 viruses of the global panel, and MLV, for sera collected from mice administered individual dMAbs and combination of the four dMAbs. Horizontal bars indicate mean, error bars represent standard error of the mean. Expression levels are representative of two experimental replicates, binding and neutralization testing performed once.
Figure 4. PGDM1400 and PGT121 express as dMAbs in NHPs.

Immune competent macaques were injected with human IgG1 (hIgG1) dMAb constructs (Day 0) and serially bled. Group 1 animals (n=4) received 6mg of PGDM1400-encoding plasmid DNA; Group 2 animals (n=4) received 3mg of PGDM1400 and 3mg of PGT121 plasmid DNA.

(A) Quantification of hIgG1 in the sera of Group 1 and Group 2 NHPs over time.

(B) Peak expression levels of total hIgG1 for each group at Day 14.

(C) Serum binding curves against HIV-1 Env trimer, BG505_MD39, using different secondary antibodies to establish the binding of PGDM1400 (human IgG1 kappa light chain, blue), and PGT121 (human IgG1 lambda light chain, green).

(D) Neutralization IC\textsubscript{50} of serum across the 12-virus global pseudotype panel using serum from peak dMAb expression (Day 14).

(E) Baseline subtracted ADCC killing activity of serum for infectious molecular clone DU151 compared to recombinant monoclonal antibodies PGDM1400 and PGT121. Horizontal bars indicate mean; error bars represent standard error of the mean. Expression levels and neutralization titers representative of two replications, all other test performed once. Two-tailed student t-test performed to determine significant difference in levels of expression between group 1 vs group 2. P < 0.05 was considered significant.
Table 4.1 Neutralization ID\textsubscript{50} of dMAb treated NHP serum across the global panel.

\textit{ID}_{50} neutralization titers for heat inactivated serum from Day 0 and Day 14 for NHP administered dMAb PGDM1400 (group 1) or PGDM1400 and PGT121 (group 2) across the global panel of pseudotype HIV-1 envelopes and non-specific MLV control.
Supplemental Figure 4.1 In vitro expression of various HIV-1 specific dMAbs. HEK293T cells were transiently transfected with plasmid dMAb constructs expressing 16 HIV-1 antibodies.

Quantification of human IgG expression in cell lysate (A) and supernatant (media) (B) using two technical replicates but representative of two experimental replicates.

(C) Example Western blot of two dMAbs, 3BNC117 and PGT128, in the media of transfected cells demonstrating expression of both the heavy and light chain. Lanes 1 and 2 for each dMAb were biological replicates.
Supplemental Figure 4. 2 Time course expression of the CD4bs, Apex, HMG and Interface dMAbs in mice.

Groups of mice (n=5) were transiently immunodepleted and delivered various dMAbs. Expression of dMAbs in the serum was followed over time for the CD4bs (A), apex and interface (B) and high mannose glycan (C) dMAbs as well as for naïve mice. Dots represent mean expression with bars displaying the standard error of the mean. Representative of two experimental replicates.
**Supplemental Figure 4. 3** Functionality of HIV-1 bNabs dMAbs produced in vivo.

(A) Binding curves of recombinant (black) and in vivo produced dMAbs (mice, Day 14, colors, n= 5) against HIV-1 Env trimer (BG505_MD39). No trimer binding was detected using naïve mouse serum using the two secondary antibodies.

(B) Individual mouse IC$_{50}$ across the 12-virus global panel (blue circles) vs values reported in the literature (red squares). Literature values gathered from Los Alamos CatNaber.
Supplemental Figure 4. Amino acid sequence modifications to HIV-1 broadly neutralizing antibody N6 improve dMAb expression in vivo without compromising N6 binding or function.

(A) Modifications to the beginning and end of the heavy and light chain amino acid sequence of human IgG1 monoclonal antibody N6 were produced. These modifications were selected to make the antibody sequence more similar to the human germline.
(B) Groups of mice (n=5) were transiently immunodepleted and injected with plasmid DNA expressing original N6, heavy chain (HC) modified + light chain (LC) original (HC$_{\text{mod}}$), HC original + LC modified (LC$_{\text{mod}}$) or both HC and LC modified (N6$_{\text{mod}}$). Expression levels of serum dMAb was determined on Day 14.

(C) Serum binding of N6$_{\text{mod}}$ (green, triangle) to HIV-1 envelope trimer BG505_MD39 were compared to binding of purified monoclonal N6 original (black, circle).

(D) Neutralization IC$_{50}$ (µg/mL) of recombinant N6 original vs N6$_{\text{mod}}$ against HIV-1 envelope pseudotype viruses representing 10 of the global panel in a TZM-bl assay. Reported values gather from Los Alamos CatNap.
A

B

C

OD595nm

Fold dilution

PGDM1400 (Anti-Kappa)

PGT121 (Anti-Lambda)
Supplemental Figure 4. 5 Individual expression of human IgG1 and ADA development in group 1 dMAb administered NHPs.

NHPs were injected on D0 with dMAb expressing PGDM1400 only. (A) Expression kinetics of human IgG1 in NHP serum for each of the four NHPs.

(B) Expression of human IgG (left y-axis) vs ADA (right y-axis) against PGDM1400 (which was administered as dMAb) and PGT121 (which was not administered to group 1) over time. Expression and ADA levels representative of two replications.

(C) Serum binding curves against HIV-1 Env trimer, BG505_MD39, using different secondary antibodies to establish the binding of PGDM1400 (human IgG1 kappa light chain, blue), and PGT121 (human IgG1 lambda light chain, green) for Day 0 pre-bleed serum.
Supplemental Figure 4. 6 Individual expression of human IgG1 and ADA development in group 2 dMAb administered NHPs.
NHPs were injected on D0 with dMAbs expressing PGDM1400 and PGT121.

(A) Expression kinetics of human IgG1 in NHP serum for each of the four NHPs.

(B) Expression of human IgG (left y-axis) vs ADA (right y-axis) against PGDM1400 (dMAb administered) and PGT121 (dMAb administered) over time. Expression and ADA levels representative of two replications.

(C) Serum binding curves against HIV-1 Env trimer, BG505_MD39, using different secondary antibodies to establish the binding of PGDM1400 (human IgG1 kappa light chain, blue), and PGT121 (human IgG1 lambda light chain, green) for Day 0 pre-bleed serum.
Supplemental Figure 4. 7 Similar neutralization titers (IC$_{50}$) for groups 1 and 2 of NHP dMAb study between in-house and collaborator assays and ADCC controls.

(A) To validate neutralization titers observed at Wistar, serum collected from NHPs at Days 0 and 14 were sent to Dr. Montefiori’s group at Duke to cross-validate data collected at Wistar. There was no significant difference between the IC$_{50}$ titers determine between the two groups for all nine viruses selected from the global panel. A modified ANOVA with post-hoc Tukey test was performed to determine significant difference in titers between the two labs. P < 0.05 was considered significant.
(B) The RSV-specific monoclonal antibody, Palivizumab, was used as a negative control and a mixture of A32, CH44, 2G12, and 7B2 monoclonal antibodies were used as positive controls for the ADCC assay.
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**Supplemental Table 4.** GenBank accession numbers used for the basis of HIV-1 dMAbs.
CHAPTER 5 Harnessing synthetic DNA and protein engineering for direct in vivo assembly of nanoparticle vaccines for enhanced immune responses

5.1 Abstract

Nanotechnologies are considered to be of growing importance to the vaccine field. Through decoration of immunogens on multivalent nanoparticles, designed nano-vaccines can elicit improved humoral immunity. However, significant practical and monetary challenges in large-scale production of nano-vaccines have impeded their widespread clinical translation. Here, we illustrate an alternative approach integrating computational protein modeling and adaptive electroporation mediated synthetic DNA delivery thus enabling direct in vivo production of nano-vaccines. We demonstrated DNA-launched nanoparticles displaying an HIV immunogen spontaneously self-assembled in vivo. DNA-launched nano-vaccines induced stronger humoral responses than their monomeric counterparts in both mice and guinea pigs, and uniquely elicited CD8+ effector T-cell immunity as compared to recombinant protein nano-vaccines. Improvements in vaccine responses were recapitulated when DNA-launched nano-vaccines with alternative scaffolds and decorated antigen were designed and evaluated. Finally, evaluation of functional immune responses induced by DLnano-vaccines demonstrated that, in comparison to control mice or mice immunized with DNA-encoded hemagglutinin monomer, mice immunized with a DNA-launched hemagglutinin nanoparticle vaccine fully survived a lethal influenza challenge, and had substantially lower viral load, weight loss and influenza-induced lung pathology. Additional study of these next-generation in vivo produced nano-vaccines may offer advantages for immunization against multiple disease targets.

5.2 Introduction

Vaccination is an extremely important public health measure that has demonstrated prophylactic and therapeutic utility against many infectious diseases(Gao et al., 2018; Lambert and Fauci, 2010; Plotkin, 2014), and impacted some forms of
cancer (Trimble et al., 2015). In the past decade, advances in material engineering has allowed for the development and study of a new generation of nanoparticle vaccines (Al-Halifa et al., 2019; Gregory et al., 2013; Zhao et al., 2014). Hepatitis B and human papillomavirus (HPV) vaccines are such examples of self-assembling virus-like particles which have impacted millions of people (Harper and DeMars, 2017; Szmuness et al., 1980). Nanoparticles may come in several shapes and forms. Inorganic materials (Cheng et al., 2012; Stone et al., 2013), nontoxic phospholipids (Giddam et al., 2012), virus-like particles (VLPs) or self-assembling protein nanoparticles (SAPN) (Fuenmayor et al., 2017; Kaba et al., 2018; Marcandalli et al., 2019; Xu and Kulp, 2019) can all scaffold and present antigens in repetitive multimeric manners to robustly stimulate immunity in animal models (Chattopadhyay et al., 2017; Marcandalli et al., 2019; Sliepen et al., 2015).

However, some intrinsic production challenges have impeded broader translation of nano-vaccines into the clinical space (Desai, 2012; Feng et al., 2019). VLP vaccines are often produced at low yields in mammalian cell lines and are difficult to purify, requiring complex reassembly processes and additional post-hoc characterization (Fuenmayor et al., 2017; Lua et al., 2014; Urakami et al., 2017). Production of HPV VLPs, for example, requires three sequential purification steps of strong cation exchange chromatography, size-exclusion chromatography, and hydroxyapatite chromatography (Jiang et al., 2011). Large-scale production of liposome-based nano-vaccines is challenging, as slight variations in the methods of production result in heterogeneity of the liposomes produced (Desai, 2012). Production of nano-vaccines for a global market could therefore require specialized pipelines which raise costs. In addition, regulatory approval of drugs for use in humans can be complex for development of multicomponent nano-medicines (Eifler and Thaxton, 2011). Technologies that would allow de novo nanoparticle assemblies in the hosts from materials that are inexpensive, simple and stable, which
bypass these complex biochemical processes and downstream purifications, may be of interest.

In this regard, computational protein engineering is an extremely powerful tool and has facilitated the design of novel biologics (Graham et al., 2019) as well as specific potent nano-vaccines (Tokatlian et al., 2019; Xu and Kulp, 2019). One such example is the eOD-GT8-60mer, which is a priming immunogen engineered to activate precursors of HIV-1 broadly neutralizing antibodies (Jardine et al., 2016; Jardine et al., 2015; Sok et al., 2016). When scaffolded with the C-terminus of the lumazine synthase (LS) enzyme from *Aquifex Aeolicus*, eOD-GT8 can assemble into a 60-mer nanoparticle to induce stronger humoral immunity and higher frequencies of antigen-specific IgG$^+$ memory B cells (Jardine et al., 2015). In terms of vaccine delivery, DNA vaccines have been studied for induction of humoral and cellular immunity (Davis et al., 2001; Dowd et al., 2016; Wang et al., 1993). Additionally, delivery of optimized DNA plasmids encoding monomeric immunogens via adaptively-controlled electroporation (EP) (Sardesai and Weiner, 2011) can result in 1000-fold enhancement of *in vivo* expression and longer-term *in vivo* production of the encoded antigens (Khoshnejad et al., 2019; Wise et al., 2019; Xu et al., 2018). The newer DNA platform is also a robust method of eliciting adaptive immune responses in humans, having demonstrated immune potency in the clinic against such diseases as ZIKA, Ebola, HIV, MERS and clinical efficacy against HPV-driven cervical dysplasia (Kalams et al., 2013; Modjarrad et al., 2019; Tebas et al., 2019; Tebas et al., 2017; Trimble et al., 2015).

While simple multimerization domains, such as heptamer domain IMX313P, have been employed to improve DNA vaccine responses (Brod et al., 2018; Tomusange et al., 2016), we explored and expanded upon this concept focusing on induction of both B- and T-cell responses to large computationally designed nanoparticles (24, 60, and 180-mers) decorated with a variety of antigens. eOD-GT8-60mer is currently being clinically
evaluated as a recombinant protein vaccine, and was examined as a prototype for DNA delivery. We discovered that the DNA-Launched nanoparticle Lumazine Synthase decorated with an anti-HIV-1 immunogen eOD-GT8 (eOD-GT8-60mer in the literature (Jardine et al., 2016), herein referred as DLnano_LS_GT8) could assemble in vivo into nanoparticles. DLnano_LS_GT8 induced stronger humoral responses than corresponding DNA-launched monomeric GT8 (DLmono_GT8) both in mice and guinea pigs, and also uniquely elicited CD8+ T-cell immunity unlike the corresponding protein nano-vaccines. We computationally designed alternative nanoparticle scaffolds and utilized different immunogens to evaluate this approach more broadly. Consistent improvements in the induction of adaptive immune responses were observed across multiple constructs, which was further shown to confer significant benefits in protecting mice from lethal influenza challenge. Synthetic DNA/EP technology can, therefore, be used to direct in vivo assembly of computationally designed nano-vaccines, which elicit more potent functional immunological responses. This combination is likely important for rapid development of vaccines and immunotherapies.

5.3 Results

5.3.1 DNA-launched GT8 nanoparticles expressed and assembled in vitro and in vivo

To determine whether DNA/EP could be used to launch structurally designed, self-assembling protein nanoparticles in vivo, we encoded the transgene eOD-GT8-60mer in the pVAX1 vector and optimized the DNA cassette for in vivo nanoparticle expression (Figure 5.1a). We first evaluated expression, secretion and assembly of plasmid encoded GT8 constructs in vitro. We engineered the GT8 constructs to incorporate an optimized human IgE-leader sequence (Xu et al., 2018) and found the in vitro intracellular expression of this construct to be strongly enhanced as compared to GT8 constructs without any leader sequence (Supplemental Figure 5.1a). We therefore used the IgE- constructs for
subsequent experiments. In addition, reducing SDS-PAGE analysis of transfection supernatants supported that both plasmid-encoded GT8-monomer and eOD-GT8-60mer could be secreted (Supplemental Figure 5.1b). Lectin-purified protein eOD-GT8-60mer eluted as a homogenous fraction by size-exclusion chromatography (SEC) (Supplemental Figure 5.1c). The assembled protein was observed to be approximately 2 MDa as determined by protein conjugate analysis with size-exclusion multiangle light scattering, SEC-MALS (Figure 5.1b). Negative stain electron microscopy (nsEM) also supported correct assembly of protein eOD-GT8-60mer with a diameter of around 32nm (Figure 5.1c).

Next, we examined the in vivo expression of both DNA-encoded GT8 monomer and nanoparticle constructs. Immunofluorescent staining of mouse muscles transfected with DNA/EP four days post injection (d.p.i) with VRC01 (a human broadly neutralizing antibody with high-affinity for GT8) showed that both DNA-encoded GT8 constructs expressed in vivo (Figure 5.1d). Reducing SDS-PAGE western analyses of muscle homogenates four d.p.i with VRC01 (in green) also confirmed in vivo expression of GT8 antigens, even though in vivo expression of DLnano_LS_GT8 was stronger and more consistent than DLmono_GT8 (Figure 5.1e). The assembly states of in vivo produced DLnano_LS_GT8 as compared to DLmono_GT8 in mouse muscle homogenates was examined with pseudo-native PAGE. Well-formed 60mer GT8-nanoparticles, as defined by the migration pattern of SEC-purified recombinant protein eOD-GT8-60mer standard (STD 60mer on gel), was observed only in DLnano_LS_GT8 treated but not in DLmono_GT8 treated mice (Figure 5.1f). Minor bands that corresponded to monomeric and oligomeric GT8 band were also observed in DLnano_LS_GT8 muscle homogenates but were significantly less intense than the 60-mer band, and may represent newly
synthesized GT8-subunits or partially assembled GT8-oligomer transiting through cellular secretory networks.

Next, we used a mannose binding lectin (MBL) labelling experiment to assess for \textit{in vivo} antigen multimerization and nanoparticle assembly. MBL is a protein that can form hexamer and preferentially bind to highly repeated glycan structures on a pathogen/antigen surface (Abbink et al., 2016). A recent study by Tokatilian et al. demonstrated that only highly multimerized glycan structures (eOD-GT8-60mer but not eOD-GT8-monomer) could bind to MBL (Tokatlian et al., 2019). In our study, we similarly showed using ELISA that while VRC01 could bind to both protein GT8-60mer and GT8-monomer, murine MBL could only bind to protein GT8-60mer (Supplemental Figure 5.1d and 5.1e). Using this assay as a multimerization readout, we demonstrated that \textit{in vivo} produced DLnano_LS_GT8, but not DLmono_GT8 could bind to MBL (Supplemental Figure 5.1f and 5.1g). Further, we observed that DLnano_LS_GT8 could be strongly labelled by endogenous murine MBL via an immune-histochemistry experiment (Figure 5.1g).

As an additional way to assess \textit{in vivo} nanoparticle formation, we employed a transmission electron microscopy-based technique, where thin sections of transfected muscles were stained with VRC01 and gold-conjugated anti-human IgG. Clusters of gold-labelled macromolecules suggestive of \textit{in vivo} launched nanoparticles decorated with multiple copies of GT8 were only observed in mice injected with DLnano_LS_GT8 but not with DLmono_GT8 (Figure 5.1h and Supplemental Figure 5.1h). In DLnano_LS_GT8 immunized mice, these clusters often had a valency greater than 10 (Figure 5.1i). We expected some reduction in labelling valency due to both steric hindrance in binding of VRC01 to individual GT8 subunits and limited solvent exposure on nanoparticle surfaces with thin sample sectioning. Quantitative measurements of the orders of clusters in
different fields of interests demonstrated that partially formed (orders between 5 and 8) and well-formed (orders no less than 9) nanoparticles were significantly more frequent in mice treated with DLnano_LS_GT8 than with DLmono_GT8, confirming in vivo assembly of these complex nano-vaccines (Supplemental Figure 5.1i).

5.3.2 DLnano_LS_GT8 elicited more rapid seroconversion and higher setpoint antibody titers than DLmono_GT8 and similar titers to protein eOD-GT8-60mer

Using immunofluorescence staining with VRC01 (green), we determined that DLnano_LS_GT8 trafficked more efficiently to the draining lymph node and co-localized with the CD35+ follicular dendritic cells (in blue) in contrast with the DLmono_GT8 seven d.p.i. (Figure 5.2a). This observation is consistent with recent findings on trafficking of recombinant protein nanoparticle vaccines (Tokatlian et al., 2019). To determine whether improved immunogen trafficking correlated with enhanced adaptive immunity, we followed humoral responses in immunized BALB/c mice. After seven d.p.i, we found that DLnano_LS_GT8 induced more rapid GT8-directed seroconversion than DLmono_GT8 (Figure 5.2b). Decoration of the GT8-antigens on the LS nanoparticle core is essential for the observed early response as co-transfection of mice muscles with 1:1 ratio of DLmono_GT8 and DNA-encoded lumazine synthase core (DLnano_LS_core) did not lead to seroconversion at this timepoint (Supplemental Figure 5.2a). We next examined if GT8 scaffolded with a simpler multimerization domain, IMX313P, would perform similarly. Heptameric DNA-encoded GT8-IMX313P (DL_GT8_IMX313P) led to limited seroconversion at seven d.p.i, but the induced antibody titer was 6.9-fold lower than that of DLnano_LS_GT8 (Supplemental Figure 5.2b). Antigen-specific circulating IgMs can play a role in protection from challenge (Bohannon et al., 2016). Here, we measured induced IgM responses and found that DLnano_LS_GT8 induced stronger IgM responses than DLmono_GT8 with two immunizations (Supplemental Figure 5.2c). Further, the IgG
titers were 1.3-log and 1.8-log higher for DLnano_LS_GT8 with single immunization (Supplemental Figure 5.2d) or two immunizations (Figure 5.2c) respectively. Consistent with this observation, we found the frequency of CD19+IgD-IgM-IgG+ GT8 antigen-specific B cells in the spleens of mice immunized with DLnano_LS_GT8 to be 5.3-fold higher relative to mice immunized with DLmono_GT8 (Figure 5.2d), even though relatively few CD19+IgD-IgM-IgG+GT8-24mer+GT8-tetramer+ B cells have been recovered per million splenocytes analyzed (Supplemental Figure 5.2e). DLnano_LS_GT8 retained folding and presentation of a key conformational epitope in vivo, as elicited murine antibodies could outcompete VRC01 binding to GT8 in competition ELISA (Supplemental Figure 5.2f and Figure 5.2e). A striking dose-sparing effect was observed for DLnano_LS_GT8. While humoral responses were remarkably attenuated for DLmono_GT8 at 2 and 10ug doses (Supplemental Figure 5.2g), DLnano_LS_GT8 given at 2, 10 or 25ug doses all induced similar levels of antibody responses (Supplemental Figure 5.2h). Importantly, differences in antibody responses induced by DLnano_LS_GT8 and DLmono_GT8 were probably not solely due to increased antigen expression for DLnano_LS_GT8 (Figure 5.1e), as DLnano_LS_GT8 still outperformed DLmono_GT8 at less than one tenth of the monomer dose (Figure 5.2f).

The ability of DLnano_LS_GT8 to improve humoral responses was observed in other animal models. Strikingly, two immunizations in C57BL/6 mice of DLmono_GT8 failed to induce seroconversion, while DLnano_LS_GT8 induced strong humoral responses (Supplemental Figure 5.2i). In genetically diverse CD1 mice, we also observed more rapid seroconversion and more robust responses for DLnano_LS_GT8 (Supplemental Figure 5.2j). Additionally, we observed DLnano_LS_GT8 significantly improved humoral responses in both female (Figure 5.2c) and male (Figure 5.2g) BALB/c mice relative to DLmono_GT8. Finally, in guinea pigs, a single 50ug intradermal
vaccination of DLnano_LS_GT8 remarkably induced seroconversion seven d.p.i and 1.2-log higher antibody titers than DLmono_GT8 over time (Figure 5.2h). We proceeded with studies of intradermal (ID) vaccination in guinea pigs as ID delivery has additional advantages of simplicity, improved tolerability, and being dose sparing (Modjarrad et al., 2019; Tebas et al., 2019).

We next compared the antibody responses induced by protein eOD-GT8-60mer and DLnano_LS_GT8. Protein eOD-GT8-60mer was subcutaneously administered in mice to be consistent with prior studies involving administration of this immunogen to mice (Jardine et al., 2015; Sok et al., 2016); further, a relative high protein dose of 10μg was used in this study as compared to prior study for protein versus DNA comparison (Tokatlian et al., 2019). We observed that two sequential immunizations of protein eOD-GT8-60mer co-formulated with Sigma Adjuvant System or DLnano_LS_GT8 in C57BL/6 mice induced similar humoral responses (Figure 5.2i). It has been recently reported that uptake and trafficking of protein-based nanoparticles are dependent on the mannose binding lectin (MBL) complement pathway (Selander et al., 2006; Tokatlian et al., 2019). We explored if DNA-launched nanoparticles depended on a similar mechanism. Similar to previous reports (Tokatlian et al., 2019), humoral responses elicited by protein-based GT8 nanoparticles in transgenic MBL and CR2 knockout mice were attenuated as compared to in the wildtype C57BL/6 mice seven d.p.i (Figure 5.2j). Strikingly, similar humoral responses were induced in the MBL or CR2 knockout mice as compared to the wildtype C57BL/6 mice by DLnano_LS_GT8 (Figure 5.2j), highlighting DLnano immunogens may act independently of MBL-complement pathway, potentially through redundant mechanisms of antigen presentation.
5.3.3 DLnano_LS_GT8 elicited superior cellular responses than DLmono_GT8 and uniquely induced CD8+ T-cell responses relative to protein eOD-GT8-60mer

We next examined the induction of antigen-specific cellular responses by DNA nano-vaccines. DLnano_LS_GT8 elicited significantly stronger antigen (GT8)-specific cellular responses than DLmono_GT8 in BALB/c mice as determined by IFN\(\gamma\)-ELIspot assays (Figure 5.3a). Intracellular cytokine staining (ICS) revealed that the scaffolding LS domain drove pre-dominantly CD4+ responses, since a higher proportion of effector memory CD3+CD4+CD44+CD62L- T-cells produced IFN\(\gamma\), TNF\(\alpha\) and IL-2 when stimulated by the LS peptides than by GT8 peptides (Figure 5.3b and Supplemental Figure 5.3a and 5.3b). In contrast, we found that effector memory CD3+CD8+CD44+CD62L- T cells induced by DLnano_LS_GT8 were more reactive to the GT8 domain than to the LS domain. DLnano_LS_GT8 induced more antigen-specific effector memory CD8+ T-cells that expressed activation cytokines IFN\(\gamma\) and exhibited effector phenotypes (CD107a+) than DLmono_GT8 in BALB/c mice (Figure 5.3c through 5.3e).

In C57BL/6 mice, we also found that DLnano_LS_GT8 elicited strong T-cell responses to the full immunogen. Both CD4+ and CD8+ responses were predominantly to the LS domain, possibly due to the lack of CD8+ T-cell epitope in the GT8 domain for this inbred strain (Supplemental Figure 5.3c through 5.3e). To determine the ability of DLnano_LS_GT8 to elicit T-cell responses to the antigenic domain in a model with more diverse HLA haplotypes, we used the outbred CD1 mice and found that DLnano_LS_GT8 induced stronger CD4+ and CD8+ effector memory T-cell responses to the GT8 domain than DLmono_GT8 (Supplemental Figure 5.3f through 5.3h). In all mice strains studied, we observed DLnano_LS_GT8 elicited significantly higher frequencies of effector memory CD8+ T-cells than could DLmono_GT8 (Figure 5.3f and Supplemental Figure 5.3i). Additionally, DLnano_LS_GT8 was observed to induce stronger CD8+ T-cell responses.
to the GT8 domain in both female (Figure 5.3d) and male (Supplemental Figure 5.3j) BALB/c mice.

In comparison to protein eOD-GT8-60mer, we observed two immunizations of DLnano_LS_GT8 induced 2.2-fold higher T-cell responses by IFNγ-ELIspot assay (Figure 5.3g). In addition, ICS revealed that while both protein and DNA-encoded GT8-nanoparticles induced CD4+ responses (Supplemental Figure 5.3k), only DNA-launched but not protein-based nanoparticles elicited potent CD8+ T-cell responses (Figure 5.3h and Supplemental Figure 5.3l). Recombinant protein nanoparticle failed to induce CD8+ T-cell responses in both WT and transgenic MBL and CR2 knockout mice; whereas DLnano_LS_GT8 induced robust CD8+ T-cell responses in these strains (Figure 5.3i), confirming our prior observations that DLnano-vaccines may act independently of the MBL-complement pathway.

5.3.4 Designed DNA-launched GT8-nanoparticles with alternative scaffolds analogously induced improved adaptive immune responses

To ensure that the observed phenomena were not limited to lumazine synthase scaffolded nanoparticles, we computationally designed additional GT8 nanoparticles. Using the crystal structures of ferritin from *Helicobacter pylori* (3BVE, a 24-mer), and PfV viral cage from *Pyrococcus furiosus* (2e0z, a 180-mer), we modeled GT8 at various geometries relative to the particle surface and designed appropriate flexible linkers. 3BVE-GT8 homogeneously assembled into spherical nanoparticles by nsEM (Supplemental Figure 5.4a and Figure 5.4a). For PfV_GT8, we observed mixed species, but the predominant peak at 9.14mL retention time, which accounted for approximately 60% of overall intensity, corresponded to torus shaped nanoparticle by nsEM (Supplemental Figure 5.4b and Figure 5.4b). To demonstrate decoration of the designed nanoparticles with GT8, recombinantly produced protein 3BVE_GT8, eOD-GT8-60mer and PfV_GT8
were all tested and observed to bind to VRC01 (Supplemental Figure 5.4c). Immunofluorescence demonstrated that both DLnano_3BVE_GT8 and DLnano_PfV_GT8 expressed \textit{in vivo} four d.p.i (Figure 5.4c), even though \textit{in vivo} expression of DLnano_PfV_GT8 was found to be stronger on average than DLnano_3BVE_GT8 by SDS-PAGE analysis (Figure 5.4d). Functionally, BALB/c mice immunized with DLnano_3BVE_GT8, DLnano_LS_GT8 and DLnano_PfV_GT8 all rapidly sero-converted seven d.p.i and mounted stronger antibody responses over the five-week period than mice immunized with DLmono_GT8 (Figure 5.4e). In addition, BALB/c mice immunized with two doses of DLnano_3BVE_GT8, DLnano_LS_GT8 and DLnano_PfV_GT8 all developed stronger CD8+ effector memory T-cell responses to the antigenic GT8 domain than those immunized with DLmono_GT8 by IFNγ ELISpot and ICS assays (Figure 5.4f, Supplemental Figure 5.4d and 5.4e).

Valency of nanoparticles was found to be relevant to the dose-sparing phenomenon observed (Figure 5.2f). At low DNA dose of 2ug, we found that 24, 60 and 180-meric DNA-launched GT8 nanoparticle vaccines but not heptameric DL_GT8_IMX313P was capable of inducing seroconversion in BALB/c mice at 7 d.p.i (Figure 5.4g). In terms of cellular immunity at this dose, we found that only 60- and 180-meric but not hepta- and 24-meric DNA-launched GT8 nano-vaccines were capable of inducing improvement in CD8+ T-cell immunity relative to DLmono_GT8 (Figure 5.4h). Overall, we observed that the aforementioned nanoparticle domains can be designed to display antigens like GT8 to elicit rapid and strong adaptive immune responses.

5.3.5 Designed DNA-launched hemagglutinin nano-vaccine induced improved functional antibody responses and stronger CD8+ T-cell immunity

To determine if these findings could be applied to an immunogen relevant to a different infectious disease, we computationally designed a LS nanoparticle to display the
receptor binding domain of the head of influenza hemagglutinin (LS_HA_NC99) based on the H1N1 strain A/New Caledonia/20/1999 and confirmed its assembly into homogenous 60-mer by both SEC, SEC-MAL and nsEM (Supplemental Figure 5.5a, Figure 5.5a and 5.5b). A dose-sparing phenomenon was observed for DLnano_LS_HA_NC99, as at a remarkably low plasmid vaccine dose of 1ug, DLnano_LS_HA_NC99 induced significantly stronger humoral responses in BALB/c mice than DLmono_HA_NC99 (Figure 5.5c). Hemagglutinin inhibition titers (HAI) against the autologous NC99 strain were found to be higher than 1:40 (which correlated with 50% reduction in the risk of infections in humans(Benoit et al., 2015)) in 100% of mice immunized with two doses of DLnano_LS_GT8 and 0% in mice immunized with two doses of DLmono_HA_NC99 (Figure 5.5d). At the final timepoint (56 d.p.i) after three immunizations, both the DLmono_HA_NC99 and DLnano_LS_HA_NC99 groups developed binding and HAI antibodies to the heterologous H1N1 influenza A/Solomon Island/3/06 strain (Supplemental Figure 5.5b and Figure 5.5e), and both the binding and HAI titers were still significantly higher for the DLnano_LS_HA_NC99 group. HAI of a more distant H1 strain, A/California/07/2009, was not detected in either group.

Additionally, in terms of elicited cellular responses, two immunizations of DLnano_LS_HA_NC99 induced 8.4-fold higher effector memory CD8+ T-cell responses than DLmono_HA_NC99 at 10ug dose in terms of CD107a and IFNγ expression, similar to our prior findings (Figure 5.5f, Supplemental Figure 5.5c and 5.5d).

Finally, we examined if homogenous in vitro assembly of the designed DLnano-vaccine was a pre-requisite to its enhanced potency. To this end, we studied the in vivo properties of a poorly folded nanoparticle. We used an alternative lumazine synthase scaffolded influenza construct, DNA-encoded LS_HA_CA09, based on the A/California/07/2009 strain which did not pass our biophysical filters, as in vitro expression
of the construct showed 3+ peaks with the two largest peaks consisting of aggregates or smaller unassembled protein by SEC (Supplemental Figure 5.5e). We found DNA-encoded LS_HA_CA09 could not induce the characteristic early sero-conversion in BALB/c mice (Supplemental Figure 5.5f). Even when the immunized mice were followed over time, the antibody responses induced by DNA-encoded LS_HA_CA09 still lagged behind those by DLnano_LS_HA_NC99, highlighting downstream success of DLnano-vaccine predicated upon preliminary computational design and biophysical characterization.

5.3.6 DNA-launched hemagglutinin nano-vaccine conferred improved protection to lethal pandemic influenza H1 A/California/07/09 challenge in mice

To further evaluate the induction of functional immune responses by DLnano-vaccines, we utilized a lethal influenza challenge model in mice. We constructed a ferritin-scaffolded receptor binding domain of hemagglutinin from H1/California/07/09 strain, DLnano_3BVE_HA_CA09, that was leader sequence, codon and mRNA-optimized as compared to a previously reported construct (Kanekiyo et al., 2019). We first confirmed its in vitro assembly into nanoparticles by SEC and nsEM (Supplemental Figure 5.6a and 5.6b). We then immunized three groups of mice twice with minimal doses (1ug) of DNA encoding either DLmono_HA_CA09, DLnano_3BVE_HA_CA09 or control backbone pVAX vector three weeks apart. We observed improved induction of binding antibody responses in mice immunized with DLnano_3BVE_HA_CA09 than those with DLmono_HA_CA09 (Figure 5.6a). Five weeks post the first immunization, we observed significant 8-fold improvement in HAI titers in mice immunized with DLnano_3BVE_HA_CA09 than those with DLmono_HA_CA09 (Figure 5.6b). We then set up two lethal influenza challenge experiments in these three groups of mice, five weeks post the final immunization. Each mouse was intranasally inoculated with 10LD$_{50}$
homologous H1/California/07/09 virus and was followed for two weeks for weight loss. Any mouse losing more than 20% of baseline body weight would have met the humane endpoint for euthanasia. In this experiment, we observed only mice immunized with DLnano_3BVE_HA_CA09 fully survived the lethal challenge (Figure 5.6c), whereas 40% (2/5) of mice immunized with DLmono_HA_CA09 or 100% (5/5) of mice immunized with control pVAX backbone succumbed to infections. Additionally, amongst mice that survived the challenge, substantially lower weight loss was observed in mice immunized with DLnano_3BVE_HA_CA09 than DLmono_HA_CA09 (Figure 5.6d).

In a separate set of experiments, we followed these three groups of immunized mice seven days post H1/CA09 challenge to determine lung viral load and pathology. It was observed, in this challenge study, that within the first seven days, 80% (4/5) of mice immunized with control pVAX vector succumbed to infection, but mice immunized with either DLmono_HA_CA09 and DLnano_3BVE_HA_CA09 survived the first seven days (Figure 5.6e), even though mice immunized with DLmono_HA_CA09 still lost substantially more weight than those immunized with DLnano_3BVE_HA_CA09 (Supplemental Figure 5.6c). Additionally, we observed significant reduction in viral load of mice immunized with DLnano_3BVE_HA_CA09 as compared to mice immunized with pVAX (2186-fold reduction) or with DLmono_HA_CA09 (156-fold reduction) (Figure 5.6f). Finally, H&E staining of lung specimens at seven days post challenge or at the time of euthanasia revealed that mice immunized with DLnano_3BVE_HA_CA09 but not with DLmono_HA_CA09 were protected from lung pathology, including the observations of eosinophilic necrotic deposits within the alveolar spaces and or thickening of alveolar septa, associated with influenza infection (Figure 5.6g and Supplemental Figure 5.6d). The two lethal challenge studies illustrated that the DLnano-vaccine could confer significant functional advantages in an infectious disease model.
5.4. Discussion

Development of vaccines can be a challenging endeavor due to poor immunogenicity of certain vaccine antigens, which results in the need to increase the number of required vaccinations, dose per vaccination, and the required interval for patients to complete the vaccine regime. Particulate vaccine formulations can help boost immunogenicity but can be slow to develop on a large scale due to manufacturing complexities. Synthetic nucleic-acid based methods for the delivery of vaccine antigens have shown great promises, as they are often produced at significantly lower costs than their protein counterparts, can be manufactured to scale and bypass complex processes of assembly (Kutzler and Weiner, 2008), offer superior safety profile (Lu et al., 2008) and demonstrate remarkable thermo-stability to allow for extended shelf-lives (Hobernik and Bros, 2018).

In this study, through the use of computational modeling and biophysical characterization, we engineered multimeric forms of HIV and influenza antigens which folded properly in vitro and displayed the desired antigenic profiles. We showed that these designer nano-vaccines are likely to assemble in vivo when delivered using synthetic DNA and adaptive electroporation, through direct evidence from pseudo-Native PAGE analysis (Figure 5.1f) and transmission electron microscopy (Figure 5.1h and 5.1i), and indirect evidence of binding of murine MBL to in vivo produced DLnano_LS_GT8 but not to DLmono_GT8 (Figure 5.1g), as well as the improved trafficking (Figure 5.2a) and the resulting immune responses (Figure 5.2b and 5.2c). The in vivo nanoparticle assembly also resulted in rapid sero-conversion, higher binding and functional HAI antibody titers yet with significant dose sparing. Enhanced antibody responses were also induced when DLnano-vaccine was administered via intradermal DNA vaccination, a newer and clinically important route of DNA vaccination (Modjarrad et al., 2019; Tebas et al., 2017).
Importantly, enhanced immune responses induced by DLnano-vaccines also conferred functional advantages. The DLnano-vaccines were more efficient at driving HAI, CD8+ T-cell responses, and ultimately generating protection to animals from intranasal influenza challenge. DNA vaccine approach can effectively synergize with structure-guided protein engineering to quickly produce *in vivo* designer nano-vaccine constructs for rapid evaluation.

This work interrogated factors which might contribute to the enhanced adaptive immune responses of DLnano-vaccines. Homogeneous *in vitro* assembly of these computationally designed DLnano-vaccines is important for their downstream success, as poorly assembled DNA-encoded LS_HA_CA09 did not elicit similarly potent immune responses (Supplemental Figure 5.5f). Homogeneous *in vitro* assembly will likely help increase the fraction of more fully assembled nanoparticles *in vivo*, contributing to the overall immunogenicity of the vaccine. It is in theory possible that the improved immunogenicity described here can be attributed to differences in levels of antigen expression. However, two observations suggest that antigen expression is not solely responsible for improved responses. First, we showed that DLnano_LS_GT8 induced stronger humoral responses than DLmono_GT8 in BALB/c mice at less than one tenth of the dose (Fig 5.2f). Second, while DLnano_PfV_GT8 expressed at higher levels *in vivo* than DLnano_3BVE_GT8 (Fig 5.4d), DLnano_PfV_GT8 and DLnano_3BVE_GT8 induced similar antibody titers and T cell responses at 25µg dose (Fig 5.4e and 5.4f). The exact contribution of nanoparticle assembly, expression and valency for the induction of optimal immune responses will require further investigation.

When DNA-launched nano-vaccines were compared to recombinant protein nano-vaccines, widely considered as an extremely potent vaccine formulation in terms of induction of antibody responses(Kanekiyo et al., 2013), we observed DLnano-vaccines
induced comparable humoral responses to recombinant protein nanovaccines, but uniquely induced potent CD8+ T-cell responses in an MBL-complement independent manner. The observation that DLnano-vaccines function independently of MBL-complement pathway is likely of interest for clinical translation of such vaccines, as approximately 5-20% of human populations have MBL deficiency (plasma MBL<100ng/mL)(Dahl et al., 2004; Zinyama-Gutsire et al., 2015). The role of T cells in immune surveillance to mediate protection provides a strong rationale for exploring this unique property of DLnano-vaccine (Zhang and Bevan, 2011), especially for such diseases as liver-stage malaria(Van Braeckel-Budimir and Harty, 2014), influenza for the elderlies(Grant et al., 2016; Koutsakos et al., 2019), and cancer(Durgeau et al., 2018).

The unique ability for DLnano vaccination to induce CD8+ T-cell responses may be related to its distinct mechanism of antigen uptake and presentation. Antigen presenting cells, such as macrophages, are known to migrate into the site of electroporation to scavenge antigens expressed through DNA cassettes associated with apoptotic cells(Kutzler and Weiner, 2008). Prior studies observed that co-delivery of DNA vaccines with pro-apoptotic mutated Caspase 2 or Fas significantly increased both CD4+ and CD8+ T-cell responses to the vaccine antigens (Chattergoon et al., 2000; Sasaki et al., 2001). Such distinct mechanism of antigen processing might lead to more efficient cross-presentation to the MHC Class I pathway. Additionally, APCs including DCs and macrophages may also be directly transfected with the inoculated DNA cassettes in vivo (Akbari et al., 1999; Chattergoon et al., 1998), and the two mechanisms may be synergistic in the induction of CD8+ T-cell immunity. Our findings also demonstrated that DLnano-vaccine could improve induced CD8+ T-cell responses by eight to ten-fold relative to their monomeric counterparts. Given that DNA-vaccines can already induce CD8+ T-cell responses in patients to cause histopathological regression of HPV-driven cervical dysplasia (Trimble
et al., 2015), the finding is relevant and whether DLnano-vaccines can further improve the clinical response rates should be explored.

Importantly, significant dose sparing can be realized with DLnano-vaccines. A dose of 1ug of plasmid DNA, a dose at which we historically would not expect to observe robust sero-conversion (Yan et al., 2018), was able to induce clear functional HAI titers in mice. Fewer immunizations of DLnano-vaccine could induce the same, if not higher, titers of antibodies. Given recent advances in the electroporation technology has improved the potency and consistency of immune responses induced by DNA vaccines in patients (Akbari et al., 1999; Kalams et al., 2013; Patel et al., 2018b; Tebas et al., 2019; Tebas et al., 2017; Trimble et al., 2015), it will be important to determine whether DLnano-vaccines can also help to reduce doses used in the clinic and lower the number of clinical visits required for vaccination. These advances may have important implications for outbreak control, and for global deployment including of vaccinations in more resource limited settings.

It will be important to build on these initial studies to improve DLnano-vaccines. For example, while it is hypothesized that cross-linking of B-cell receptors through multivalent antigen display can improve B-cell responses(Xu and Kulp, 2019), studies to examine other mechanisms to improve B-cell responses and the mechanisms for the improved CD8+ T-cell responses for DLnano-vaccines relative to their monomeric counterparts are also important. Due to the unique ability of DLnano-vaccine to elicit strong CD8+ T-cell immunity, new DLnano-vaccines should be designed and evaluated to target diseases like cancer and T-cell dependent infectious diseases. The combined advantages of a simplified cost-effective temperature-stable platform, with the ability to retain in vivo structural integrity may be of value for the development of additional vaccines for HIV, influenza as well as other infectious diseases.
5.5. Conclusion

This work demonstrates that advances in synthetic DNA and adaptive electroporation technologies have allowed for \textit{in vivo} assembly of complex computationally designed particulate nano-vaccines to induce improved humoral and cellular responses, and to confer functional protective benefits in a survival study. As DNA can be rapidly manufactured to scale with low costs, it can be envisioned that computationally designed nano-vaccines can be rapidly evaluated to expedite clinical translational and global deployment of various promising vaccine candidates.

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Figure 5. 1 Expression and assembly of *in vitro* produced protein eOD-GT8-60mer and GT8-monomer and *in vivo* produced DLnano_LS_GT8 and DLmono_GT8.
a. Predicted structure of eOD-GT8-60mer, LS inner scaffold is shown in purple, decorated GT8 shown in green and N-linked glycans are represented as blue sticks.

b. SECMAL trace showing the calculated molecular weight of SEC purified eOD-GT8-60mer.

c. Negative stain electron microscopy images of purified eOD-GT8-60mer.

d. *In vivo* expression of DLmono_GT8 or DLnano_LS_GT8 in BALB/c mice four d.p.i, as probed by VRC01 and anti-human Alexa Fluor 488, nuclei staining with DAPI is shown in blue.

e. Reducing SDS PAGE western analysis to determine *in vivo* expression of DLmono_GT8 and DLnano_LS_GT8 four d.p.i in muscle homogenates with VRC01 (in green); GAPDH (in red) is used as the loading control.

f. Pseudo-native PAGE analysis comparing migration of *in vivo* produced DLmono_GT8 and DLnano_LS_GT8 to *in vitro* produced SEC purified recombinant GT8-monomer (labelled as STD mono) and eOD-GT8-60mer (labelled as STD nano) protein standards.

g. Murine MBL labelling of naïve mouse muscles or muscles transfected with DLmono_GT8 and DLnano_LS_GT8 seven d.p.i.

h. Transmission electron microscopy (TEM) images of muscle sections from mice injected with DLmono_GT8 or DLnano_LS_GT8 seven d.p.i that were immunolabelled with VRC01 and gold anti-human IgG. Red arrows highlight VRC01 staining.

i. TEM image of muscle section showing an example of high-valency GT8 nanoparticle assembled *in vivo*. 80ug plasmid DNA dose of DLmono_GT8 or DLnano_LS_GT8 used in d-i.
Figure 5.2 Characterization of in vivo trafficking of DLnano_LS_GT8 and humoral responses induced by DLnano_LS_GT8 versus DLmono_GT8.
a. Trafficking of DLnano_LS_GT8 and DLmono_GT8 seven d.p.i in the draining lymph nodes, as determined by VRC01 staining (green) and anti-CD35-BV421 staining (blue) for co-localization analyses.

b. ELISA binding against monomeric GT8 using serum from female BALB/c immunized with DLmono_GT8 or DLnano_LS_GT8 seven d.p.i.

c. Endpoint titers to GT8 over time using serum from female BALB/c receiving two immunizations of DLmono_GT8 or DLnano_LS_GT8 three weeks apart.

d. Frequencies of CD19+IgM-IgD-IgG+ GT8 specific B-cells in the spleen of naïve female BALB/c mice or female BALB/c mice immunized with two doses of DLmono_GT8 or DLnano_LS_GT8 five weeks post the second immunization.

e. Percentage inhibition of VRC01-GT8 binding by naïve mice sera or post-immune sera from the DLmono_GT8 or DLnano_LS_GT8 vaccinated mice at 1:200 dilution.

f. Comparison of GT8 endpoint titers for female BALB/c mice receiving two doses of DLmono_GT8 at 25ug dose or DLnano_LS_GT8 at 2ug dose.

g. Comparison of GT8 endpoint titers for male BALB/c mice receiving two doses of DLmono_GT8 or DLnano_LS_GT8 at 25ug dose.

h. Comparison of endpoint titers in guinea pigs receiving single 50ug intradermal immunization of DLmono_GT8 or DLnano_LS_GT8.

i. Comparison of humoral responses induced by protein eOD-GT8-60mer adjuvanted by Sigma Adjuvant System or DLnano_LS_GT8 as assessed in C57BL/6 mice.

j. Humoral responses in wildtype C57BL/6, MBL KO or CR2 KO mice to protein eOD-GT8-60mer (purple) and DLnano_LS_GT8 vaccinations (red) seven d.p.i. 80ug of plasmid DNA
used in a, 25ug plasmid DNA and 10ug recombinant protein used elsewhere in the figure unless otherwise specified. Each group except in j includes five animals; each group in j include four animals; each dot represents an animal; error bar represents standard deviation; arrow below the plot represents an immunization; two-tailed Mann-Whitney Rank Test used to compare groups; p-values were adjusted for multiple comparison where appropriate; *, p-value<0.05.
Figure 5.3 Characterization of cellular responses induced by DLnano_LS_GT8 versus DLmono_GT8 in BALB/c mice and by protein eOD-GT8-60mer and DLnano_LS_GT8 in C57BL/6 mice.

a. ELISPOT responses to the LS peptides and GT8 peptides in BALB/c mice immunized with two doses of DLmono_GT8 or DLnano_LS_GT8 at specified doses.

b. Effector memory CD4+ T-cell responses (CD3+CD4+CD44+CD62L-) in immunized BALB/c mice as in a.

c-e. Effector memory CD8+ T-cell responses (CD3+CD8+CD44+CD62L-) in immunized BALB/c mice in terms of IFNγ expression in d. and CD107a expression in e.
f. Comparison for the frequencies of CD8+ effector memory T-cell responses induced by DLmono_GT8 or DLnano_LS_GT8 immunizations in BALB/c mice.

g. T-cell responses as determined by IFN-γ ELISpot assays for protein eOD-GT8-60mer and DLnano_LS_GT8 immunized C57BL/6 mice.

h. CD4+ effector memory T-cell responses for protein eOD-GT8-60mer and DLnano_LS_GT8 immunized C57BL/6 mice as determined by ICS.

i. Comparisons of CD8+ T-cell responses induced by protein eOD-GT8-60mer (purple) versus DLnano_LS_GT8 vaccinations (red) in wildtype C57BL/6, MBL KO or CR2 KO mice. 25ug plasmid DNA and 10ug recombinant protein used in the figure unless otherwise specified. Each group except in i includes five mice; each group in i includes four animals; each dot represents a mouse; error bar represents standard deviation; two-tailed Mann-Whitney Rank Test used to compare groups; p-values were adjusted for multiple comparison where appropriate; *, p-value<0.05.
Figure 5.4 Design and evaluation of new DLnano GT8-vaccines with alternative scaffolds.

a. nsEM image of SEC-purified fraction of \textit{in vitro} produced 3BVE-GT8 nanoparticles.

b. nsEM image of SEC-purified fraction of \textit{in vitro} produced PfV-GT8 nanoparticles.

c. \textit{In vivo} expression of DLnano\_3BVE\_GT8 and DLnano\_PfV\_GT8 in transfected mouse muscles as determined by immunofluorescence; VRC01 labelling is shown in green and nuclei labelling shown in blue.
d. Reducing SDS PAGE western analysis to determine in vivo expression of DLnano_3BVE_GT8 and DLnano_PfV_GT8 four d.p.i in muscle homogenates with VRC01 (in green); GAPDH (in red) is used as the loading control.

e. Humoral responses in BALB/c mice immunized with two 25ug doses of DLmono_GT8, DLnano_3BVE_GT8, DLnano_LS_GT8 and DLnano_PfV-GT8.

f. CD8+ effector memory CD107a+ T-cell responses to GT8 domain in BALB/c mice immunized with DLmono_GT8, DLnano_3BVE_GT8, DLnano_LS_GT8 and DLnano_PfV-GT8 as in e.

g. Humoral responses in BALB/c mice immunized with 2ug doses of DLmono_GT8, DL_GT8_IMX313P, DLnano_3BVE_GT8, DLnano_LS_GT8 and DLnano_PfV-GT8 seven d.p.i. h. CD8+ effector memory CD107a+ T-cell responses to GT8 domain in BALB/c mice immunized twice with 2ug DLmono_GT8, DL_GT8 IMX313P, DLnano_3BVE_GT8, DLnano_LS_GT8 and DLnano_PfV-GT8 three weeks apart. 80ug of plasmid DNA used in c and d; 25ug plasmid DNA used elsewhere in e and f; 2ug plasmid DNA used in g and h. Each group contains five mice; each dot represents a mouse; error bar represents standard deviation; arrow below the plot represents an immunization; two-tailed Mann-Whitney Rank Test used to compare groups; p-values were adjusted for multiple comparison where appropriate; *, p<0.05.
Figure 5.5 Design and evaluation of new DLnano influenza hemagglutinin vaccine.

a. SECMAL trace of lectin and SEC purified LS_HA_NC99.

b. nsEM image of SEC-purified fraction of *in vitro* produced protein LS_HA_NC99 nanoparticles.

c. Humoral responses in BALB/c mice that received DLnano_LS_HA_NC99 or DLmono_HA_NC99 at 1ug dose.

d. Autologous HAI titers against the H1 NC99 strain at D0, D42 (post-dose #2) and D56 (post-dose #3) for mice treated with 1ug DLmono_HA_NC99 or DLnano_LS_HA_NC99.

e. Heterologous HAI titers against the H1 SI06 strain at 56 d.p.i for mice treated with 1ug DLmono_HA_NC99 or DLnano_LS_HA_NC99.

f. CD8+ effector memory IFNγ+ T-cell responses to NC99 HA domain in naïve BALB/c mice or mice immunized with two doses of 10ug DLmono_HA_NC99 or
DLnano_LS_HA_NC99. Each group contains five mice; each dot represents a mouse; error bar represents standard deviation; arrow below the plot represents an immunization; two-tailed Mann-Whitney Rank Test used to compare groups; p-values were adjusted for multiple comparison where appropriate; *, p<0.05.
Figure 5.6 Functional evaluations of DLmono_HA_CA09 versus DLnano_3BVE_HA_CA09 in H1 A/California/07/09 lethal challenge model.

a. Binding endpoint titers to HA(CA09) over time in BALB/c mice immunized with two 1ug doses of pVAX, DLmono_HA_CA09 or DLnano_3BVE_HA_CA09 three weeks apart.

b. HAI titers to the autologous A/California/07/09 strain in BALB/c mice immunized with 1ug pVAX, DLmono_HA_CA09 or DLnano_3BVE_HA_CA09 five weeks from their first vaccination.
c. Percentages of vaccinated mice surviving the lethal 10LD₅₀ H1/A/California/07/09 challenge over two-week period.

d. Weight changes in mice immunized with pVAX, DLmono_HA_CA09 or DLnano_3BVE_HA_CA09 over two-week period following 10LD₅₀ H1/A/California/07/09 challenge.

e. Percentages of vaccinated mice surviving the lethal 10LD₅₀ H1/A/California/07/09 challenge over seven-day period in a separate study.

f. Lung viral load in challenged mice at seven days post challenge or at the time of euthanasia as determined by RT-qPCR.

g. H&E stain for lung histo-pathology in mice seven days post viral challenge or at the time of euthanasia, normal lung histology is shown for comparison; scale bar represents 100um. Each group contained 10 mice in panels a and b; each group contained five in the remaining panels; each dot represents a mouse; error bar represents standard deviation; arrow below the plot represents an immunization; two-tailed Mann-Whitney Rank Test used to compare groups; p-values were adjusted for multiple comparison where appropriate; *, p<0.05.
**Supplemental Figure 5. 1** *In vitro* expression of protein eOD-GT8-60mer and GT8-monomer and *in vivo* expression and assembly of DLnano_LS_GT8 and DLmono_GT8.

a. Immunofluorescence analyses of intracellular expression of protein eOD-GT8-monomer and -60mer with or without IgE leader sequence in transfected HEK293T cells as determined by staining with VRC01 (green) and DAPI (blue) staining.

b. Reducing SDS-PAGE analysis of Expi293F transfection supernatants of pVAX backbone plasmid, protein GT8-monomer, protein eOD-GT8-60mer.

c. SEC trace of lectin column purified Expi293F transfection supernatant of eOD-GT8-60mer.

d-e. Binding of *in vitro* produced protein eOD-GT8-monomer and eOD-GT8-60mer to VRC01 (d.) and MBL (e.) in ELISA assays.

f-g. Binding of *in vivo* expressed DLmono_GT8 and DLnano_LS_GT8 seven d.p.i to VRC01 (f.) and MBL (g.) in the ELISA assays.

h. Additional TEM images of muscle sections from mice injected with DLmono_GT8 and DLnano_LS_GT8.

i. Quantitative determination of the frequencies of clusters of different orders in the TEM images; *, p<0.05. 80ug of plasmid DNA used *in vivo* for panels f through i.
Supplemental Figure 5.2 Humoral responses induced by DLnano_LS_GT8 versus DLmono_GT8 vaccination in female BALB/c, C57BL/6 and CD1 mice.
a. ELISA binding against monomeric GT8 using serum from BALB/c immunized with 1:1 ratio (25ug each) of DLmono_GT8 with pVAX backbone plasmid, DLmono_GT8 with DLnano_LS(core) or DLnano_LS_GT8 with pVAX backbone seven d.p.i.

b. Endpoint titers at seven d.p.i in BALB/c mice immunized with DLmono_GT8, DL_GT8_IMX313P, or DLnano_LS_GT8.

c. IgM endpoint titers to GT8 over time in BALB/c mice immunized with two doses of DLmono_GT8, or DLnano_LS_GT8.

d. Endpoint titers to GT8 over time using serum from BALB/c receiving single immunizations of DLmono_GT8 or DLnano_LS_GT8.

e. Flow plot demonstration of gating of antigen-specific GT8-Tetramer-APC+ GT8-24mer-FITC+ CD19+IgM-IgD-IgG+ B-cells in the spleens of BALB/c mice immunized with two doses of DLmono_GT8 or DLnano_LS_GT8 five weeks post the second immunization.

f. ELISA data showing competition of VRC01 binding at its corresponding EC_{70} concentration to GT8 by week five post-immune sera from mice immunized with two doses of DLmono_GT8 or DLnano_LS_GT8.

g. Endpoint titers to GT8 using serum for BALB/c receiving two immunizations of varying doses of DLmono_GT8.

h. Endpoint titers to GT8 using serum from BALB/c mice receiving two immunizations of varying doses of DLnano_LS_GT8.

i. Humoral responses in C57BL/6 mice immunized with two doses of DLmono_GT8 or DLnano_LS_GT8.
Humoral responses in CD1 mice immunized with two doses of DLnano_LS_GT8 or DLmono_GT8. 25μg of plasmid DNA used in these experiments unless otherwise specified. n=5 for BALB/c and C57BL/6 mice, n=10 for CD1 mice; each line represents an animal; error bar represents standard deviation; arrow below the plot represents an immunization; *, p<0.05.
Supplemental Figure 5. 3 Generalizability of improved cellular responses of DLnano_LS_GT8 in BALB/c, C57BL/6 and CD1 mice strains; comparison of induced CD8+ T-cell responses by protein eOD-GT8-60mer versus DLnano_LS_GT8 in C57BL/6 mice.

a and b. Frequencies of TNFα and IL-2 expressing CD4+ effector memory T-cells specific to either the LS and GT8 domains in female BALB/c mice immunized twice with DLmono_GT8 or DLnano_LS_GT8.

c. Comparisons of total cellular responses to the LS and GT8 domains as assessed by IFN-γ ELISpot assay in female C57BL/6 versus BALB/c mice.

d. and e. Comparison of CD4+ (d.) and CD8+ (e.) effector memory T-cell responses to the LS and GT8 domains in female C57BL/6 and BALB/c mice.

f-h. Overall T-cell (f), CD4+ effector memory (g), and CD8+ effector memory (h) T-cell responses in female CD1 mice immunized with two doses of DLnano_LS_GT8 as compared to DLmono_GT8.

i. Frequencies of effector memory CD8+ T-cells in female C57BL/6 and CD1 mice immunized twice with DLnano_LS_GT8 and DLmono_GT8.

j. Comparison of frequencies of GT8-specific CD8+ T-cell responses induced by two immunizations of DLnano_LS_GT8 versus DLmono_GT8 in male BALB/c mice.

k. CD4+ effector memory T-cell responses induced by protein eOD-GT8-60mer and DLnano_LS_GT8 in C57BL/6 mice as determined by ICS.

l. Flow plot demonstrating induction of CD8+ effector memory T-cell responses by DLnano_LS_GT8 in comparison to protein eOD-GT8-60mer in C57BL/6 mice as
25ug plasmid DNA and 10ug recombinant protein used in the figure unless otherwise specified. n=5 for BALB/c and C57BL/6 mice, n=10 for CD1 mice; each dot represents an animal; error bar represents standard deviation; two-tailed Mann-Whitney Rank Test used to compare groups; *, p<0.05; ***, p<0.0005.
**Supplemental Figure 5. 4** Characterization of biophysical profiles and immune responses induced by newly designed DLnano GT8-vaccines with alternative scaffolds.

**a.** SEC trace of recombinantly produced designed 3BVE-GT8.

**b.** SEC trace of designed PfV_GT8 immunogen shows partial assembly into the 180-mer form.

**c.** Binding of *in vitro* produced protein GT8-monomer, 3BVE_GT8, eOD-GT8-60mer and PfV_GT8 to VRC01 by ELISA.

**d** and **e.** Effector memory T-cell responses to GT8 domain in BALB/c mice immunized with two doses of 25μg DLmono_GT8, DLnano_3BVE_GT8, DLnano_LS_GT8 and DLnano_PfV_GT8 by IFNγ ELIsots (d) and ICS for CD8+ T-cells (e). n=5 per group; each dot represents an animal; error bar represents standard deviation; two-tailed Mann-
Whitney Rank Test used to compare groups; p-values were adjusted for multiple comparison where appropriate; *, \( p<0.05 \).
Supplemental Figure 5. Characterization of biophysical profiles and immune responses induced by newly designed DLnano influenza hemagglutinin-based vaccines.

a. SEC trace of recombinantly produced designed LS_HA_NC99 nanoparticles.

b. Binding of sera from BALB/c mice immunized with 1µg DLnano_LS_HA_NC99 or DLmono_HA_NC99 at 56 d.p.i (post-dose #3) to heterologous recombinant H1 (SI06) hemagglutinin protein.

c and d. CD8+ effector memory T-cell responses to NC99 HA domain in BALB/c mice immunized with two 10µg doses of DLmono_HA_NC99 or DLnano_LS_HA_NC99 in terms of IFNγ (c) and CD107a (d) expression.

e. SEC trace of lectin purified recombinantly produced LS_HA_CA09.
f. Comparison of humoral responses induced by two 10ug doses of DLnano_LS_HA_NC99, which homogeneously assembled and by DNA-encoded LS_HA_CA09, which did not homogeneously assemble in vitro. n=5 per group; each dot represents an animal; error bar represents standard deviation; two-tailed Mann-Whitney Rank Test used to compare groups; p-values were adjusted for multiple comparison where appropriate; *, p<0.05.
Supplemental Figure 5.6 Improved protection from lethal H1/CA09 challenge in mice with DLnano_3BVE_HA_CA09 vaccination.

a. SEC trace for lectin-purified recombinantly produced 3BVE_HA_CA09 nanoparticles.

b. nsEM image of SEC-purified 3BVE_HA_CA09 nanoparticles.

c. Weight changes in mice immunized with pVAX, DLmono_HA_CA09 or DLnano_3BVE_HA_CA09 over seven-day period following 10LD_{50} H1/A/California/07/09 challenge as in Figure. 5.6e.

d. H&E stain for lung histo-pathology in remaining 12 mice seven days post viral challenge or at the time of euthanasia as in Figure. 5.6e. 1ug DNA dose used in vivo for c and d. n=5 per group; each line represents an animal.
CHAPTER 6 Designing synthetic DNA launched nanoparticle vaccines against tumor associated antigens for *in vivo* tumor clearance

6.1 Abstract

Cytolytic T cells (CTL) play a pivotal role in defense against intracellular pathogens and surveillance against tumors. Induction of CTL response by vaccination may be challenging as it requires direct transduction of target cells, or special adjuvants to promote cross presentation. Here, we observed that combined DNA electroporation (EP) vaccinations of two different nanoparticle vaccines, but not vaccinations with corresponding recombinant protein nanoparticles, induced CTL responses. DNA/EP vaccinations uniquely induced tissue apoptosis and influx of antigen presenting cells, including pro-inflammatory M1 macrophages to scavenge antigens. Induced tissue apoptosis was observed to be transient and local to immunization site. Systemic depletion of macrophages prior to DNA vaccinations significantly attenuated CTL response. Finally, DNA but not recombinant protein vaccinations of nanoparticles scaffolding Gp100 and Trp2 epitopes induced CTL responses to these epitopes and suppressed tumor growth in mouse melanoma tumor models. Transient physical stress induced through vaccination can therefore be harnessed to create pro-inflammatory environments that promote infiltration, antigen uptake and cross-presentation by antigen-presenting cells for unique and robust induction of CTL against cancer.

6.2 Introduction

Cytolytic T cells (CTL) is an extremely important branch of adaptive immune system, which can selectively kill target cells through release of cytokines, granzyme and perforin, as well as mediating target cell apoptosis through Fas and Fas-Ligand interaction(Halle et al., 2017). T cells engineered with artificial receptors for anti-tumor activity is an exciting area of research but may come with limitations in throughput and costs. Therefore, induction of broad endogenous antigen-specific CTL through vaccination may, therefore, be an important approach to treat cancer(Farhood et al., 2019).
However, induction of CTL through vaccination can be challenging as it requires antigen presenting cells (APCs) to present HLA-restricted epitopes to the MHC I pathway and provide costimulatory signal to prime CD8+ T cells (Chen and Flies, 2013). Using viral vectors modified to encode target antigens is an approach to drive CTL responses, potentially through direct transduction of target cells (Tan et al., 2013). Additionally, special adjuvants can be used to facilitate cross-presentation of target antigens, which to varying degrees promote phago-lysosomal escape and retro-translocation of target antigens into the cytosol (Gros and Amigorena, 2019). Alternatively, dendritic cells (DCs) can be loaded \textit{ex vivo} with saturating concentrations of peptides and adoptively transferred back into the host to prime CD8+ T cells (Saxena and Bhardwaj, 2018). Each approach has certain drawback: relatively few adjuvants have been demonstrated to help prime CD8+ T-cell responses in clinic (Cheng et al., 2012), viral-vectored vaccine can be limited by pre-existing immunity against the viral vector (Fausther-Bovendo and Kobinger, 2014), and manufacturing issues with large-scale production of DC vaccines.

DNA vaccination has also been observed to elicit CD8+ T-cell responses both in preclinical animal models and in clinical trials (Trimble et al., 2015), plausibly through a combination of direct transfection of target cells (Akbari et al., 1999) along with cross presentation of DNA-encoded antigens (Fu et al., 1997). Previously we have observed DNA vaccination of antigens scaffolded by \textit{in vivo} assembled nanoparticles induced significantly improved CD8+ T-cell responses compared to DNA-encoding non-scaffolded antigens. In this work, we compared the ability of DNA and protein vaccinations of identical nanoparticle antigens to induce CTL responses in mice. We discovered that intramuscular DNA vaccination, but not RIBI adjuvanted protein vaccination, was capable of inducing robust CTL responses against influenza and HIV antigens. Mechanistic studies revealed that in comparison to protein vaccination, DNA launched nanoparticle vaccines uniquely
induced apoptosis of transfected muscle tissues, which was followed by infiltration of antigen presenting cells (APCs), including pro-inflammatory M1 macrophages, to scavenge antigens. Systematic depletion of macrophages significantly attenuated induced CTL response. Finally, DNA launched nanoparticle vaccines induced potent CTL responses against tumor-associated antigens Trp2 and Gp100 and facilitated tumor rejection in 80% of mice in the prophylactic B16-F10 melanoma model. This work therefore provides a demonstration that induction of transient physical stress through DNA-launched nanoparticle vaccination may be a safe, easy and robust way to facilitate antigen cross presentation and CTL priming to target cancer.

6.3 Results

6.3.1 DNA-launched but not recombinant protein nanoparticle vaccines induced CD8+ T-cell responses to HIV and influenza antigens

First, we compared adaptive immune responses induced by DNA vaccination and protein vaccination adjuvanted by Sigma Adjuvant System (or RIBI). We utilized two model antigens: a priming HIV antigen eOD-GT8-60mer and an influenza hemagglutinin (H1 A/NewCaledonia/20/1999) 60mer antigen scaffolded by lumazine synthase (Jardine et al., 2016). **DNA-Launched nanoparticle Lumazine Synthase** decorated with an anti-HIV-1 immunogen eOD-GT8 (**DLnano_LS_GT8**, Supplemental Fig. 6.1a) and protein eOD-GT8-60mer induced similar antibody titers in BALB/c mice after two immunizations, even though antibody titer induced by DLnano_LS_GT8 was slightly higher after a single immunization (Fig. 6.1a). Epitope mapping conducted in prior studies identified that CD4+ T-cell responses elicited by LS-scaffolded DLnano-vaccines were predominantly specific for the LS domain. In this study, CD4+ T-cell responses to the LS domain after two vaccinations, as measured by intracellular cytokine staining (ICS) of splenocytes following peptide stimulations, were similar between DLnano_LS_GT8 and protein eOD-GT8-
60mer (Fig. 6.1b). Strikingly, we observed robust induction of CD8+ T-cell responses to the GT8 domain in mice immunized with DLnano_LS_GT8 but not in those vaccinated with protein eOD-GT8-60mer as determined by ICS (Fig. 6.1c and 6.1d) and IFNγ ELISpot assay (Fig. 6.1e and 6.1f).

We determined if the observation could be replicated when an alternative nanoparticle-scaffolded influenza antigen was evaluated. Indeed, two vaccinations of DLnano_LS_HA(NC99) and protein HA(NC99)_60mer induced similar binding antibody titers to recombinant NC99 hemagglutinin (Fig. 6.1g) as well as hemagglutination inhibition titers against the autologous A/NewCaledonia/20/1999 virus (Fig. 6.1h). CD4+ T-cell responses induced by DLnano_LS_HA(NC99) and protein HA(NC99)_60mer were also similar (Supplemental Fig. 6.1b). However, similar to what was observed for the HIV antigen, only DLnano_LS_HA(NC99) but not protein HA(NC99)_60mer induced CD8+ T-cell response to the HA domain as measured by ICS (Supplemental Fig. 6.1c and Fig. 6.1i) and by IFNγ ELISpot (Supplemental Fig. 6.1d and Fig. 6.1j). In addition, we determined that when protein antigens were administered in combination with EP, they induced slightly improved humoral responses (Supplemental Figs. 6.1e and 6.1f) and yet failed to induce CD8+ T-cell responses (Supplemental Fig. 6.1g). Thus, we have demonstrated that induction of CD8+ T-cell immunity, in this context, required expression of antigens in host cells from DNA-cassettes.

6.3.2 Induction of tissue apoptosis and APC infiltration by DNA-launched nanoparticle vaccines were important for CD8+ T-cell priming

We hypothesized distinct modality of antigen uptake and presentation for DNA-launched nanoparticle vaccine as compared to protein nanoparticle vaccine may attribute to unique induction of CTL responses by DLnano-vaccines. Previous studies observed that co-administration of DNA-vaccines with DNA-cassettes encoding pro-apoptotic genes
led to significantly improved induced CTL responses (Chattergoon et al., 2000). We, therefore, hypothesized that DNA/EP vaccinations could create a pro-inflammatory environment at the site of transfection through the induction of tissue apoptosis, thereby promoting infiltration of APCs and antigen uptake.

We first assessed the extent of muscle tissue damage upon vaccine administration with an immunofluorescence assay to stain for cleaved caspase 3, which would be present in cellular cytosol following apoptotic signaling events (Porter and Janicke, 1999). In C57BL/6 mice, four days post injection (d.p.i), hypercellularity and expression of cleaved caspase 3 were only observed in the muscles of mice immunized with DLnano_LS_GT8 combined with EP but not in those vaccinated with protein eOD-GT8-60mer co-formulated in RIBI or in naïve mice (Fig. 6.2a). Alternatively, we used an additional Terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) assay to assess for double stranded DNA breaks, a surrogate marker for apoptosis (Kyrylkova et al., 2012), in the vaccinated muscle tissues (Fig. 6.2b). To ensure robustness of staining, we generated a positive control specimen by incubating muscle specimen with DNAse I, which generated double-stranded DNA breaks throughout the specimen, resulting in brown staining of all nuclei in the specimen. The negative control sample was generated by staining of the specimen in the absence of TdT, resulting in green appearance of all nuclei because of methyl green counterstain (Fig. 6.2b). Four d.p.i, brown nuclei suggestive of cellular apoptosis were only observed in muscle tissues treated with DLnano_LS_GT8 and electroporation but not in muscle sections from naïve mice or those treated with protein eOD-GT8-60mer formulated in RIBI. At a higher level of magnification, nuclei fragmentation was also observed in the DNA group (Fig. 6.2b).

We performed a time course experiment and observed that DLnano_LS_GT8 induced tissue apoptosis was transient, peaked at 6 d.p.i and was fully resolved by 14
d.p.i (Supplemental Fig. 6.2a). Additionally, tissue apoptosis should be limited locally to the injection sites. Indeed, we did not observe indicators of tissue damage such as significant elevations of serum lactose dehydrogenase (LDH) and creatine kinase (CK) levels in mice immunized with DLnano_LS_GT8 or protein eOD-GT8-60mer relative to untreated control mice post injections (Supplemental Fig. 6.2b and 6.2c).

We next examined the consequences of induced tissue apoptosis in terms of tissue APC infiltration. Indeed, seven d.p.i, we observed increased influx of CD11b+F4/80+ macrophages into muscles of mice treated with DLnano_LS_GT8 and EP compared to protein eOD-GT8-60mer formulated in RIBI (Fig. 6.2c and 6.2d). Furthermore, infiltrating macrophages were observed to be predominantly pro-inflammatory M1 macrophages rather than anti-inflammatory M2 macrophages (Supplemental Fig. 6.2d and 6.2e). Increased influx of CD11c+MHC Class II+ dendritic cells (DCs) was also observed for mice treated with DLnano_LS_GT8 compared to those treated with protein eOD-GT8-60mer; however, infiltrating DCs are significantly less abundant than macrophages by approximately 20-fold (Fig. 6.2e). Finally, staining of antigen-presenting cells with VRC01 (broadly neutralizing antibody with high affinity for GT8)-FITC demonstrated that infiltrating macrophages induced by DLnano_LS_GT8 but not those induced by protein eOD-GT8-60mer had taken up the GT8 antigen (Fig. 6.2f and Supplemental Fig. 6.2f).

To determine the functional roles played by macrophages in priming of CD8+ T-cell responses by DNA-launched nanoparticle vaccines, we systemically depleted the macrophage populations in C57BL/6 mice prior to DNA vaccinations. Depletion was carried out through intravenous (IV) injection of clodrosome, which specifically depleted phagocytic macrophages (Weisser et al., 2012). Single IV infusion of clodrosome specifically depleted CD11b+F4/80+ macrophages but not CD11c+MHC Class II+ DCs in
the spleens of animals one d.p.i (Supplemental Fig. 6.2g-6.2i). Depletion of infiltrating macrophages in the muscles post vaccination required increased doses of clodrosome on -3, 0, and 3 d.p.i (with respect to DNA vaccination), which significantly reduced macrophage infiltration into the muscles by approximately four-fold (Fig. 6.2g). Importantly, this depletion scheme significantly attenuated induced CD8+ T-cell responses to the GT8 domain following one vaccination of DLnano_LS_GT8 14 d.p.i by approximately three-fold (Fig. 6.2h), highlighting the importance of infiltrating macrophage populations in priming of CD8+ T-cells in DNA vaccination with in vivo launched nanoparticle vaccines.

6.3.3 DNA-launched nanoparticle vaccines scaffolding Trp2\textsubscript{188} and Gp100\textsubscript{25} peptides mediated protection against melanoma challenge in mice

To demonstrate the functional relevance of CD8+ T-cell priming by DNA-launched nanoparticle vaccines in the treatment of cancer, we designed novel nanoparticle vaccines displaying CD8+ T-cell epitopes. The self-assembling nanoparticles were engineered using a structure-guided process. In our preliminary experiments, Lumazine Synthase on its own does not express well in mammalian cell lines and we hypothesized that it could not be used on its own to scaffold anti-tumor peptide antigens. We engineered a new version of DLnano_LS_GT8 capable of displaying peptide antigens. In this construction, the heavily glycosylated GT8 domain facilitates solubilization and secretion of designed nanoparticles and could potentially be replaced by other heavily glycosylated domains. To ensure epitope accessibility and homogenous nanoparticle assembly, N-linked glycans in proximity to the C-terminus of GT8 were removed by mutations in the PNGS sequence; additionally, a 15 amino acid linker was also incorporated to the C-terminus of GT8 upstream of the scaffolded anti-tumor peptide(s). We designed nanoparticles presenting 60 copies of Trp2\textsubscript{188} peptide (DLnano_LS_Trp2\textsubscript{188}) and Gp100\textsubscript{25} peptide.
Homogenous *in vitro* assemblies of DLnano_LS_Trp2\textsubscript{188} and DLnano_LS_Gp100\textsubscript{25} were observed by Size Exclusion Chromatography, SEC, (Fig. 6.3a and Supplemental Fig. 6.3a) and by negative stain Electron Microscopy, nsEM (Fig. 6.3b and Supplemental Fig. 6.3b). Groups of C57BL/6 mice were immunized with designed DNA-launched nanoparticle Trp2\textsubscript{188} or Gp100\textsubscript{25} vaccines individually, and were observed to induce significantly improved CD8\textsuperscript{+} T-cell responses to the Trp2\textsubscript{188} and Gp100\textsubscript{25} peptides compared to DNA vaccines encoding monomeric GT8-scaffolded Trp2\textsubscript{188} and Gp100\textsubscript{25} respectively (DLmono_Trp2\textsubscript{188} and DLmono_Gp100\textsubscript{25}, Fig. 6.3c and 6.3d). To determine if we could deliver both epitopes at once, we performed a follow-up experiment where we co-administered DLnano_LS_Trp2\textsubscript{188} and DLnano_LS_Gp100\textsubscript{25} in separate sites in a single animal. The co-administration resulted in improved elicitation of CD8\textsuperscript{+} T-cell responses to both Trp2 and Gp100 peptides as compared to DLmono_Trp2\textsubscript{188} and DLmono_Gp100\textsubscript{25} (Supplemental Fig. 6.3c).

We next determined whether B16-F10 melanoma bearing mice can mount endogenous CD8\textsuperscript{+} T-cell responses to Trp2 (SVYDFFVWL) and Gp100 (EGPRNQDWL) epitopes, and whether protein versus DNA vaccinations of nanoparticles scaffolding 60 copies of Trp2 and Gp100 peptides could induce CD8\textsuperscript{+} T-cell responses to confer protection against tumor growth. Anti-PD1 treatments were administered to three groups of mice (Groups 2-4) in the study three days post tumor inoculation and weekly thereafter (Fig. 6.3e) (Moynihan et al., 2016). We observed that 14 days post subcutaneous inoculation of $10^5$ B16-F10 cells and following two combined vaccine/anti-PD1 treatments, CD8\textsuperscript{+} T-cell responses to Trp2 was only observed in mice treated with DNA vaccinations but not those receiving anti-PD1 treatment alone or anti-PD1 with protein vaccinations (Fig. 6.3f). Induced CD8\textsuperscript{+} T-cells specific for Trp2 were observed to exhibit effector phenotypes, IFN\textgreek{y}+CD107a\textsuperscript{+} (Fig. 6.3g), and were also poly-functional as determined by
co-expression of IFNγ, TNFα and IL2 (Fig. 6.3h). Similar observations were also made in terms of CD8+ T-cell responses to Gp100, even though lower frequency of epitope-specific poly-functional CD8+ T-cell was observed upon DNA vaccination (Supplemental Fig. 6.3d-6.3f). In the therapeutic model (vaccine, anti-PD1 treatments administered three days post tumor inoculation), DNA but not protein vaccination of Trp2188 and Gp10025-60mer was observed to suppress B16-F10 tumor growth in mice (Fig. 6.3i). Additionally, DNA but not protein vaccination was observed to significantly prolong median survival of mice in the therapeutic model by 11 days (p-value=0.0177 by Log-Rank test) (Fig. 6.3j). The effect was even more pronounced when mice were vaccinated prior to tumor inoculation. Two vaccinations of DLnano_LS_Trp2188 and DLnano_LS_Gp10025 administered prior to 10^5 B16-F10-Luc cell challenge completely prevented tumor growth in 80% (4/5) mice; whereas tumor growth was observed in all pVAX control mice or mice treated with protein Trp2188 and Gp10025-60mer (Fig. 6.3k). Additionally, while all mice in the pVAX and protein groups died, 80% of mice in the DNA group had tumor-free survival until the end of the study (Fig. 6.3l), highlighting therapeutic utility of CTL priming by DLnano-vaccines.

6.4 Discussion

CTL can play an extremely important role in surveillance against intracellular pathogens and tumors. In the cancer space, presence of tumor infiltrating CD8+ T-cell is correlated with improved prognosis in cancer patients (Gooden et al., 2011; Shimizu et al., 2019). Induction of CD8+ T-cell responses also corresponds to improved tumor clearance and survival in various preclinical animal studies (Beyranvand Nejad et al., 2019). Such observations have led to the use of T-cell based therapies, such as DC vaccines or in vitro expansion and adoptive transfer of tumor infiltrating lymphocytes (TIL), in cancer patients, achieving varying degrees of success (Rohaan et al., 2018; Wu et al., 2012).
Induction of CTL responses by vaccination can be challenging and not readily achieved by vaccination with protein or inactivated virus. For example, the most advanced malaria vaccines under development, RTS,S, which contains repeat and T-cell epitopes of CircumSporozoite Protein fused to hepatitis B surface antigen to form protein particles, could induce antibody but not CD8+ T-cell response to the vaccine antigens (Moorthy and Ballou, 2009).

DNA vaccines have been shown to elicit CD8+ T-cell responses both in preclinical animal models and in clinical trials (Duperret et al., 2018b; Trimble et al., 2015). Prior mechanistic studies highlighted plausible contributions by direct transfection of DCs and macrophages with DNA cassettes (Akbari et al., 1999). Additionally, cross presentation of DNA-cassettes encoded antigens was also believed to be important for priming CTL through more indirect evidence (Fu et al., 1997). In this work, we observed DNA/EP mediated tissue apoptosis followed by macrophage infiltration and antigen uptake, which may subsequently transfer sequestered peptides to cDCs for priming of CD8+ T cells (Huang et al., 2019). Induced tissue apoptosis was observed to be transient and local, as we would expect given strong safety profile of DNA vaccines in clinic (Trimble et al., 2015).

We also demonstrated the importance of CD8+ T-cell priming by DNA-launched nanoparticle vaccines in a melanoma challenge model. We designed a novel and generalizable nanoparticle platform for displaying CD8+ T cell epitopes to various tumor antigens. As a proof-of-principle, we demonstrated nanoparticle vaccines scaffolding 60 copies of CD8+ epitopes from Trp2 and Gp100 could be engineered. Future studies should be explored to examine alternative nanoparticle sizes and shapes. Here, the anti-melanoma LS-nanoparticles scaffolding Trp2 and Gp100 demonstrated significantly improved CTL responses could be elicited to both targets. DNA but not protein vaccinations of Trp2\textsubscript{188} and Gp100\textsubscript{25} 60mer elicited CTL responses in mice, suppressed
tumor growth and prolonged overall survival in the therapeutic tumor challenge model, and conferred 80% protection when vaccines were prophylactically administered. The work provided a demonstration that robust CTL responses can be generated with great simplicity by harnessing physical injury to create a pro-inflammatory environment that favored APC infiltration, antigen uptake and cross-presentation. Induced CTL responses can be directed to whole antigens, such as to GT8 or to influenza (Fig. 6.1), or to selected peptides (Fig. 6.3), creating possibility of developing whole antigen or neoantigen peptide-oriented DNA-launched nanoparticle vaccines against various cancer targets and creating a viable and attractive strategy in the exciting era of cancer immunotherapy.

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Figure Legends

Figure 6. 1 Comparison of immune responses induced by DLnano_LS_GT8 and protein eOD-GT8-60mer, as well as DLnano_LS_HA(NC99) versus HA(NC99)-60mer in BALB/c mice.

a. Endpoint titers to GT8-monomer induced by DLnano_LS_GT8 in comparison with RIBI adjuvanted protein eOD-GT8-60mer.

b. CD4+ IFNγ responses to the LS domain induced by DLnano_LS_GT8, protein eOD-GT8-60mer or in naïve mice.
c and d. Flow cytometry plots and combined statistics for CD8+ IFNγ responses to the GT8 domain induced by DLnano_LS_GT8, protein eOD-GT8-60mer or in naïve mice.

e and f. IFNγ ELISpot images and combined statistics for overall T-cell responses to the GT8 domain induced by DLnano_LS_GT8, protein eOD-GT8-60mer or in naïve mice.

g. Endpoint titers to NC99 hemagglutinin induced by DLnano_LS_HA(NC99) in comparison with RIBI adjuvanted protein HA(NC99)-60mer.

h. HAI titers against autologous H1 A/NewCaledonia/20/1999 induced by DLnano_LS_HA(NC99), protein HA(NC99)-60mer or in naïve mice.

i. ICS determination of CD8+ IFNγ responses to the HA domain induced by DLnano_LS_HA(NC99), protein HA(NC99)-60mer or in naïve mice.

j. IFNγ ELISpot assays for overall T-cell responses to the HA domain induced by DLnano_LS_HA(NC99), protein HA(NC99)-60mer or in naïve mice. Each group includes five mice; each dot represents an animal; error bar represents standard deviation; arrow below the plot represents an immunization; two-tailed Mann-Whitney Rank Test used to compare groups; p-values were adjusted for multiple comparison where appropriate; *, p-value<0.05.
Figure 6.2 Determination of the role of tissue apoptosis and APC infiltration upon DNA vaccination in C57BL/6 mice.
a. Immunofluorescence staining for cleaved caspase 3 (red) or nuclei (blue) for muscle sections from naïve mice or those treated with protein eOD_GT8-60mer or DLnano_LS_GT8.

b. TUNEL assay to determine presence of double-stranded DNA breaks (brown) or intact DNA (green) for muscle sections from naïve mice or those treated with protein eOD_GT8-60mer or DLnano_LS_GT8.

c and d. Flow cytometry plots and combined statistics for frequency of muscle infiltrating CD11b+F4/80+ macrophages in naïve mice, or those treated with protein eOD-GT8-60mer or DLnano_LS_GT8 seven d.p.i.

e. Flow determination of muscle infiltrating CD11c+MHC Class II+ DCs in naïve mice, or those treated with protein eOD-GT8-60mer or DLnano_LS_GT8 seven d.p.i.

f. Flow determination for GT8-uptake by VRC01-FITC staining for muscle macrophages in naïve mice, or mice treated with protein eOD-GT8-60mer or DLnano_LS_GT8 seven d.p.i.

g. Comparison for changes in frequencies of muscle infiltrating macrophages four d.p.i in mice treated with DLnano_LS_GT8 four days post DNA vaccination; the mice also received three doses of IV clodrosome or control encapsosome on D-3, 0 and 3.

h. CD8+ T-cell responses induced by DLnano_LS_GT8 or protein eOD-GT8-60mer in mice that did or did not receive systematic macrophage depletion with clodrosome. Each group includes four mice in c-f and five mice in g-i; each dot represents an animal; error bar represents standard deviation; two-tailed Mann-Whitney Rank Test used to compare groups; p-values were adjusted for multiple comparison where appropriate; *, p-value<0.05.
Figure 6.3 Characterization of functional importance of CD8+ T-cell priming by DNA versus protein vaccination in melanoma challenge model in C57BL/6 mice.
a. SEC trace for designed LS_Trp2_{188}-60mer.

b. nsEM image of SEC purified LS_Trp2_{188}-60mer nanoparticles.

c. Comparison of CD8+ IFN\(\gamma\) T-cell responses to Trp2_{188} peptide in naïve mice or mice immunized with DLmono_Trp2_{188} or DLnano_LS_Trp2_{188}.

d. Comparison of CD8+ IFN\(\gamma\) T-cell responses to Gp100_{25} peptide in naïve mice or mice immunized with DLmono_Gp100_{25} or DLnano_LS_Gp100_{25}.

e. Treatment and vaccination schemes used to study CD8+ T-cell responses to both Trp2_{188} and Gp100_{25} peptides in naïve mice, B16F10-tumor bearing mice that received anti-PD1 treatment alone, or anti-PD1 treatment in combination with protein or DNA vaccination of LS-GT8 scaffolded 60mer nanoparticles presenting Trp2_{188} and Gp100_{25}.

f, g and h. Induced IFN\(\gamma\)+, IFN\(\gamma\)+CD107a+, or IFN\(\gamma\)+TNF\(\alpha\)+IL-2+ CD8+ T-cell responses to Trp2_{188} in naïve tumor-free mice or B16-F10 bearing mice that received anti-PD1 treatment alone, or anti-PD1 treatment in combination with protein Trp2_{188} and Gp100_{25}-60mer or DLnano_LS_Trp2_{188} and DLnano_LS_Gp100_{25}.

i and j, tumor growth (i) and overall survival (j) in mice challenged with \(10^5\) B16-F10 cells and then treated with received anti-PD1 treatment alone, or anti-PD1 treatment in combination with protein Trp2_{188} and Gp100_{25}-60mer or DLnano_LS_Trp2_{188} and DLnano_LS_Gp100_{25} three days post tumor challenge.

k. IVIS to determine \textit{in vivo} tumor growth in the minimal disease model where mice first received two vaccinations of pVAX vector, combination of protein Trp2_{188} and Gp100_{25}-60mer, or combination of DLnano_LS_Trp2_{188} and DLnano_LS_Gp100_{25} and were then challenged with \(10^5\) B16-F10-Luc cells seven days post second immunization.
I. Survival curves for mice shown in k. Each group includes five mice; each dot represents a mouse; error bar represents standard deviation; two-tailed Mann-Whitney Rank Test used to compare groups; p-values were adjusted for multiple comparison where appropriate; *, p-value<0.05.
Supplemental Figure 6. 1 Comparison of immune responses induced by DLnano_LS_HA(NC99) versus HA(NC99)-60mer in BALB/c mice.

a. Layout of all plasmids used in this study.

b. CD4+ IFNγ responses to the LS domain induced by DLnano_LS_HA(NC99), HA(NC99)-60mer or in naïve mice.

c. Flow cytometry plots for CD8+ IFNγ responses to the HA domain induced by DLnano_LS_HA(NC99), HA(NC99)-60mer or in naïve mice.
d. IFNγ ELIspot images for overall T-cell responses to the HA domain induced by DLnano_LS_HA(NC99), HA(NC99)-60mer or in naïve mice.

e. Humoral responses in mice immunized with protein eOD-GT8-60mer with or without electroporation.

f. Humoral responses in mice immunized with protein HA(NC99)-60mer with or without electroporation.

g. CD8+ T-cell responses in mice immunized with protein HA(NC99)-60mer or eOD-GT8-60mer with electroporation. Each group includes five mice; each dot represents an animal; error bar represents standard deviation; arrow below the plot represents an immunization; two-tailed Mann-Whitney Rank Test used to compare groups; p-values were adjusted for multiple comparison where appropriate; *, p-value<0.05.
Supplemental Figure 6.2 Characterization of muscle infiltrating APC induced by DNA vaccination.
a. Time course immunofluorescence images showing cleaved caspase 3 expression (red) in transfected muscle tissue over time; images from two mice were shown at each timepoint; nuclei staining with DAPI is shown in blue.

b and c. Serum LDH and CK enzymatic activity in mice immunized with DLnano_LS_GT8, RIBI adjuvanted protein eOD-GT8-60mer or untreated mice following injection.

d. Flow plot for determination of macrophage polarization (M1 versus M2) by staining CD11b+F4/80+ populations with CD206 and CD11c.

e. Frequencies of M1 versus M2 macrophages in the muscles as determined by flow four days post DLnano_LS_GT8 vaccination.

f. Flow plot by VRC01-FITC staining for determination of GT8-uptake in muscle macrophages from naïve mice, or mice treated with protein eOD-GT8-60mer or DLnano_LS_GT8 seven d.p.i.

g. Flow plots for splenic CD11c+MHC Class II+ DC populations upon IV treatment of clodrosome or control encapsosome.

h. Flow plots for splenic CD11b+F4/80+ macrophage populations upon IV treatment of clodrosome or control encapsosome.

i. Comparison for changes in frequencies of splenic macrophages and DCs in Naïve mice upon IV treatment of clodrosome or control encapsosome. Each group includes five mice in b-i; each dot represents an animal; error bar represents standard deviation; arrow below the plot represents an immunization; two-tailed Mann-Whitney Rank Test used to compare groups; p-values were adjusted for multiple comparison where appropriate; *, p-value<0.05.
Supplemental Figure 6. 3 Characterization of CD8+ T-cell responses induced by DLnano_LS_Trp2188 and DLnano_LS_Gp10025.

a. SEC trace for designed LS_Gp10025-60mer.

b. nsEM image of SEC purified LS_Gp10025-60mer nanoparticles.

c. Comparison of CD8+ IFNγ T-cell responses to both Trp2188 and Gp10025 peptide in naïve mice or mice immunized with both DLmono_Gp10025 and DLnano_LS_Trp2188 or DLnano_LS_Gp10025 and DLnano_LS_Trp2188.

d, e and f. Induced IFNγ+, IFNγ+CD107a+, or IFNγ+TNFα+IL-2+ CD8+ T-cell responses to Gp10025 in naïve tumor-free mice or B16-F10 bearing mice that received anti-PD1 treatment alone, or anti-PD1 treatment in combination with protein Trp2188 and Gp10025-60mer or DLnano_LS_Trp2188 and DLnano_LS_Gp10025. Each group includes five mice; each dot represents an animal; error bar represents standard deviation; arrow below the
plot represents an immunization; two-tailed Mann-Whitney Rank Test used to compare groups; *, p-value<0.05.
CHAPTER 7 Conclusion and Future Directions

7.1 Synthetic DNA/EP enables *in vivo* delivery of complex anti-HIV-1 biologic eCD4-Ig, simultaneous expression of multiple bNAb s, as well as their *in vivo* post-translational modifications through co-delivery of an DNA-encoded enzyme.

We have demonstrated that a single treatment of DNA-encoded eCD4-Ig in immunodeficient mice (transient immune-modulation through the passive transfer of antibody that blocks CD40L signaling) would enable robust systemic expression of the construct, with a peak level close to 100ug/mL and persistent expression for more than half a year. Furthermore, simultaneous delivery of plasmid eCD4-Ig with plasmid Golgi-resident enzyme IgE-TPST2 would enable potent *in vivo* sulfation of eCD4-Ig to significantly enhance its potency in terms of neutralization against the global panel of pseudoviruses. Through engineering of the enzyme to traffic to trans-Golgi network (TGN) via incorporation of a stronger secretion signal (IgE leader sequence), the enzyme demonstrates superior colocalization with another TGN marker and achieves high catalytic efficiency, with only 1/1000 plasmid copies of IgE-TPST2 required for full *in vivo* sulfation of eCD4-Ig (Xu et al., 2018). This work demonstrates that combined DNA/EP enables robust and prolonged *in vivo* expression of complex biologic and enzyme, highlighting utility of such approach for *in vivo* delivery of additional biologics against diseases of interests.

We further explored the use of DNA/EP for simultaneous *in vivo* expression of four distinct HIV-1 bNAb s targeting four different epitopes on HIV Env (PGT121, PGDM1400, 3BNC117, and PGT151). In mice that are transiently immune depleted with anti-CD4/CD8 antibodies, single treatment of four distinct HIV DMAb plasmids enables potent coverage of the entire global panel of HIV-1 pseudoviruses with significantly improved IC$_{50}$ neutralization titers in animals' sera as compared to mice treated with an HIV DMAb.
monotherapy. Two of the constructs PGT121 and PGDM1400 were further evaluated in NHPs. A single round of DMAb treatment enables potent level of expression for both DMAbs (higher than 10μg/mL) and confers broadly neutralizing as well as ADCC activity to the sera of the NHPs at the peak timepoint. Unfortunately, as the study involved delivery of human monoclonal antibodies into NHPs, anti-antibody responses were observed seven days after treatment, which ultimately led to decrease in sera DMAb levels beyond 28 days of treatment (Wise et al., 2019). This study demonstrates the feasibility of co-delivery of multiple biologics in a single animal to improve breadth and potency of coverage, and additionally, that DNA/EP may be a potent vehicle for in vivo delivery for both small and large mammals.

A major limitation for this approach is the decline in serum expression level over time even in immune incompetent mice. In the case of eCD4-Ig, peak expression usually occurred 14 days post injection, and expression level tended to decline (though still remained detectable) by approximately 40-fold over time. Waning systemic expression of the biologic may reduce potency of protection conferred by the biologics and could potentially be related to DNA methylation in the CMV promoter region of the plasmid, which subsequently contributed to silencing of the transgene expression (Mehta et al., 2009). Additionally, in fully immunocompetent animals, rate of decline in systemic DMAb expression is even higher as animals develop ADA responses. Several approaches may be used to address these limitations. For example, a combinatorial use of both early (CMV) and late (chicken β-actin, CBA) promoters may be used for enhanced longer term DMAb pharmacokinetic profile (Gray et al., 2011). Additionally, human germline modification of the encoded monoclonal antibodies, as well as analysis and knockout of potential CD4+ helper epitopes in the protein sequence of the antibodies are both viable strategies to reduce immunogenicity of encoded DMAb by decreasing the antigenicity of the encoded biologics (Pratt, 2018). Overall, additional DMAb and DNA-encoded immunoadhesin
technologies may be needed for their successful translation into the clinical spaces. However, these two studies demonstrate the utility of DNA/EP technology to promote *in vivo* folding and assembly of complex protein domains, thus paving the way for us to investigate *in vivo* DNA-delivery of next-generation nanoparticle vaccine.

7.2 Synthetic DNA/EP enables *de novo* assembly of macromolecular nanoparticle protein complexes to induce significantly enhanced functional adaptive immune responses

We have harnessed insights obtained through our DMAb studies to engineer significantly more complex nanoparticle vaccines for *in vivo* expression. Using three complementary techniques (transmission electron microscopy and gold-conjugated antibody based immune-labelling, pseudo-Native PAGE, and lectin binding immunohistochemistry), we determined that DNA/EP could support direct *de novo* assembly of HIV-1 priming antigen eOD-GT8-60mer. As compared to DNA-encoded monomeric antigen, DNA-launched nanoparticle vaccines can induce extremely rapid sero-conversion seven days post the initial immunization in both mice and guinea pigs, and higher set-point antibody titers. DNA-launched nano-vaccines are extremely dose-sparing, supporting robust induction of humoral responses at 1/50 to 1/10 of the conventional DNA vaccine doses. At an extremely low dose (1µg of DNA plasmid), DNA-launched nanoparticle scaffolding influenza hemagglutinin (HA) antigen, but not DNA-encoded monomeric HA, offered complete protection to vaccinated mice challenged with lethal doses of pandemic H1/California/07/09 virus (Xu et al., 2020).

Additionally, DNA-launched nano-vaccines also induced significantly enhanced antigen-specific CTL responses, in terms of expression of intracellular Th1 cytokines (IFNγ, TNFα and IL-2) in splenocytes of the animals following antigen-specific peptide stimulation. This finding was harnessed for the design of novel nano-vaccine scaffolding tumor associated antigens (melanoma associated antigen Gp100 and Trp2). Treatments
of B16F10-bearing mice with a combination of anti-PD1 and Gp100/Trp2 DNA-nanoparticle vaccines significantly prolonged median survival of animals as compared to those treated with anti-PD1 alone. Additionally, prophylactic vaccination of mice with DNA-launched nano-vaccines followed by B16F10 challenge resulted in complete response in 80% of the vaccinated mice, highlighting the functional importance of CTL responses induced by these DNA-launched nanoparticle vaccines.

I performed a head-to-head comparison between protein nanoparticle vaccines and DNA-launched nanoparticle vaccines encoding eOD-GT8-60mer immunogen. While both protein and DNA-nanoparticle vaccines induced potent humoral responses, in terms of rate of sero-conversion and overall magnitudes, only DNA-launched nano-vaccine but not corresponding protein nano-vaccine adjuvanted by RIBI could elicit CTL responses. Mechanistic studies demonstrate that DNA/EP delivery of nano-vaccine induced unique pro-inflammatory environment in transfected muscle tissues, by causing transient apoptosis of transfected muscle fibers and infiltration of APCs, including pro-inflammatory M1 macrophages. Infiltrating APCs can subsequently scavenge cell-associated antigen for cross-presentation and CTL priming. Consequently, only DNA-launched nano-vaccines but not protein nano-vaccines against tumor-associated antigens Trp2 and Gp100 elicited CTL responses, and conferred protection to mice challenged with B16F10 melanoma cells. This study not only demonstrates that DNA/EP can be used for in vivo assembly of complex nanoparticle vaccines but also that unique immunological features can be driven by DNA-launched nanoparticles (dose-sparing, quick sero-conversion and induction of CTL responses). As such, we can envision that DNA-launched nano-vaccines represent a versatile platform with likely importance for different diseases and which could facilitate rapid clinical translation of promising next-generation vaccine candidates designed through protein engineering.
7.3 Future direction and Conclusion

In this work, we have demonstrated that DNA/EP may be a potent tool for in vivo folding and assembly of complex protein domains and may be a versatile platform for sustained in vivo expression of biologics or delivery of next-generation vaccines that can induce unique immunological features (Figure 7.1). Additional studies may be pursued to further evaluate and demonstrate utility of DNA-encoded immunotherapies or DNA-launched nano-vaccines in a highly clinically relevant model. For example, HIV-1 or SHIV challenge studies can be pursued to further evaluate potency of anti-HIV-1 immunoadhesin eCD4-Ig or HIV-1 bNAb to mediate immediate and/or persistent protection in a humanized mouse model or an NHP model respectively. Additional optimizations and developments may also be required to further enhance utility of this technology. Optimizations in the choice of plasmid promoters, as well as modifications to immune-silence delivered biologics (potentially through glycan masking), would be needed to further improve pharmacokinetic properties of in vivo expressed biologics. Additional strategies, such as DNA-launched RNA replicon technology, may also be included to further increase in vivo DNA-encoded biologic expression to enable potent and biologically relevant levels of expression in larger mammals and in humans (Zimmer, 2010).

In the case of DNA-launched nanoparticle vaccine DLnano_LS_GT8, further study can be pursued evaluating this immunogen in an animal model with a relevant human antibody repertoire (VRC01 germline transgenic mice, or H2L2 mouse developed by Harbor Biomed) to examine whether DNA-launched nano-vaccine is capable of priming and inducing the appropriate precursor antibody response, and whether that response can be further boosted sequentially with more native-like Envelop immunogens to increase breadth of neutralization. The proof-of-concept that DNA-launched influenza nano-vaccine can provide protection to mice from challenge at a low dose is likely important, and
suggests that additional DNA-launched nano-vaccines scaffolding influenza HA head immunogens (against H3N2 and influenza B) should be designed and evaluated, either individually and in combination. Additionally, it will also likely be important to evaluate DNA-launched influenza nano-vaccines in larger animals (rabbits, NHPs and pigs) to determine whether the DNA-launched nano-vaccine technology can overcome attenuation in vaccine immunogenicity when a DNA vaccine candidate is advanced from a smaller mammal to a larger mammal. Additionally, nanoparticle-scaffolding antigens against additional diseases may be pursued to further validate generalizability of the technology. Additional strategies should be considered to facilitate computational design of nanoparticle vaccines that will be able to spontaneously assemble when expressed, such that the technology can be rapidly deployed to target emerging infectious disease in an outbreak scenario. For instance, a bi-component nanoparticle assembly strategy has garnered significant interests recently and could be explored using the DNA technology (Georgiev et al., 2018).

In the cancer space, DNA-launched nano-vaccines against melanoma-associated antigen appears to provide excellent protection to animals in a prophylactic vaccination setting but lacks potency to mediate durable control of tumors in a therapeutic vaccination setting when the tumor microenvironment is already well established. To address such limitation, a multi-modal treatment approach, which has been demonstrated to be effective in suppressing tumor growth, should be explored (Moynihan et al., 2016). It, therefore, can be envisioned a combinatorial approach in which DNA-encoded antibodies (checkpoint blockade, or antibody against tumor-associated antigen) maybe combined with DNA-launched nano-vaccines (against tumor-associated antigens or neo-antigens) for potential synergy in the treatment of cancer (Duperret et al., 2018a).
With further optimizations/diversifications of the technology, DNA/EP may potentially facilitate rapid translation/evaluation of promising vaccine/immunotherapy candidates into human testing, for the betterment of both human and animal health.
Figure 7.1 Summary of major findings in the thesis with regards to DNA-encoded immunotherapies and DNA-launched nanoparticle vaccines.
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