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Engineered DNA-Mediated Antibody Gene Transfer for Prophylaxis Against Infectious Diseases

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Abstract
Monoclonal antibodies (mAbs) have become important therapeutic and prophylactic agents for a number of indications, including infectious diseases. However, due to many issues, particularly the high cost of antibody production, mAb therapies are limited to the world's richest populations. Furthermore, lengthy product development programs mean only a small number of mAb products can be produced at any one time. Engineering novel, low-cost, and simple methods of developing and delivering mAbs would be highly advantageous, potentially expanding the utility of antibody approaches into a wider array of applications. Here, we describe an approach to deliver human IgG neutralizing mAbs in vivo using DNA plasmid-mediated antibody gene transfer. This approach, which we term DNA mAb (DMAb) delivery, generates biologically relevant levels of mAbs after a single intramuscular injection of antibody-encoding DNA followed by in vivo electroporation (EP). First, we developed antibody-encoding DNA plasmids that could reproducibly deliver human mAbs to mouse serum. We show that these plasmid-encoded antibodies have similar binding capacity and functionality to in vitro-produced purified antibodies. Then, we use a mouse model to show that intramuscular delivery of pDVSF-3 LALA, which encodes a human anti-Dengue virus (DENV) IgG1 neutralizing antibody modified with a mutation that abrogates Fcγ receptor binding, produces anti-DENV antisera capable of binding to and neutralizing DENV1-3. Importantly, mice receiving pDVSF-3 LALA, but not the unmodified pDVSF-3 WT, were protected from both virus-only disease and antibody-enhanced lethal disease. To build upon these initial findings, we evaluated targeted genetic approaches and alternative delivery regimens in order to increase DMAb expression in vivo. Using DMAbs encoding human IgG1 antibodies against Borrelia burgdorferi (the causative agent of Lyme disease) as a model, we show that specific amino acid modifications to the framework regions of antibody variable domains confer increased in vitro and in vivo DMAb expression levels compared to the original DMAb sequences. Of note, these modifications were found to have no detrimental effect on the antibody's borreliacidal activity. Lastly, we observed that pre-treatment of the DMAb injection site with hyaluronidase resulted in a 2.4 to 6.4-fold increase in human IgG concentration levels in vivo compared to mice receiving EP-mediated DMAb delivery only. Taken together, these data establish DNA plasmid-based antibody gene transfer as a safe, effective means of delivering tailored, protective monoclonal antibodies to hosts.

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ABSTRACT

ENGINEERED DNA-MEDIATED ANTIBODY GENE TRANSFER FOR PROPHYLAXIS AGAINST INFECTIOUS DISEASES

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Monoclonal antibodies (mAbs) have become important therapeutic and prophylactic agents for a number of indications, including infectious diseases. However, due to many issues, particularly the high cost of antibody production, mAb therapies are limited to the world's richest populations. Furthermore, lengthy product development programs mean only a small number of mAb products can be produced at any one time. Engineering novel, low-cost, and simple methods of developing and delivering mAbs would be highly advantageous, potentially expanding the utility of antibody approaches into a wider array of applications. Here, we describe an approach to deliver human IgG neutralizing mAbs *in vivo* using DNA plasmid-mediated antibody gene transfer. This approach, which we term DNA mAb (DMAb) delivery, generates biologically relevant levels of mAbs after a single intramuscular injection of antibody-encoding DNA followed by *in vivo* electroporation (EP). First, we developed antibody-encoding DNA plasmids that could reproducibly deliver human mAbs to mouse serum. We show that these plasmid-encoded antibodies have similar binding capacity and functionality to *in vitro*-produced purified antibodies. Then, we use a mouse model to show that intramuscular delivery of pDVSF-3 LALA, which encodes a human anti-Dengue virus (DENV) IgG1 neutralizing antibody modified with a mutation that abrogates Fcγ receptor binding, produces anti-DENV antisera capable of binding to and neutralizing DENV1-3. Importantly, mice

receiving pDVSF-3 LALA, but not the unmodified pDVSF-3 WT, were protected from both virus-only disease and antibody-enhanced lethal disease. To build upon these initial findings, we evaluated targeted genetic approaches and alternative delivery regimens in order to increase DMAb expression *in vivo*. Using DMAbs encoding human IgG1 antibodies against *Borrelia burgdorferi* (the causative agent of Lyme disease) as a model, we show that specific amino acid modifications to the framework regions of antibody variable domains confer increased *in vitro* and *in vivo* DMAb expression levels compared to the original DMAb sequences. Of note, these modifications were found to have no detrimental effect on the antibody's borreliacidal activity. Lastly, we observed that pre-treatment of the DMAb injection site with hyaluronidase resulted in a 2.4 to 6.4-fold increase in human IgG concentration levels *in vivo* compared to mice receiving EP-mediated DMAb delivery only. Taken together, these data establish DNA plasmid-based antibody gene transfer as a safe, effective means of delivering tailored, protective monoclonal antibodies to hosts.

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CHAPTER 1 – Introduction

Humoral immunity and the basis for vaccination

The humoral immune system is one of the first lines of defense against invading pathogens; the generation of antibodies by B lymphocytes plays a vital role in preventing infection and protecting the host against disease. Typically, antibody responses are generated either by exposure to an encountered pathogen or through the process of vaccination. Indeed, antibody responses are responsible for the efficacy of almost all of the vaccines currently in use (S. A. Plotkin, 2009). Vaccination has had an immeasurably positive impact on human health, resulting in the complete elimination of smallpox (Breman & Arita, 1980) and the drastically reduced incidence of measles, mumps, rubella, poliomyelitis, tetanus, rotavirus, and more (Moxon & Siegrist, 2011). Modern vaccines, typically comprised of live-attenuated microorganisms, killed viral particles, or recombinant viral proteins, elicit the production of specific antibodies that bind superficial microbial structures on the target pathogen. Unfortunately, immunological pressure or imprecise genome replication can cause certain pathogens to accumulate mutations that limit the effectiveness of antibodies originally generated against the pathogen. Typically, antibody responses generated by traditional vaccines only target the specific antigens found in the inoculum, and are poorly able to control similar pathogens that carry either subtle or gross changes to the antigen. As such, many pathogens that have adapted complex immune evasion mechanisms – such as human immunodeficiency virus, influenza, dengue, and malaria – have proven to be difficult targets for traditional vaccination (Karlsson Hedestam et al., 2008; Man John Law, Landi, Magee, Lorne Tyrrell, & Houghton, 2013).

Passive immunity and the rise of monoclonal antibody therapy

While the roots of vaccination can be traced back to the 18th century Chinese practice of variolation against smallpox (Stanley A. Plotkin, Orenstein, & Offit, 2013), it wasn't until the late 19th century that the development of antibody passive transfer therapy began to take shape. In 1890, Emil von Behring and Shibasaburo Kitasato demonstrated that naïve animals could acquire immunity against tetanus or diphtheria after being injected with serum obtained from animals immunized with the respective toxins (von Behring & Kitasato, 1991). This early success paved the way for what became known as serum therapy, in which sera from immune animals or patients are passively administered to a recipient host in order to confer immunity to a pathogen. Serum therapy was generally effective and widely used, yet the practice was beset with a number of issues, such as lot-to-lot variability, dosing uncertainty, and allergic reactions (Casadevall, 1999). While there have been improvements in the passive transfer of polyclonal antibodies over the preceding decades, especially in regards to the purification of antibodies from serum before passive administration, the advent of antibiotics in the 1940s ultimately reduced the usage of serum therapy (Casadevall & Scharff, 1994). However, there are still 11 FDA-approved polyclonal antibody preparations used in the clinic, primarily for immune compromised individuals and post-exposure prophylaxis (Robbins, Schneerson, & Szu, 1996).

Cesar Milstein and Georges Köhler's pioneering development of hybridoma technology in 1975 greatly expanded the possibilities of specific antibody transfer (Kohler & Milstein, 1975). This new methodology, in which splenocytes from an immunized mouse are fused with a mouse myeloma cell line, allowed for continuous production of monoclonal antibodies (mAbs) against the immunogen. A little over a decade later, the FDA approved the first mAb therapy, murine-derived muromonab-CD3,

ushering in a new age of antibody-based therapeutics (Todd & Brogden, 1989). However, the murine antibodies produced by hybridomas limited their clinical effectiveness, as anti-mouse antibody immune responses in patients led to the rapid clearance of drug from the body, causing issues with drug readministration (Wilde & Goa, 1996). Fortunately, advances in recombinant DNA technology and antibody discovery techniques have led to the development of chimeric, humanized, and fully human mAbs that are less immunogenic than their murine counterparts (Lonberg, 2005; Traggiai et al., 2004; Vaughan, Osbourn, & Tempest, 1998). As a result, there are now over 20 FDA-approved mAb drugs on the market, with hundreds in the development pipeline (Keizer, Huitema, Schellens, & Beijnen, 2010).

While the successes of mAb therapy have been admirable, there are a number of issues in current production and delivery methods that limit the modality's clinical potential and the populations that these drugs can reach. On the production side, clinical antibody development is prohibitively expensive: investment costs for a commercial antibody production facility can range anywhere from $40 million to $650 million depending on the size of the facility, among other factors (Farid, 2007). Furthermore, the half-life of therapeutic mAbs *in vivo* is typically between a few days and 3-4 weeks (Keizer et al., 2010), necessitating frequent administrations of drug to maintain effective antibody concentrations in patients. This partially explains the exorbitant prices for mAb therapies, which can cost cancer patients $35,000 annually for effective antibody treatment (Farid, 2007). Additionally, traditional antibody delivery mechanisms are time intensive for recipients and are often performed via intravenous infusions that require trained medical personnel to administer properly (Keizer et al., 2010). For these reasons and more, mAb therapies are generally restricted to a subset of patients who may benefit from such treatments.

Antibody gene transfer for infectious diseases

An alternative to traditional passive transfer techniques is the use of vector-mediated antibody gene transfer for the delivery of well-characterized mAbs. This approach effectively directs tissues targeted by the vector to act as antibody-producing depots such that transfected tissues in the host ectopically express the genes of a desired mAb *in vivo*, resulting in the secretion of mAbs into the circulation. This approach has been assessed with an array of viral- and non-viral vectors (Appendix, Table A1), each with unique characteristics worth exploring.

Adenoviral-mediated antibody gene transfer

Adenovirus (Ad) vectors have a long history as gene delivery vehicles and have been among the most commonly used viral vectors in gene therapy (Wilson, 1996). In regards to their use in antibody gene transfer, Ad vectors are generally known for their rapid, yet transient, expression profiles. In one study, a group C serotype 5 Ad (Ad5) was used to deliver a murine mAb against the protective antigen (PA) component of *Bacillus anthracis*, the bacteria responsible for anthrax. Mice given the Ad vector showed serum anti-PA antibodies as early as one day after administration, eventually peaking at 7 days post-injection. Furthermore, mice were protected as quickly as 1 day after Ad administration, with protection lasting 8 weeks before completely disappearing by six months (De, Hackett, Crystal, & Boyer, 2008). In another study, mice were given an Ad5 vector expressing a murine version of the anti-respiratory syncytial virus (RSV) mAb palivizumab, followed one week later by intranasal RSV challenge. Not only did mice receiving the Ad vector show anti-RSV serum titers within 3 days, but they also had 5.4-fold lower RSV titers in the lungs compared to control animals after RSV challenge (Skaricic et al., 2008). Soon after, Ad5 served as the vector of choice to deliver an anti-

Yersinia pestis mAb to mice, of which 80% were protected when challenged 4 days post-Ad administration (Sofer-Podesta et al., 2009). Ad5 vectors were also used to deliver a camelid single-domain antibody (sdAb) against the influenza hemagglutinin (HA) protein, protecting mice when given between 24 hours and 14 days before lethal influenza challenge (Tutykhina et al., 2013). More recently, another antibody against *Bacillus anthracis* – this time a bispecific camelid mAb – was delivered by Ad5-mediated antibody gene transfer and protected 100% of mice up to 11 days post-Ad administration (De et al., 2008; Moayeri et al., 2016). Taken together, Ad-mediated antibody gene transfer is rapid, but short-lived, means of delivering mAbs *in vivo*.

However, a major caveat to the use of Ad-based vector systems is the widespread pre-existing anti-Ad immunity in the human population, particularly against the widely used human Ad5 serotype; pre-existing human Ad immunity can significantly reduce the clinical efficacy of Ad-mediated gene transfer (Fausther-Bovendo & Kobinger, 2014). Therefore, it is generally felt that alternative vector systems will be necessary for clinically effective vector-based antibody gene transfer.

Adeno-associated virus-mediated antibody gene transfer

Much of the pioneering work in the antibody gene transfer field began with a seminar study from Anne Lewis and colleagues, in which a recombinant adeno-associated virus serotype 2 (rAAV2) vector was used to deliver the human anti-HIV broadly neutralizing antibody (bnAb) b12 to Rag1 immunodeficient mice via intramuscular injection (Lewis, Chen, Montefiori, Johnson, & Clark, 2002). Using a dual-promoter system to deliver the heavy and light chain transgenes with a single vector, mice produced serum-detectable levels of human IgG over the course of the 6-month study that were capable of neutralizing T cell line-adapted (TCLA) and primary HIV-1

isolates. These studies were later extended to delivering anti-simian immunodeficiency virus (SIV) antibody-like immunoadhesins to rhesus macaques using self-complementary AAV1 (scAAV1) vectors, resulting in long-lasting neutralizing activity and protection against intravenous SIV challenge (Johnson et al., 2009). A similar approach, known as Vectored Immunoprophylaxis (VIP), has been successfully employed in mouse models of HIV (both intravenous and mucosal challenges) (Balazs et al., 2012) influenza (Balazs, Bloom, Hong, Rao, & Baltimore, 2013), malaria (Deal et al., 2014), and hepatitis C (de Jong et al., 2014). Recent work also suggests that VIP is efficacious in nonhuman primates, as AAV-mediated delivery of a simianized version of the human anti-HIV bnAb VRC07, in combination with the immunosuppressive drug cyclosporine, led to substantial expression of simian VRC07 antibody in rhesus macaques for nearly 4 months and protection against simian-human immunodeficiency virus (SHIV) infection 5.5 weeks after AAV injection (Saunders et al., 2015). Another study employed intranasal delivery of a rAAV9 vector encoding an anti-influenza HA bNAb FI6 and showed protection against lethal influenza in mice and ferrets (Limberis, Adam, et al., 2013), showing the range of delivery methods available for effective AAV-mediated antibody gene transfer.

In addition to prophylactic applications of AAV-mediated antibody gene transfer, questions of antibody expression kinetics and therapeutic efficacy have also been explored. A number of studies have compared the kinetics of AAV-mediated antibody gene transfer to that of Ad-mediated antibody gene transfer, and the results generally suggest that AAV delivery has slower but longer lasting expression kinetics compared to the rapid but transient nature of Ad-mediated antibody gene transfer (De, Hackett, Crystal, & Boyer, 2008(Skaricic et al., 2008). Regarding the therapeutic potential of this antibody delivery platform, one interesting study looked at a murine model of HIV

treatment and showed that treatment of humanized mice with antiretroviral therapy (ART) drugs, followed by AAV-mediated expression of an anti-HIV bnAb, resulted in durable viremic control after ART was stopped (Horwitz et al., 2013). Taken together, these results suggest that AAV-mediated antibody gene transfer is capable of slower but durable expression of high concentrations of mAbs in a variety of animal models.

As with Ad vectors, anti-vector immunity against AAV presents many challenges for its use as a gene delivery vehicle in humans. AAV infection is common in humans, resulting in high seroprevalence of IgG antibodies to multiple AAV serotypes. Additionally, most sera positive for anti-AAV antibodies were found to have some neutralization properties (Boutin et al., 2010), which have been found to inhibit vector transduction in animals and humans at titers as low as 1:5 (Jiang et al., 2006; Manno et al., 2006; Scallan et al., 2006). Clinical trials have shown that prior exposure to the AAV capsid through natural infection may generate a pool of AAV-specific memory T cells that are reactivated upon AAV vector-mediated gene transfer (Manno et al., 2006; Mingozzi et al., 2007) and that these T cell responses are dose-dependent (Mingozzi et al., 2009). One study showed that AAV2 capsid-specific human T cells proliferated upon exposure to other AAV serotypes, suggesting that simply altering AAV serotype usage for gene transfer may not evade the anti-vector immune response (Mingozzi et al., 2007). However, a recent clinical study demonstrated that intramuscular delivery of an AAV1 vector expressing M-type α-1 antitrypsin was capable of long-term transgene expression despite the presence of a CD8+ T cell response, perhaps due to the concomitant activation of a small natural T regulatory cell population to the AAV capsid (Mueller et al., 2013). These data collectively suggest that immunity against the AAV vector is a major concern clinically, with manifestations of anti-vector immunity being

dependent on multiple factors, including pre-existing immunity, vector dose, and delivery route.

DNA plasmid-mediated antibody gene transfer

While the majority of antibody gene transfer studies have employed viral vectored methods, notable drawbacks such as anti-vector immunity (either pre-existing or newly developed as a result of viral vector administration) make DNA plasmid-mediated antibody gene transfer an attractive alternative (Appendix, Figure A1). Numerous preclinical and clinical studies have shown that DNA plasmid delivery does not elicit anti-vector immunity (Klinman et al., 2000), a benefit that has often been used to administer multiple doses of DNA vaccines without generating an immune response to the DNA plasmid vector (Klinman et al., 2000; MacGregor, Boyer, Ciccarelli, Ginsberg, & Weiner, 2000). Additionally, continued advancements in DNA plasmid delivery technology have greatly improved the prospects of DNA plasmid-mediated gene delivery in both animal and clinical settings. For example, plasmid delivery via *in vivo* electroporation (EP) improves plasmid delivery by a factor of 10-1,000 fold over naked DNA delivery alone (Sardesai & Weiner, 2011). The advantages of DNA plasmid-mediated gene transfer are further buttressed by the platform's ease of use and large-scale production, relatively low costs, and strong safety profile. Despite these advantages, DNA plasmid-mediated antibody gene transfer is a relatively underexplored field.

Tjelle and colleagues were the first to use EP-delivered DNA plasmids for antibody gene transfer (Tjelle et al., 2004). In this study, mice were injected intramuscularly with DNA plasmids encoding chimeric (mouse-human) IgG3 antibodies. When naked DNA plasmids were injected without EP, antibody levels were similar to

baseline levels in control mice; however, DNA plasmid injection followed by EP led to serum-detectable levels of human IgG ranging from 50-200 ng/mL. Mice injected with fully murine antibody-encoding plasmids followed by EP showed greater mAb serum levels that were detectable for at least 7 months post-injection. When sheep were injected with murine antibody-encoding plasmids, six of seven animals showed serum-detectable murine antibody levels ranging between 30-50 ng/mL for up to 4 weeks post-injection.

This initial proof-of-concept study showed that DNA plasmids could indeed be used to deliver antibody-encoding genes intramuscularly, but it was not until 2011 that Yamazaki *et al*. published the first report showing *in vivo* protective functionality of DNA plasmid-mediated antibody gene transfer against an infectious disease in mice. Animals intramuscularly injected with DNA encoding an anti-influenza HA murine mAb followed by EP were protected against a lethal influenza dose when challenged nearly three weeks after DNA plasmid-mediated antibody gene transfer (Yamazaki et al., 2011). Furthermore, these murine mAbs were detectable at least 70 days post-injection, showing the long-term expression potential of DNA plasmid vectors.

Given the dearth of studies using DNA plasmid-mediated antibody gene transfer, we began to explore ways to optimize this delivery method. In our initial studies, mice were given highly optimized DNA plasmids expressing Fab fragments of the human anti-HIV-1 bnAb VRC01 (Muthumani et al., 2013). When delivered with EP, a single administration of the optimized plasmid constructs resulted in the generation of Fab molecules in mouse sera possessing gp120-binding and HIV-1 neutralizing activity against diverse HIV-1 isolates for at least 7 days. Importantly, this delivery method resulted in serum-detectable production of human Fab within 48 hours, suggesting that

DNA plasmids possess the rapidity of Ad vector-mediated antibody gene transfer without the anti-vector immunity.

Goals of this thesis project

Monoclonal antibody therapy carries great promise, and the advent of new antibody discovery techniques has greatly increased the array of potent neutralizing antibodies that target a vast number of infectious diseases. Yet the existing mechanisms for mAb delivery preclude the use of these drugs in many patient populations, either due to high costs or insufficient health care infrastructure. Furthermore, viral vector-mediated antibody gene transfer carries issues of vector immunogenicity, which can limit patient populations or prohibit readministration of additional vectored antibodies. A major goal of my thesis project was to investigate the use of highly optimized synthetic DNA plasmids as a possible delivery vehicle for antibody gene transfer *in vivo*. For our initial studies, we developed DNA mAb-encoding plasmids (DMAbs) to express neutralizing human IgG mAbs against dengue virus. We then incorporated targeted genetic modifications to the antibody Fc region of the anti-DENV DMAb to assess the effect of Fcγ receptor binding on DMAb functionality *in vitro* and in a mouse model of severe dengue disease. Finally, we explored methods to increase antibody production levels *in vivo* through targeted antibody framework modifications of DMAb-encoded IgGs and alternative DNA delivery regimens. Ultimately, the primary goal of this thesis project was to determine the feasibility of DNA-plasmid mediated antibody gene transfer *in vivo* as an alternative to traditional and viral vector-based antibody delivery methods.

CHAPTER 2 – Materials and Methods

Cell lines. Vero cells were kindly provided by Professor Robert Doms (Children's Hospital of Philadelphia, Department of Pathology and Laboratory Medicine) and cultured in Medium 199 (Invitrogen) supplemented with 5% FBS and antibiotics (Invitrogen; 100 units/mL penicillin and 100 µg/mL streptomycin). K562 cells were purchased from ATCC and grown in Iscove's Modified Dulbecco's Medium (Invitrogen) supplemented with 10% FBS and antibiotics (Invitrogen; 100 units/mL penicillin and 100 µg/mL streptomycin). HEK293T cells were purchased from ATCC and grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) with 10% FBS and antibiotics (Invitrogen; 100 units/mL penicillin and 100 µg/mL streptomycin).

Dengue viruses. Dengue virus types 1 (TH-S-man; ATCC VR-1586), 2 (New Guinea C; ATCC VR-1584), 3 (Philippines/H87/1956; BEI Resources NR-80), and 4 (H241; BEI Resources NR-86) were amplified in Vero cells cultured at 37^{o}C in Medium 199 supplemented with 2% FBS and antibiotics (Invitrogen; 100 units/mL penicillin and 100 µg/mL streptomycin).

Antibody plasmid construction. The DNA plasmids pDVSF-3 and pDVSF-3-LALA encode fully human IgG1 mAbs whose variable regions were derived from the anti-DENV1-3 human mAb DV87.1 [Genbank accession numbers: DV87.1 VH KC294015, DV87.1 VL KC294016]. The DNA plasmids p319-44wt and p319-44mod1 encode fully human IgG1 mAbs whose variable regions were derived from the anti-hisOspA antibody 319-44. Each transgene consisted of the heavy and light chain genes separated by a furin cleavage site coupled with a P2A self-processing sequence. The transgenes were

codon and RNA optimized for expression in humans, synthesized by GenScript and cloned into modified pVax1 mammalian expression vectors (Invitrogen) under the control of the human cytomegalovirus immediate-early promoter.

In vitro *transfection.* For transfection of DMAb plasmids, 293T cells were plated at 2.5 x 10^5 cells/well in flat-bottom 6-well plates. The next day, a transfection mixture, consisting of 5 μg of DMAb plasmid incubated with 20 μL of Turbofectin 8.0 transfection reagent (OriGene) and 100 μL of DMEM, was then added to the 293T cells. After 2 days at 37^{o}C, supernatant was harvested from the cells and the human IgG concentration was measured by performing an anti-human IgG ELISA.

Western blots. For protein analysis, human antibodies were purified from supernatants of transfected cells with a Protein A antibody purification kit (Montage Antibody Purification Kit with PROSEP-A media, Millipore). Purified antibodies were separated in precast Bis-Tris gels (Invitrogen) under either reducing or nonreducing conditions. Proteins were transferred to Immobilon-FL PVDF transfer membranes (Millipore). Membranes were blocked for 1 hour in Odyssey Blocking Buffer (Li-Cor Biosciences), incubated with a goat anti-human IgG 680RD antibody (Li-Cor Biosciences), and washed. Protein bands were visualized on the Li-Cor Odyssey CLx.

ELISAs.

***Human IgG quantification ELISA*.** For quantification of total human IgG, ELISA plates were coated with 1 μg/well of goat anti-human IgG-Fc fragment antibody (Bethyl) overnight at 4^{o}C. Plates were blocked with 10% FBS in PBS for 1 hour at room temperature. After washing, samples were diluted in 1% FBS in PBS-T, added to the

plate, and incubated for 1 hour at room temperature. Plates were washed, and HRP-conjugated goat anti-human kappa light chain (Bethyl) was added for 1 hour at room temperature. Sample was detected with SIGMA*FAST* OPD (Sigma-Aldrich). A standard curve was generated using purified human IgG/Kappa (Bethyl).

DENV E protein binding ELISA. For determination of DENV E protein binding, ELISA plates were coated with 1 µg/mL of recombinant E protein from DENV1-4 (Fitzgerald Industries International) overnight at 4^{o}C. Plates were blocked with 10% FBS in PBS for 1 hour at room temperature. After washing, samples were diluted in 1% FBS in PBS-T, added to the plate, and incubated for 1 hour at room temperature. Plates were washed, and HRP-conjugated goat anti-human IgG-Fc fragment antibody (Bethyl) was added for 1 hour at room temperature. Sample was detected with SIGMA*FAST* OPD (Sigma-Aldrich).

B. burgdorferi *hisOspA binding ELISA*. For determination of antibody binding to his-tagged OspA protein (hisOspA), ELISA plates were coated with 1 µg/mL of recombinant hisOspA protein from *B. burgdorferi* (courtesy of MassBiologics) overnight at 4^{o}C. Plates were blocked with 10% FBS in PBS for 1 hour at room temperature. After washing, samples were diluted in 1% FBS in PBS-T, added to the plate, and incubated for 1 hour at room temperature. Plates were washed, and HRP-conjugated goat anti-human IgG-Fc fragment antibody (Bethyl) was added for 1 hour at room temperature. Samples were detected with SIGMA*FAST* OPD (Sigma-Aldrich).

Flow Cytometry.

DENV titration assay. FACS infectious units (IU)/mL were quantified by a flow cytometry-based viral titration assay. Briefly, Vero cell monolayers were infected with serial dilutions of DENV for 24 hours, after which infected cells positive for intracellular

expression of the dengue E protein were enumerated by intracellular staining with the monoclonal antibody 4G2.

Intracellular staining of DENV-infected cells. Vero cells were infected with DENV1 TH-Sman (ATCC VR-1586), DENV2 New Guinea C (ATCC VR-1584), DENV3 H87 (BEI Resources) or DENV4 H241 (BEI Resources) at an MOI of 0.01. After 5 days, cells were harvested, then were fixed, permeabilized, and washed with Cytofix/Cytoperm and Cytoperm/Cytowash (BD Biosciences). Cells were incubated with sample containing human anti-DENV antibodies diluted in Cytoperm/Cytowash for 1 hour on ice, washed, and then stained with goat anti-human IgG Fc FITC (Abcam) for 1 hour on ice. After washing, cells were analyzed on a LSRII (BD Biosciences).

DENV neutralization assay. Vero cells were seeded in 100 µL of Medium 199 supplemented with 5% FBS and 1% PenStrep (Invitrogen) and plated at 5 x 10^3 cells/well in flat-bottom 96-well plates. The next day, different dilutions of mAb-containing sample were incubated with 200 pfu/well of DENV for 1 hour at 37^{o}C. The neutralization mixture was then added to Vero cells. After 3 days, cells were harvested, then were fixed, permeabilized, and washed with Cytofix/Cytoperm and Cytoperm/Cytowash (BD Biosciences). Cells were stained with 4G2 diluted in Cytoperm/Cytowash for 1 hour on ice, washed, and then stained with goat anti-mouse IgG Fc FITC (Abcam) for 1 hour on ice. After washing, cells were analyzed on a LSRII (BD Biosciences).

DENV antibody enhancement assay. To assess antibody-dependent enhancement, K562 cells were seeded in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS at 5 x 10^3 cells/well in flat-bottom 96-well plates and incubated with neutralization mixture formulated as above. After 3 days, cells were fixed, permeabilized, and washed as above. Cells were stained with 4G2, washed, and then

stained with goat anti-mouse IgG Fc FITC before a final series of washes. Cells were then analyzed on a LSRII as above.

Animals. Wild type 129/Sv and C3H/HeNCrl mice were purchased from Charles River Laboratories. 129/Sv mice lacking both the IFN-α/β and γ receptors (AG129) were bred and housed at the La Jolla Institute for Allergy and Immunology (LIAI) Animal Facility. B6.Cg-Foxn1nu/J and C57BL/6 mice were purchased from The Jackson Laboratory. All animal housing and experimentation were approved by and conducted in accordance with the guidelines set by the NIH and the Perelman School of Medicine at the University of Pennsylvania or La Jolla Institute for Allergy and Immunology Institutional Animal Care and Use Committees.

DMAb injection strategies.

DENV DMAb studies. Mice were administered a single 50 μL intramuscular (IM) injection of plasmid into the quadriceps followed by *in vivo* electroporation (EP) using a CELLECTRA adaptive constant current electroporation device (Inovio Pharmaceuticals Inc.) as previously described (Flingai et al., 2015). Serum samples were collected pre-injection and at various times after plasmid administration to determine human IgG antibody concentration, binding ability, and neutralization capacity.

Lyme DMAb studies. Mice were administered 30 μL intramuscular (IM) injections of DMAb plasmid into either the quadriceps muscle or the tibialis anterior (TA) muscle followed by *in vivo* electroporation unless stated otherwise. Serum samples were collected pre-injection and at various times after plasmid administration to determine human IgG antibody concentration and binding ability.

Hyaluronidase pre-treatment. For hyaluronidase pre-treatment of mouse muscle, bovine hyaluronidase (Sigma-Aldrich) at a concentration of 0.4 U/μL in deionized water (dH_2O) was injected in a volume of 30 μL into either the TA or quadriceps muscle of anesthetized mice 30 minutes prior to DMAb injection and *in vivo* electroporation. A CELLECTRA adaptive constant current electroporation device (Inovio Pharmaceuticals Inc.) was used, as previously described(Flingai et al., 2015).

DENV challenge. All DENV challenge experiments were performed five days after DNA administration in AG129 mice. For DENV2 virus-only challenge experiments, 5 to 6 week-old AG129 mice were infected intravenously (via the tail vein) with $1x10^9$ genome equivalents (GE) of DENV2 strain S221 diluted in a total volume of 200 μL PBS with 10% FCS. For DENV2 enhanced disease challenge experiments, AG129 mice were administered 5 μg of the non-neutralizing anti-DENV mAb 2H2 intraperitoneally 30 minutes prior to infection with an intravenous $1x10^9$ GE dose of DENV2 strain S221.

Statistical analyses. All graphs were prepared using GraphPad Prism 6 (GraphPad Software). Survival data were expressed using Kaplan-Meier survival curves. A two-way ANOVA test was used to determine differences between multiple groups.

CHAPTER 3 – Protection against dengue disease by DNA plasmid-mediated antibody gene transfer

*The majority of the results described in this chapter have been published in: Flingai S, Plummer EM, Patel A, Shresta S, Mendoza JM, Broderick KE, Sardesai NY, Muthumani K, Weiner DB. (2015). Protection against dengue disease by synthetic nucleic acid antibody prophylaxis/immunotherapy. *Sci Rep* 5: 12616.

INTRODUCTION

Dengue virus (DENV) is the most important mosquito-borne viral infection in humans. In recent years, the number of cases and outbreaks has dramatically increased worldwide. While vaccines are being developed, none are currently available that provide balanced protection against all DENV serotypes. Advances in human antibody isolation have uncovered DENV neutralizing antibodies (nAbs) that are capable of preventing infection from multiple serotypes. Yet delivering monoclonal antibodies using conventional methods is impractical due to high costs. Engineering novel methods of delivering monoclonal antibodies could tip the scale in the fight against DENV. Here we demonstrate that simple intramuscular delivery by electroporation of synthetic DNA plasmids engineered to express modified human nAbs against multiple DENV serotypes confers protection against DENV disease and prevents antibody-dependent enhancement (ADE) of disease in mice. This synthetic nucleic acid antibody prophylaxis/immunotherapy approach may have important applications in the fight against infectious disease.

Nearly 400 million dengue infections occur each year (Bhatt et al., 2013), and cases of dengue fever (DF) and the potentially fatal dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) have grown in recent decades. The geographical reach of

dengue has expanded to include over 100 countries, resulting in a significant health and economic burden worldwide (Bhatt et al., 2013; Gubler, 2012). While primary DENV infection is thought to elicit persistent and effective immunity against reinfection with the same serotype, only short-term protection is elicited against other DENV serotypes (Rothman, 2004). Disease severity is associated with subsequent heterotypic infection, during which non- or sub-neutralizing levels of cross-reactive antibodies from prior infection form immune complexes with DENV that lead to increased infection of Fcγ receptor (FcγR)-bearing monocytes and macrophages (Halstead, 1979; Peiris, Gordon, Unkeless, & Porterfield, 1981; Peiris & Porterfield, 1979). This phenomenon, known as antibody-dependent enhancement (ADE), gives rise to one of the greatest challenges in developing a dengue vaccine: eliciting balanced, neutralizing immunity across multiple serotypes while minimizing the risk of ADE. A recent live-attenuated, quadrivalent vaccine candidate from Sanofi has shown promising protective efficacy against DENV1, 3, and 4, but underwhelming protection against DENV2 (Capeding et al., 2014; Sabchareon et al., 2012; Villar et al., 2014), a serotype frequently associated with severe disease from secondary infections (Leitmeyer et al., 1999). Furthermore, whether vaccine-induced humoral responses can overcome the threat of ADE in vaccinees over time remains to be seen.

Passive immunization studies have shown that neutralizing monoclonal or polyclonal antibodies can provide cross-serotype protection against DENV infection in mice (Beltramello et al., 2010; Kaufman, Summers, Dubois, & Eckels, 1987; Kyle, Balsitis, Zhang, Beatty, & Harris, 2008; Lai et al., 2007; Shrestha et al., 2010; Sukupolvi-Petty et al., 2010) and non-human primates (NHPs) (Lai et al., 2007). Yet monoclonal antibody delivery in humans is incredibly expensive, creating cost-prohibitive barriers for most regions of the world where such therapy would be needed. Developing new

methods for delivering cross-reactive, neutralizing monoclonal antibodies into the circulation may provide rapid, complete protection against DENV-associated disease.

One such approach involves vector-mediated gene transfer of monoclonal antibodies. Several studies have demonstrated the effectiveness of this delivery strategy in protecting NHPs against SIV (Johnson et al., 2009), humanized mice against HIV (Balazs et al., 2012; Balazs et al., 2014), and mice and ferrets against influenza (Balazs et al., 2013; Limberis, Adam, et al., 2013; Limberis, Racine, et al., 2013). While these studies have employed intramuscular or intranasal administration of adeno-associated virus (AAV) vectors to produce protective antibodies, our interest in DNA plasmids has led us to explore whether such vectors can be used to deliver neutralizing monoclonal antibodies into the circulation. DNA plasmids represent an interesting vector model for gene transfer: they have an excellent safety profile, and unlike viral vectors, have no vector-associated serology, allowing for repeat delivery (Klinman et al., 2000; Kutzler & Weiner, 2008; MacGregor et al., 2000). As a proof of concept, we previously constructed optimized DNA plasmids capable of expressing Fab fragments of the HIV-1 broadly neutralizing antibody VRC01 in mice after intramuscular injection and *in vivo* electroporation (EP), resulting in mouse sera that neutralized multiple strains of HIV-1 (Muthumani et al., 2013). To date, however, no vector system has been used to deliver neutralizing, protective anti-DENV IgG antibodies into any animal model.

Here, we describe an approach to delivering cross-reactive neutralizing antibodies against DENV into the circulation using DNA plasmid-mediated antibody gene transfer. This synthetic DNA-encoded antibody approach (DMAb) produces biologically relevant levels of mAbs after a single intramuscular injection of antibody-encoding DNA. As this approach allows for genetic tailoring of the exact features of the desired antibody, we further studied the role of Fc region modifications on protection. We demonstrate

that intramuscular delivery of a DNA plasmid encoding an anti-DENV human IgG1 nAb, with an Fc region mutation that abrogates FcγR binding, protects mice from both virus-only infection and antibody-enhanced lethal infection.

RESULTS

DMAb optimization and *in vitro* characterization. The expression of human IgG antibodies from DNA-based vectors has briefly been explored in the past (Tjelle et al., 2004) and resulted in low levels of serum-detectable antibodies *in vivo*. However, subsequent genetic optimizations to DNA plasmids and accompanying delivery systems, particularly EP, have resulted in increased expression of desired proteins (Sardesai & Weiner, 2011). With this in mind, we systematically optimized DMAb DNA through the creation of two- and single-plasmid antibody-encoding DNA cassettes, with the aim of increasing human IgG production from DMAb *in vivo* (Figure 1). The two-plasmid DMAb system consisted of two plasmids encoding either the heavy chain or light chain gene sequence of a human IgG mAb, each plasmid driven by the CMV promoter. Meanwhile, the single plasmid DMAb system is comprised of the heavy and light chain gene sequences of a human IgG mAb separated by a furin cleavage site and a P2A self-processing peptide (Figure 2), cloned into an expression plasmid driven by the CMV promoter. For our initial human IgG characterization studies, we assessed two-plasmid DMAb delivery *in vivo*, building upon the foundation laid by our previously published anti-HIV-1 VRC01 human Fab two-plasmid delivery work (Muthumani et al., 2013). Dosage studies of two-plasmid DMAb delivery in C57BL/6 mice showed a dose-dependent increase in human IgG concentration in the serum; detectable human IgG levels were observed as quickly as two days post-injection in mice receiving 100μg of total DNA

(Figure 3). In order to compare two- and single-plasmid DMAb constructs, we moved to an *in vitro* expression system for a more high-throughput comparison of plasmid designs. We transfected the two- or single-plasmid DMAb plasmids into human embryonic kidney (HEK) 293T cells, and secreted antibody levels in the supernatant were quantified after 48 hours by ELISA. We observed a slight increase in human IgG production from the single-plasmid condition (Figure 4), which led us to use the single plasmid DMAb design for all subsequent experiments.

We designed and constructed two highly optimized single plasmid DMAbs against DENV: pDVSF-3 WT, which encodes for the human IgG1 mAb DV87.1, a well-characterized mAb capable of neutralizing DENV1-3 (Beltramello et al., 2010); and pDVSF-3 LALA, which encodes for an Fc region-modified version of DV87.1 with abrogated FcγR binding by way of two leucine-to-alanine (LALA) mutations in the CH2 region (Hessell et al., 2007) that have been shown to eliminate antibody-dependent enhancement[14]. Each transgene was genetically optimized, synthesized, and subcloned into a modified pVax1 mammalian expression vector. In order to further optimize the single plasmid DMAb constructs, we compared different human signal sequences fused to the IgG heavy and light chain genes, assessing human IgG expression levels *in vitro*. The use of human IgG or IgE heavy and light chain signal sequences led to comparable pDVSF-3 WT expression *in vitro*, while the human growth hormone (HGH) signal sequence showed reduced human IgG expression (Figure 5). Due to these results, the human IgG signal sequences were used for all subsequent DMAbs.

In order to assess whether the LALA mutation had any effect on DMAb production, we compared human IgG expression levels between pDVSF-3 WT and pDVSF-3 LALA *in vitro*. Both pDVSF-3 WT and pDVSF-3 LALA resulted in 600 ng/mL of human IgG, confirming that the LALA mutation has no effect on antibody expression

levels *in vitro* (Figure 6). To confirm proper antibody assembly, DVSF-3 WT and DVSF-3 LALA antibodies were collected from supernatants of transfected HEK293T cells and separated by SDS-PAGE gel for Western blot analysis (Figure 7). The heavy and light chain proteins were at their expected molecular weights, suggesting proper protein cleavage and antibody assembly.

To assess the biological activity of the antibodies, we first performed a binding ELISA assay that measures whether the antibody-containing supernatant can bind to recombinant DENV1-3 E proteins. The supernatants of HEK293T cells that secreted either DVSF-3 WT or DVSF-3 LALA antibodies were able to recognize DENV1-3 E proteins, while DENV4 went unrecognized, as expected (Figure 8). Additionally, DVSF-3 WT- and DVSF-3 LALA-containing supernatants were able to stain Vero cells infected with DENV1-3, whereas Vero cells infected with DENV4 were not stained by the supernatants (Figure 9). To assess the capacity of the LALA mutation to eliminate Fcγ receptor binding, we performed a binding ELISA that measured DVSF-3 WT and DVSF-3 LALA Fc binding to immobilized FcγR1a, a receptor found on macrophages and monocytes that has been known to mediate ADE (Kontny, Kurane, & Ennis, 1988). DVSF-3 WT antibody secreted from HEK 293T cells were bound by the FcγR1a receptor, whereas the DVSF-3 LALA antibody not bound by the receptor (Figure 10). To evaluate whether this Fcγ receptor binding difference between the WT and LALA DMAbs is manifested in differences in ADE capabilities, we used an ADE *in vitro* assay in which DVSF-3 WT or LALA DMAbs, after incubation with dengue virions, were then incubated with human monocytic K562 cells, which contain Fcγ receptors but are only susceptible to DENV infection in the presence of enhancing antibodies. Importantly, K562 cells exposed to DVSF-3 WT and virus showed substantial DENV infection, whereas cells

exposed to DVSF-3 LALA and virus had no such infection *in vitro*, suggesting that DVSF-3 WT is capable of enhancing DENV infection (Figure 11).

DMAb results in long-term expression of neutralizing DENV antibodies in mouse serum. In order to investigate antibody production kinetics *in vivo*, we assessed the duration of DNA plasmid-encoded human IgG expression in nude mice, which would model antibody expression in an immune-accommodating host. For our initial long-term *in vivo* kinetics studies, B6.Cg-Foxn1nu/J mice were injected intramuscularly with 100 µg of a DNA plasmid encoding another human IgG1 anti-DENV antibody, DVSF-1 WT (derived from DV82.11, a human IgG1 mAb that targets the DII fusion loop of the E protein and has been well characterized for its ability to neutralize DENV1-4 (Beltramello et al., 2010)), followed immediately by EP. Human IgG concentrations in the serum were detectable within 5 days of injection, with peak levels of ~1000ng/mL at two weeks post-injection (Figure 12). Duration of human IgG expression lasted at least 19 weeks, showcasing the sustained expression levels attainable with DNA plasmids. Given that the mouse DENV challenge model uses mice from the 129/Sv background, we sought to determine whether the antibody-encoding DNA plasmid constructs could produce serum-detectable levels of DVSF-3 WT or LALA in this background strain. Upon comparing DVSF-3 LALA expression levels in various immune competent and immunodeficient mouse strains, we found that 129/Sv DMAb expression of DVSF-3 LALA was comparable to expression in B6.Cg-Foxn1nu/J immunodeficient mice at Day 7 post-injection (Figure 13). Interestingly, there was variability across different immune competent and immunodeficient mouse strains, suggesting that DMAb expression levels are not simply a function of the DMAb plasmid itself, nor a function of immune competence vs. immunodeficiency. As we observed *in vitro,* serum from 129/Sv mice receiving either pDVSF-3 WT or pDVSF-3 LALA showed comparable human IgG levels

(Figure 14) and stained Vero cells infected with DENV1-3 (Figure 15). Additionally, whereas naïve sera were unable to neutralize virus in a neutralization assay, both WT and LALA-containing serum were capable of neutralizing DENV1-3 (Figure 16).

Dengue DMAb delivery of DVSF-3 LALA protects against enhanced dengue disease in mice. To assess whether mice expressing DNA plasmid-encoded anti-DENV neutralizing mAbs would be protected from DENV challenge, we employed the AG129 mouse model, which lacks type I and type II interferon (IFN) receptors and, upon DENV infection, recapitulates many aspects of human disease (Shresta, Sharar, Prigozhin, Beatty, & Harris, 2006; Zellweger, Prestwood, & Shresta, 2010). Importantly, these mice have been shown to exhibit ADE, with low doses of serotype-specific as well as cross-reactive antibodies both enhancing infection (Zellweger et al., 2010). For these studies, mice were infected with the mouse-adapted DENV2 strain S221, which, in the presence of sub-neutralizing amounts of the anti-DENV mAb 2H2, causes antibody-enhanced severe disease and acute lethality (4-6 days post-infection) in AG129 mice at sublethal doses (Zellweger et al., 2010).

For challenge studies, AG129 mice were given a single intramuscular injection of pDVSF-3 WT or pDVSF-3 LALA followed immediately by EP. Negative controls received a single intramuscular injection of pVax1 empty vector followed by EP. Five days later, the mice were challenged with a sub-lethal dose ($1x10^9$ GE) of DENV2 S221 in the presence (ADE) or absence (virus-only infection) of exogenous anti-DENV mAb 2H2. Mice in the pDVSF-3 WT, pDVSF-3 LALA, and pVax1 cohorts had mean human IgG concentrations of 750 ng/mL, 1139 ng/mL, and undetectable levels, respectively, one day before challenge (Figure 17; $p \leq 0.0930$ for comparison between pDVSF-3 WT and pDVSF-3 LALA). Under virus-only infection conditions, we expect pDVSF-3 WT-treated mice to experience ADE and acute lethality, as immune complexes formed by DVSF-3

WT antibodies with DENV should lead to increased infection (Beltramello et al., 2010). Our *in vitro* antibody enhancement assay results (Figures 10 and 11), which showed that DVSF-3 WT enhances DENV infection of monocytic cells, suggests that DVSF-3 WT has the potential to cause ADE *in vivo*. Conversely, we expect pVax1- and pDVSF-3 LALA-treated mice to survive, being unable to enhance disease. Indeed, five of six pDVSF-3 LALA-treated mice and all five pVax1 mice showed no lethal disease enhancement; all pDVSF-3 WT-treated mice succumbed to disease by day 5 (Figure 18; $p \leq 0.0084$ for comparison between pDVSF-3 LALA and pDVSF-3 WT), demonstrating the non-enhancing functionality of pDVSF-3 LALA against virus-only infection. Under ADE conditions, we expect both pDVSF-3 WT- and pVax1-treated mice to experience acute lethality due to enhanced infection, whereas pDVSF-3 LALA-treated mice should be protected from severe disease. All five mice receiving pDVSF-3 LALA survived under ADE conditions, while those receiving either pDVSF-3 WT or pVax1 empty vector succumbed to acute, antibody-enhanced disease within 4-5 days (Figure 19; $p \leq 0.0072$ for comparison between pDVSF-3 LALA and pDVSF-3 WT). Taken together, these data show that injection of pDVSF-3 LALA does not cause lethal enhancement after virus-only infection and protects against severe disease in ADE conditions, supporting the concept of muscle correctly processing and expressing functional antibodies from this platform.

DMAb delivery of multiple antibodies increases human IgG concentration and breadth of viral coverage in mice. Given that DENV serotypes have been shown to escape neutralization (Sukupolvi-Petty et al., 2010), it is likely that an antibody cocktail targeting multiple epitopes on the DENV virion would produce an ideal prophylactic strategy. DNA plasmids have been shown in numerous experiments to be delivered in multi-plasmid formulations (Moore, Kong, Chakrabarti, & Nabel, 2002; Villarreal et al.,

2014), suggesting that delivery of multiple antibody-encoding plasmids is feasible. To test this concept, we injected 129/Sv mice with multiple anti-DENV DMAbs in a multi-site injection regimen (Figure 20). Mice received a 100 μg intramuscular injection of pDVSF-1 LALA, pDVSF-2 LALA (derived from DV22.3, a human IgG1 mAb that targets DI/DII of the E protein and has been well characterized for its ability to neutralize DENV4 (Beltramello et al., 2010)), pDVSF-3 LALA, or some combination thereof. To prevent antibody heavy and light chain recombination events, each DMAb was injected in a different leg muscle – either the quadriceps or the TA muscle of the left or right leg. Whereas mice injected with pDVSF-3 LALA were only capable of seroconverting against DENV1-3, mice injected with both pDVSF-2 LALA and pDVSF-3 LALA at different sites were able to seroconvert to all four serotypes, and mice injected with all three antibodies had even greater binding against all four serotypes (Figures 21 and 22). These data suggest that delivery of DMAbs can ultimately increase breadth of protection against infectious diseases.

DISCUSSION

The rising global health burden of dengue has created an enhanced urgency to develop a safe, inexpensive, and effective DENV vaccine that prevents both initial infection and ADE-induced severe disease. Here, a single intramuscular injection of a DNA plasmid encoding a modified human anti-DENV1-3 neutralizing antibody was capable of protecting mice against antibody-enhanced DENV disease without enhancing virus-only infection. The ability of DNA plasmids to encode protective Fc region-modified LALA antibodies is significant due to the inability of our immune system to produce ADE-preventing antibody variants upon DENV vaccination or natural infection. Since current vaccine candidates generate traditional antibodies, the potential of vaccines to

inadvertently promote ADE, especially as vaccine protection wanes, is a serious concern. Delivering Fc region-modified LALA antibodies by DMAb that protect against both dengue fever and ADE-induced severe disease could be a unique alternative or addition to traditional vaccine approaches.

The protection conferred by neutralizing anti-DENV mAbs expressed by DMAb is very rapid, with complete survival in mice challenged less than a week after pDVSF-3 LALA administration – significantly more rapidly than vaccine-driven protection, which can take weeks to months to reach peak efficacy levels. The rapid induction of immunity may be advantageous to travelers, as well as the elderly or other populations who respond poorly to vaccines. Travelers to endemic regions frequently receive a number of intramuscular vaccinations prior to travel; as such, we could envision this approach being included alongside normal travel immunization regimens.

We demonstrated that plasmid-encoded DVSF-3 LALA serum levels of 1 μg/mL were protective against lethal enhanced dengue disease in mice. Previous work from Beltramello and colleagues showed that i.p. delivery of 1 to 5 μg of purified DV87.1 antibody 24 hours prior to DENV challenge was able to protect mice from lethal disease[14]; the protective levels observed in their study support our results here. Importantly, we demonstrated the delivery of multiple DENV DMAb plasmids in mice, which increased human IgG levels as well as the amount of serotypes targeted. This novel strategy could be used to increase the breadth of protective coverage against not just DENV, but also other infectious diseases. Furthermore, as monoclonal antibodies have proven to be efficacious against specific cancers or autoimmune disorders, employing DMAb to deliver monoclonal antibodies could be beneficial in such therapeutic antibody treatments and allow many such therapies to reach underserved

populations. In summary, DMAb provides a rapid, novel delivery system for biologically relevant functional full-length monoclonal antibodies *in vivo*.

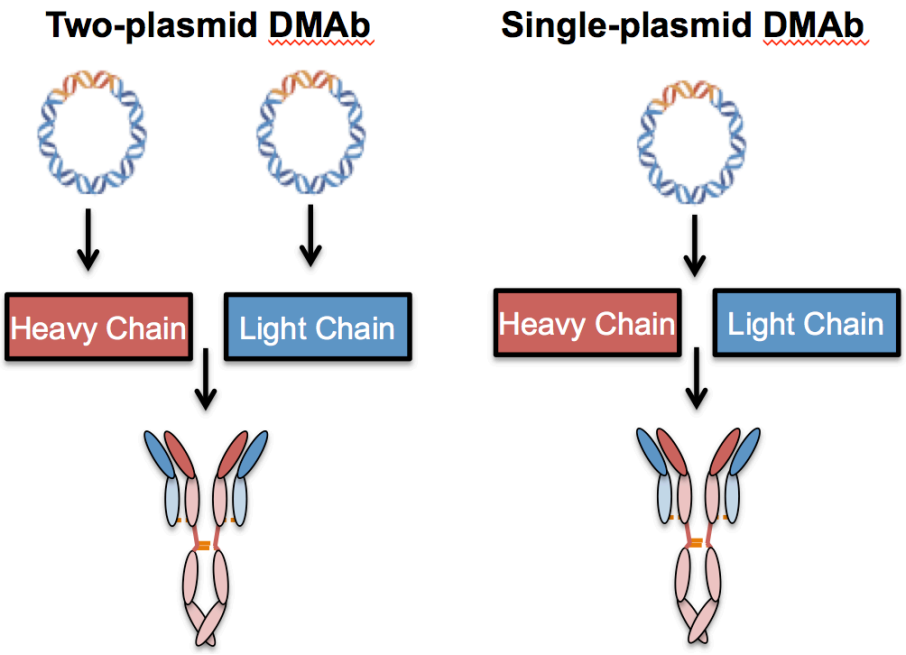


Figure 1. Schematic of two-plasmid and single-plasmid antibody-encoding DNA cassettes for human IgG expression.
The two-plasmid system (left) consists of two plasmids encoding the heavy or light chain gene sequence of a human IgG monoclonal antibody, while the single-plasmid system (right) expresses the heavy and light chain genes from a single open reading frame by separating the two genes with a furin cleavage site and P2A self-processing peptide.

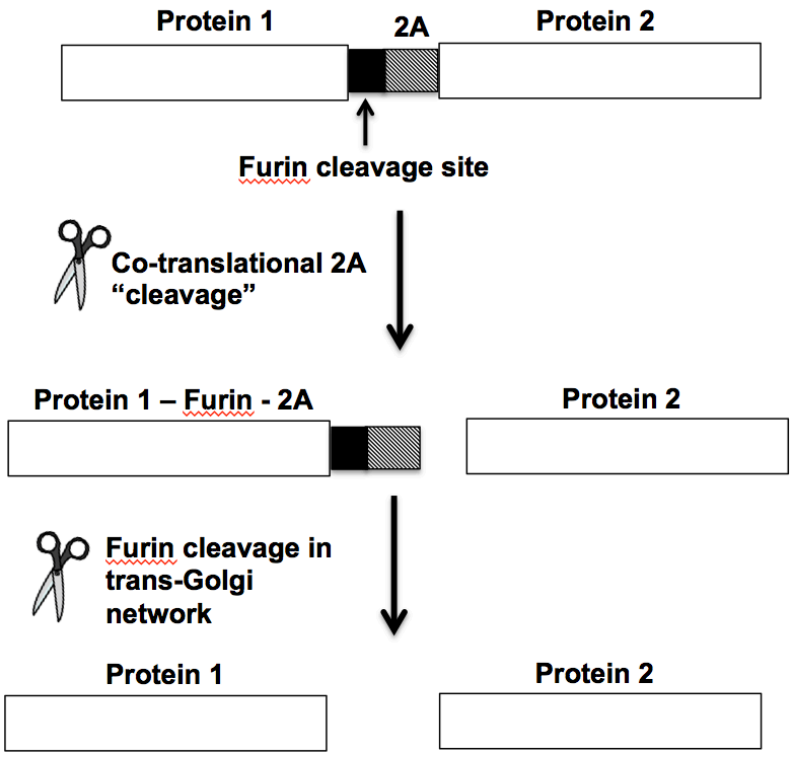


Figure 2. Protein cleavage mechanisms used for single-plasmid DMAb design. The 2A peptide sequence provides the initial location for protein cleavage, occurring co-translationally via a "ribosomal skip" mechanism. The preceding furin cleavage site provides not only a backup cleavage event further down the secretion pathway (in the trans-golgi network), but also allows for the complete removal of the residual 2A peptide from the upstream protein product.

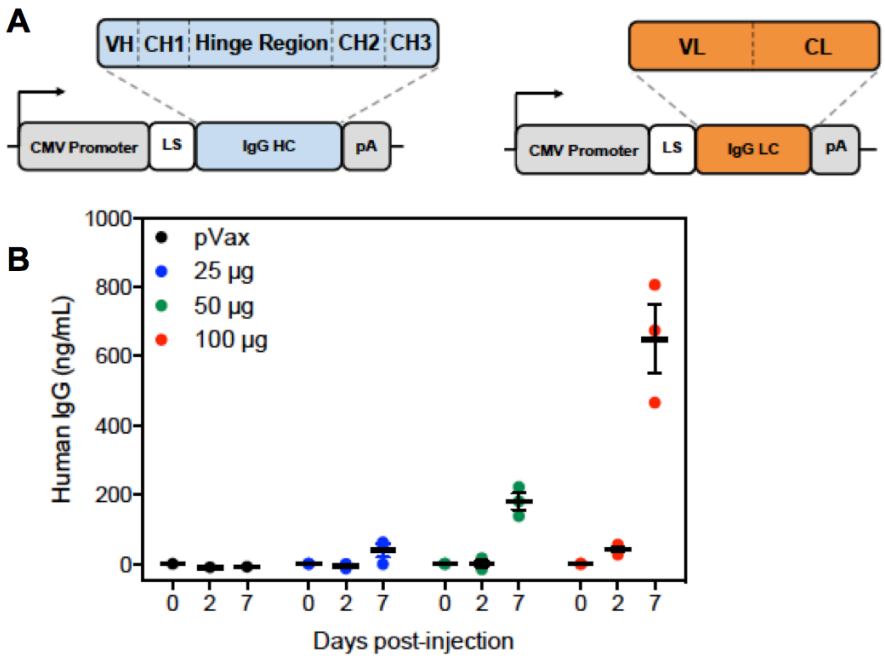


Figure 3. Development and optimization of two-plasmid DMAb.
(A) Schematic illustration of initial two-plasmid DMAb delivery system; human IgG antibody heavy and light chain sequences were expressed on separate plasmids co-delivered either *in vitro* or *in vivo* in a single formulation. (B) Dosage study for two-plasmid DMAb delivery of human IgG in C57B/6 mice measured by ELISA; DNA amounts indicate total DNA injected intramuscularly followed by EP (100 µg = 50 µg IgG heavy chain DNA + 50 µg IgG light chain DNA; n = 3 mice per group).

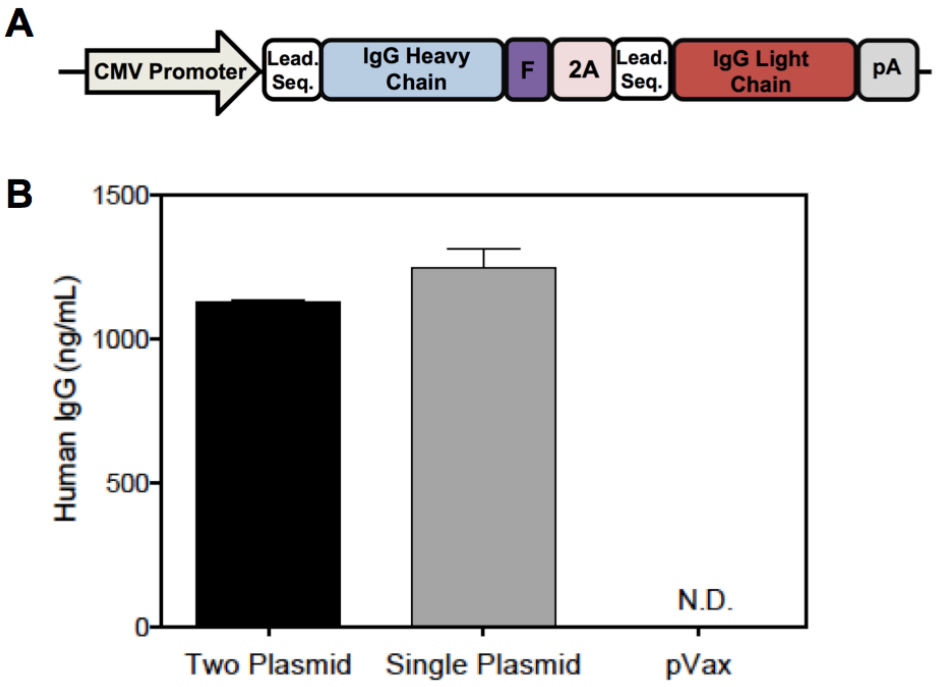


Figure 4. Comparison of two-plasmid and single-plasmid DMAb constructs in vitro.
(A) Schematic illustration of single plasmid DMAb delivery system; antibody heavy and light chain sequences are separated by a combination of furin and 2A cleavage sites. (B) Comparison of human IgG levels *in vitro* by ELISA from two-plasmid DMAb or single plasmid DMAb (samples run in duplicate; data representative of two independent experiments). N.D., not detectable.

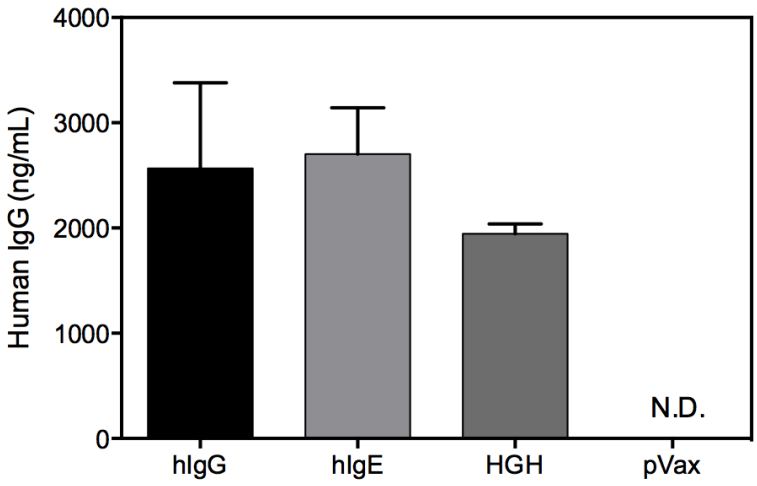


Figure 5. Comparison of human leader sequences on DMAb expression in vitro. The human IgG1 (hIgG), human IgE (hIgE), and human growth hormone (HGH) leader sequences were inserted into single plasmid DMAb constructs, and their effect on human IgG expression was assessed by human IgG quantification ELISA (samples run in duplicate; data representative of three independent experiments). N.D., not detectable.

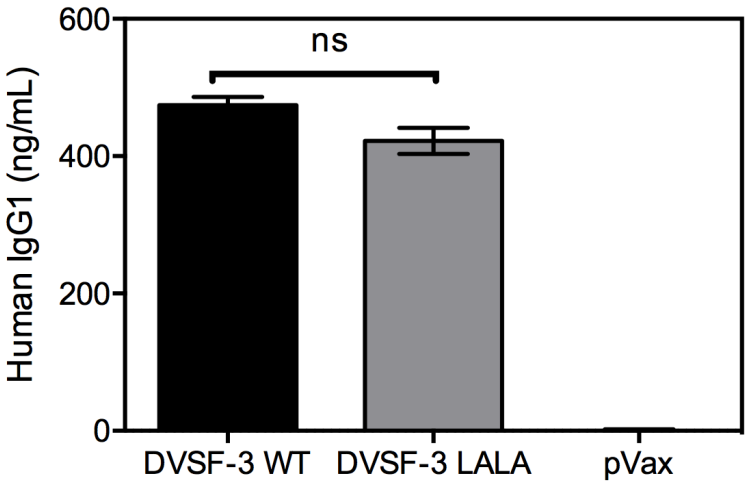


Figure 6. LALA mutation has no detrimental effect on human IgG expression levels in vitro.
ELISA quantification analysis of human IgG in supernatants of pDVSF-3 WT- or LALA-transfected 293T cells. The data displayed are the mean of triplicate values +/- standard error of the mean (SEM) and are representative of three independent experiments.

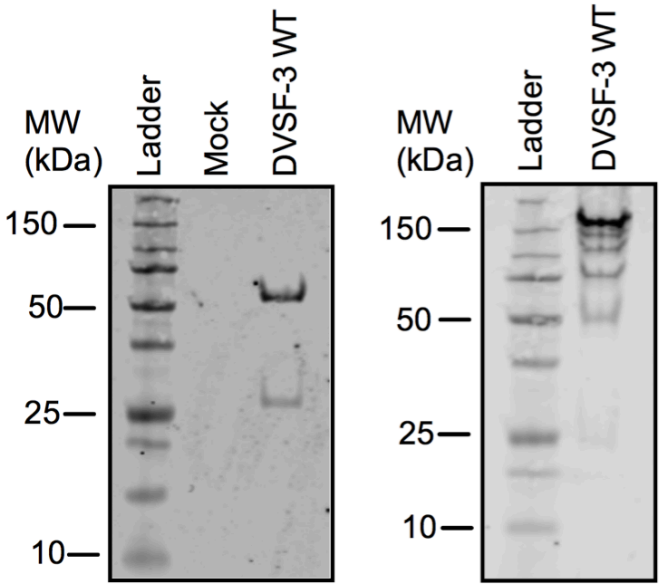


Figure 7. Single-plasmid DMAb construct produces human IgG heavy and light chains.
Western blot analysis of pDVSF-3 WT-transfected 293T supernatants containing DVSF-3 WT. Antibodies were purified by Protein A spin columns and separated by SDS-PAGE under reducing (left) and non-reducing (right) conditions.

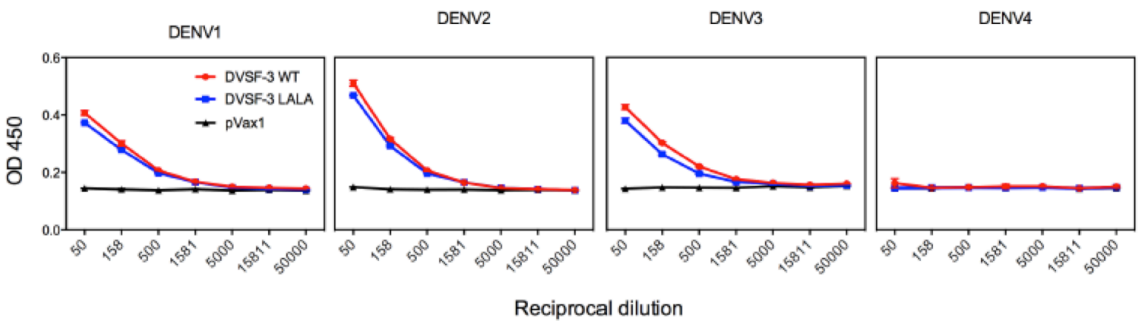


Figure 8. Supernatants from pDVSF-3 WT and LALA transfectants show comparable E protein binding.
ELISA binding analysis of human IgG in supernatants of pDVSF-3 WT- or LALA-transfected 293T cells against purified recombinant DENV E proteins (samples run in duplicate; data representative of two independent experiments).

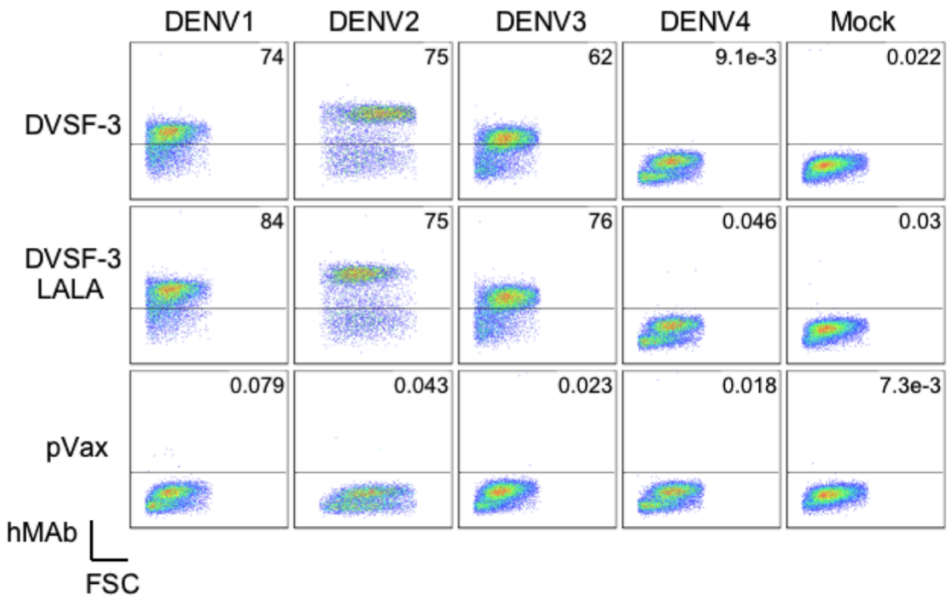


Figure 9. Supernatants from pDVSF-3 WT and LALA transfectants bind to DENV1-3 E protein in virally-infected Vero cells.
Vero cells were either uninfected (Mock) or infected by DENV1, 2, 3, or 4, then fixed, permeabilized, and stained with supernatants of pDVSF-3 WT- or LALA-transfected 293T cells. The data displayed are representative of two independent experiments.

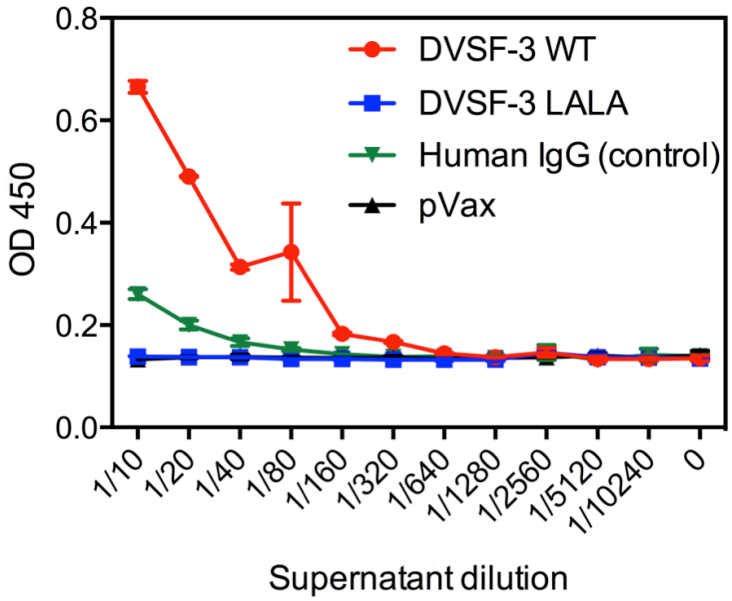


Figure 10. LALA mutation eliminates antibody binding to FcγRIa.
ELISA binding analysis of supernatants of pDVSF-3 WT- or LALA-transfected 293T cells against immobilized FcγR1a, a receptor found on macrophages and monocytes that has been known to mediate ADE. The data displayed are representative of two independent experiments.

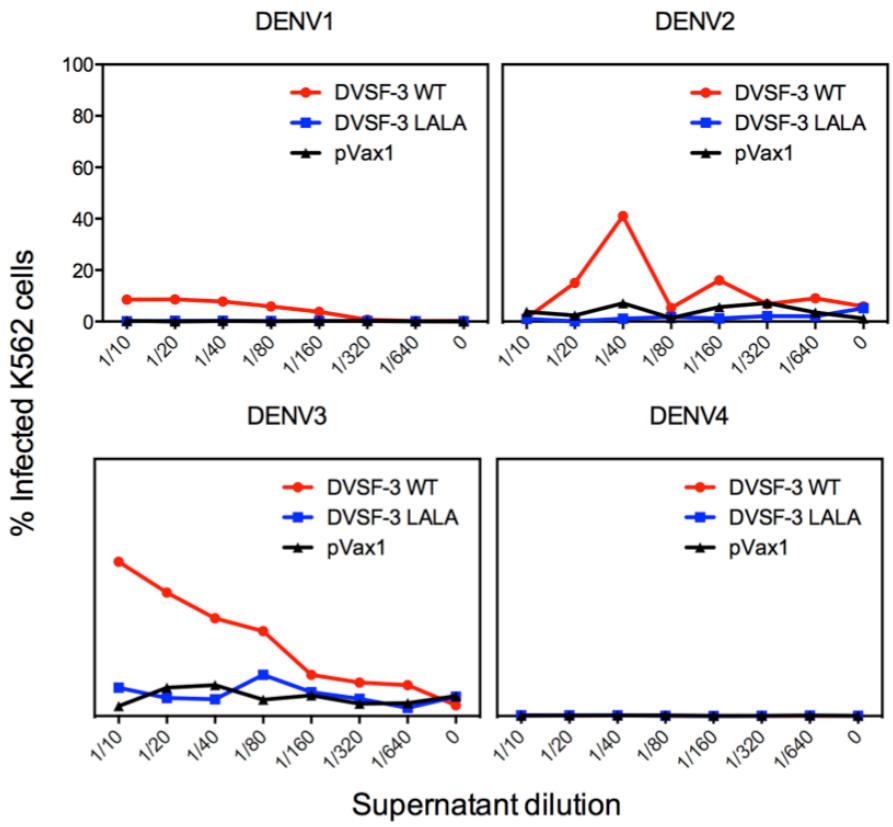


Figure 11. Supernatants from pDVSF-3 LALA do not enhance DENV infection in vitro.

Antibody-dependent enhancement was assessed by incubating DENV1, 2, 3, or 4 with serial dilutions of supernatants of pDVSF-3 WT- or LALA-transfected 293T cells before addition to K562 cells. The percentage of infected cells is shown (samples run in duplicate; data representative of two independent experiments).

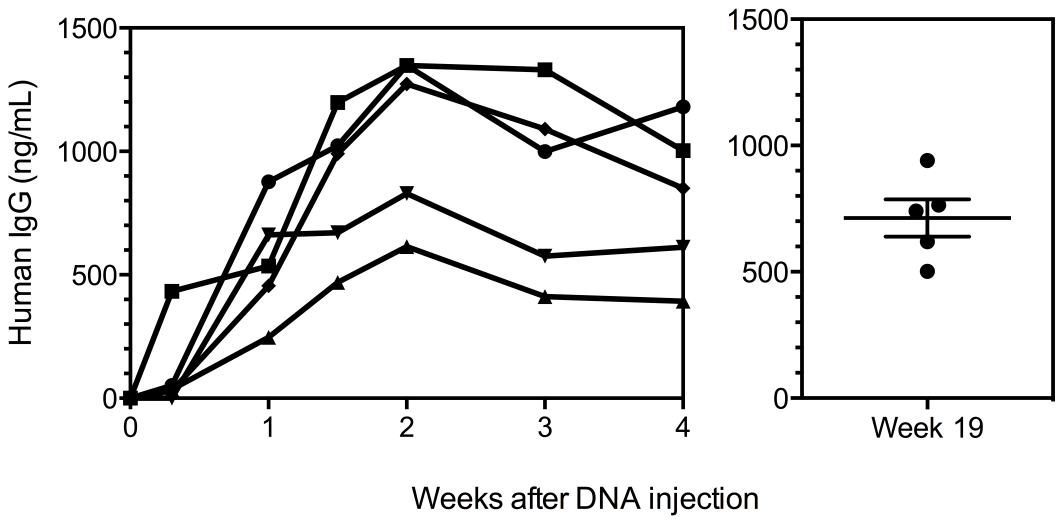


Figure 12. Human IgG concentration in immunodeficient mouse serum after single DMAb injection.
Total serum-detectable levels of human IgG were measured by ELISA after a single intramuscular injection of 100 µg DNA plasmid encoding the anti-DENV human IgG antibody DVSF-1 into B6.Cg-Foxn1nu/J immunodeficient mice. Human IgG levels between weeks 0-4 (left) and at week 19 (right; error bars display the mean of values from five animals +/- SEM). Each line (left) or dot (right) represents an individual mouse (n = 5 mice).

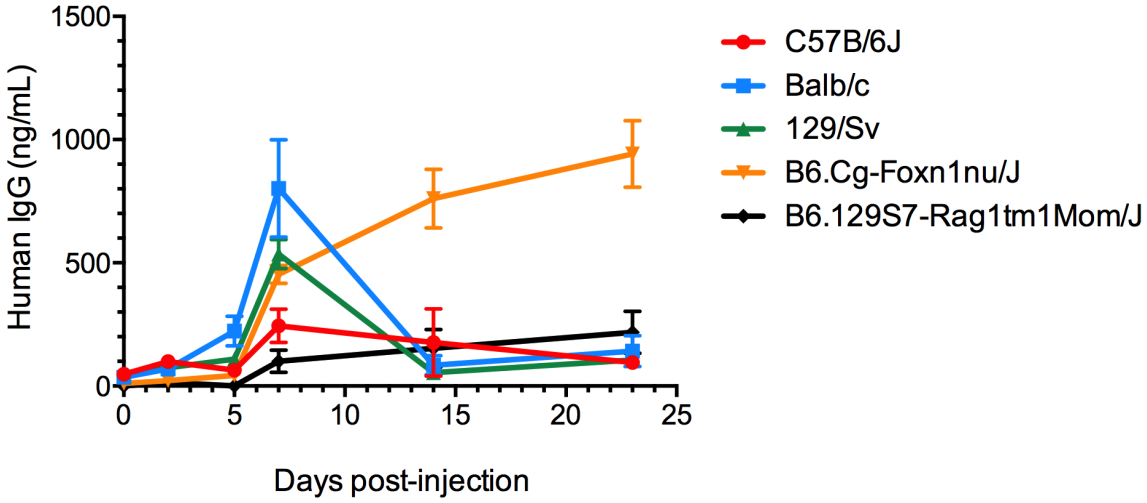


Figure 13. DVSF-3 LALA DMAb expression differs across various immune competent and immunodeficient mouse models.
Total serum-detectable levels of human IgG were measured by ELISA after a single intramuscular injection of 100 µg DNA plasmid encoding DVSF-3 LALA into immune competent (C57B/6J, Balb/c, 129/Sv) and immunodeficient (B6.Cg-Foxn1nu/J, B6.129S7-Rag1tm1Mom/J) mice (n = 5 mice per group, data displayed are the mean +/- SEM of each group's animals and are representative of two independent experiments).

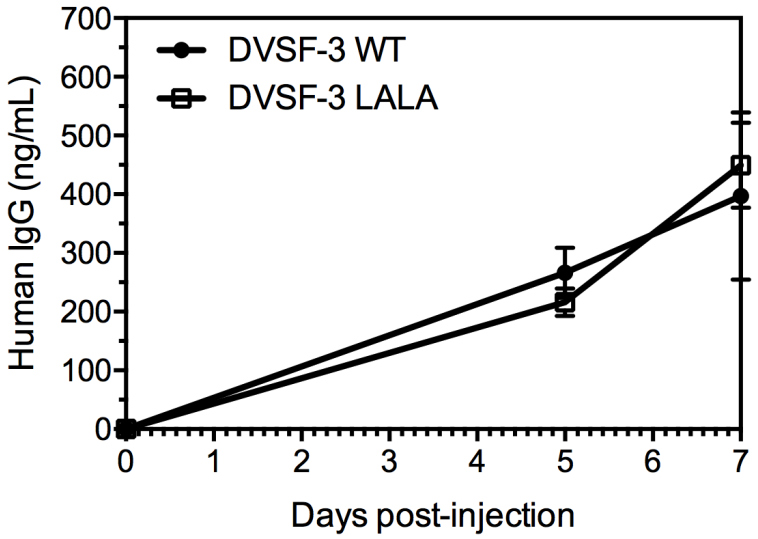


Figure 14. DVSF-3 WT and LALA DMAbs show comparable human IgG concentration levels in mouse serum after intramuscular injection.
Total human IgG in serum was measured by ELISA after intramuscular injection of pDVSF-3 WT or pDVSF-3 LALA plasmids in 129/Sv mice (n = 4-5 mice per group, data displayed are the mean +/- SEM of each group's animals and are representative of two independent experiments).

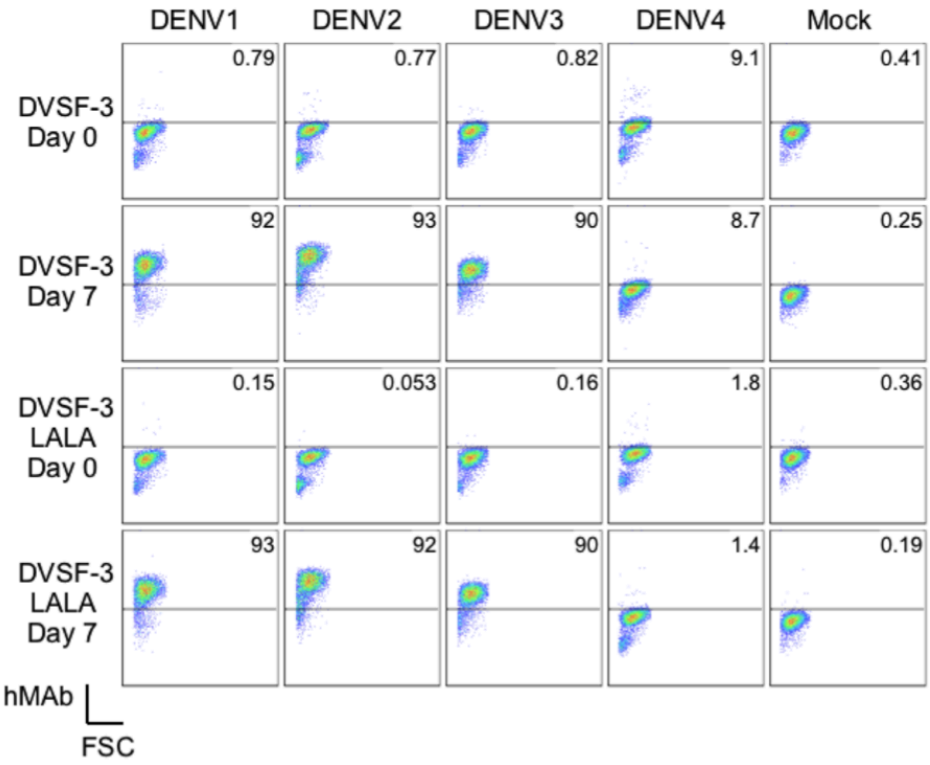


Figure 15. Mice receiving pDVSF-3 WT or pDVSF-3 LALA seroconvert within one week post-DMAb injection.
Vero cells were either uninfected (Mock) or infected by DENV1, 2, 3, or 4, then fixed, permeabilized, and stained with 129/Sv mouse serum taken at days 0 or 7 post-DNA injection of pDVSF-3 WT or pDVSF-3 LALA (n = 5 mice per group, data representative of two independent experiments).

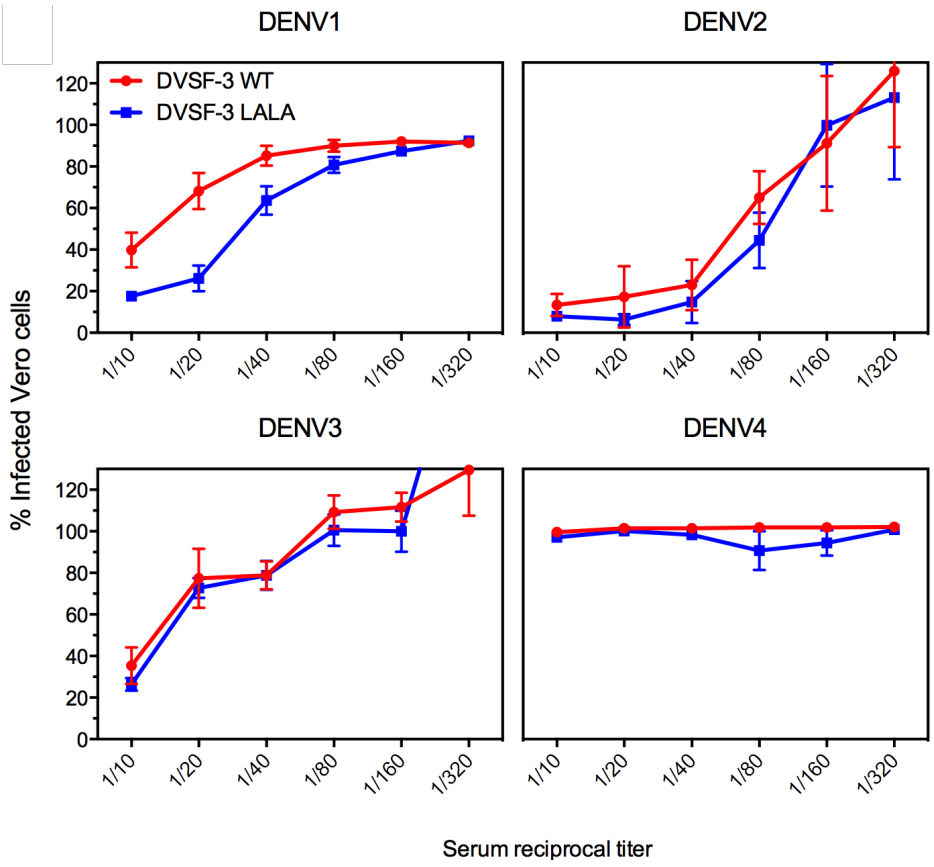


Figure 16. Mice receiving pDVSF-3 WT or pDVSF-3 LALA show comparable serum DENV neutralization capacity upon seroconversion.
Neutralization was assessed by incubating DENV1, 2, 3, or 4 with serial dilutions of 129/Sv mouse serum taken at day 7 post-DNA injection of either pDVSF-3 WT or pDVSF-3 LALA (n = 5 mice per group) before addition to Vero cells. The percentage of infected cells is shown; error bars are the mean +/- SEM of each group's animals).

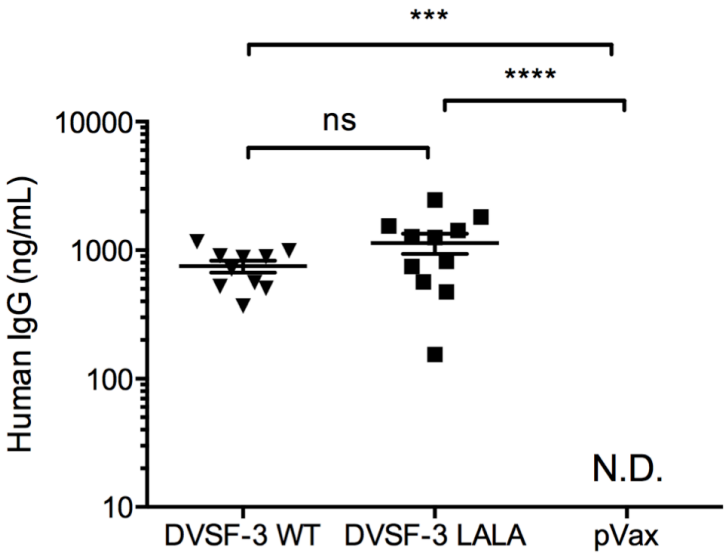


Figure 17. Pre-challenge levels of anti-DENV human IgG levels in AG129 mice after DMAb delivery.

Total human IgG in serum was measured by ELISA 4 days after DNA intramuscular injection (one day before DENV2 challenge) and EP of pDVSF-3 WT, pDVSF-3 LALA, or pVax empty vector plasmids in AG129 mice (n = 10-11 mice per group: $p \leq 0.0005$ for comparison between pDVSF-3 WT and pVax; $p \leq 0.0001$ for comparison between pDVSF-3 LALA and pVax). N.D., not detectable.

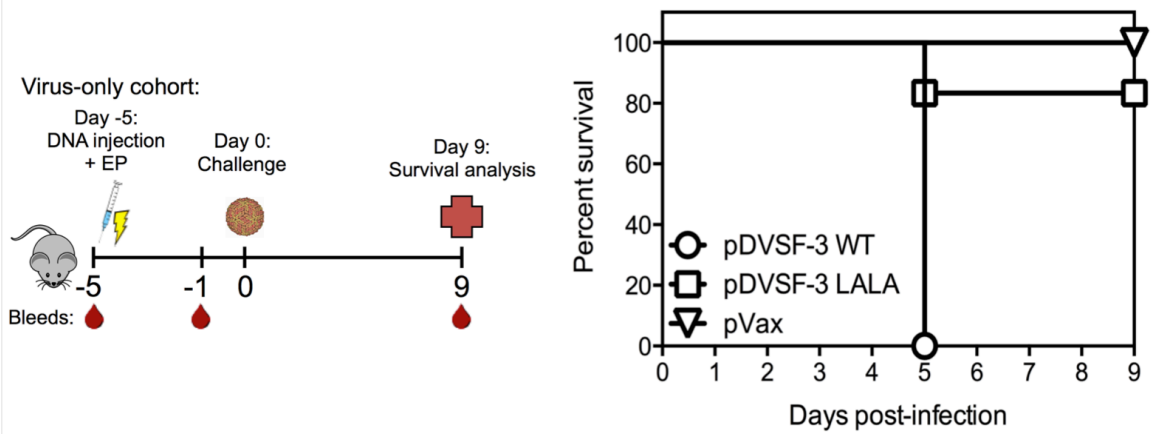


Figure 18. Mice receiving pDVSF-3 LALA show protection against DENV2 virus-only challenge.
AG129 mice received an intramuscular injection of either pDVSF-3 WT, pDVSF-3 LALA, or pVax empty vector five days prior to challenge with a sublethal dose of DENV2 S221 (n = 5-6 mice per group; $p \leq 0.0084$ for comparison between pDVSF-3 LALA and pDVSF-3 WT). A Kaplan-Meier survival curve is shown.

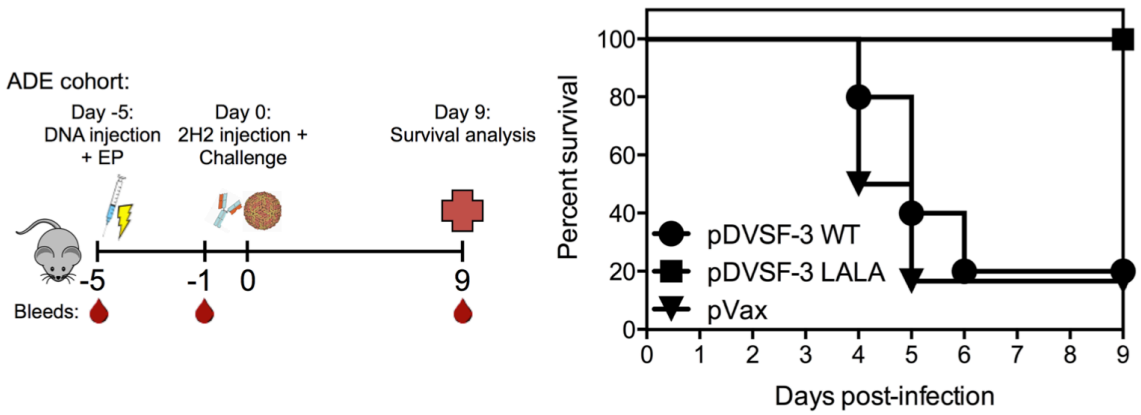


Figure 19. Mice receiving pDVSF-3 LALA are fully protected from antibody-dependent enhancement DENV2 challenge.
AG129 mice received an intramuscular injection of pDVSF-3 WT, pDVSF-3 LALA, or pVax empty vector five days prior to administration of an enhancing dose of the non-neutralizing anti-DENV mAb 2H2. Thirty minutes later, mice were challenged with a sublethal dose of DENV2 S221 (n = 5-6 mice per group; $p \leq 0.0072$ for comparison between pDVSF-3 LALA and pDVSF-3 WT). A Kaplan-Meier survival curve is shown.

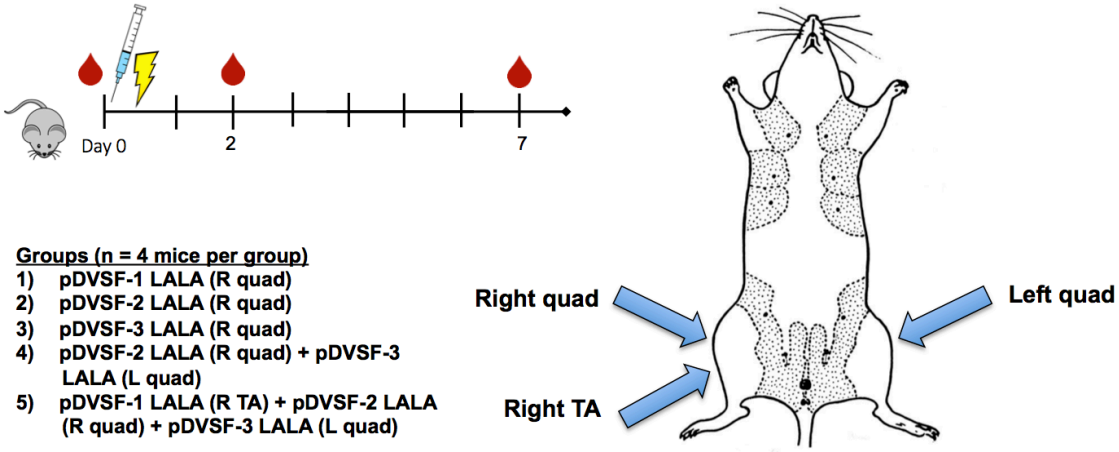


Figure 20. Delivery of multiple DMAbs.
129/Sv mice received a 100 µg intramuscular injection of pDVSF-1 LALA, pDVSF-2 LALA, pDVSF-3 LALA, or some combination thereof in either the quadriceps or tibialis anterior muscles. To prevent potential antibody recombinations between various heavy and light chains, each muscle site was injected with one DMAb.

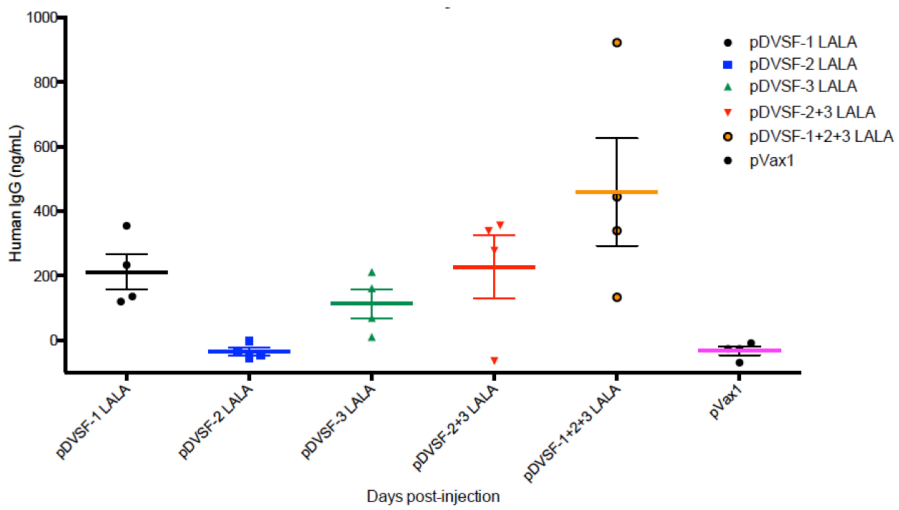


Figure 21. Multi-site DMAb injections of different anti-DENV DMAbs results in elevated human IgG concentrations in mouse serum.
Total human IgG of DVSF-1 LALA, DVSF-2 LALA, DVSF-3 LALA, DVSF-2 LALA and pDVSF-3 LALA, or DVSF-1 LALA, DVSF-2 LALA, and DVSF-3 LALA in serum was measured by ELISA 7 days after DNA intramuscular injection and EP of 100 µg of respective plasmids in 129/Sv mice (n = 4 mice per group; error bars represent standard error of the mean).

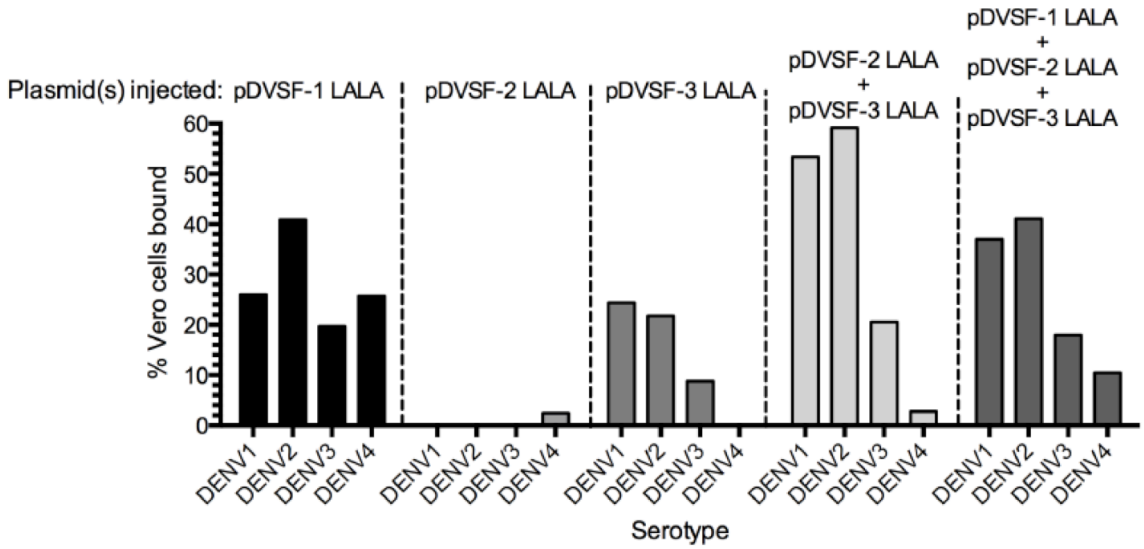


Figure 22. Mice receiving multiple anti-DENV DMAbs seroconvert and display increased breadth of DENV binding.
Bar graph representing the breadth of 129/Sv mouse serum staining of Vero cells infected with DENV1, 2, 3, or 4 after injection of single (pDVSF-1 LALA, pDVSF-2 LALA, or pDVSF-3 LALA) or multiple (pDVSF-2 LALA + pDVSF-3 LALA, or pDVSF-1 LALA + pDVSF-2 LALA + pDVSF-3 LALA) plasmids (n = 4 mice per group; data representative of all mice from each respective group).

CHAPTER 4 – Optimization of DMAb delivery though targeted antibody framework modifications and hyaluronidase pre-treatment

INTRODUCTION

Lyme disease, a tick-borne zoonotic illness caused by the spirochete *Borrelia burgdorferi sensu lato* (henceforth referred to as *B. burgdorferi*), is a major public health concern across the Northern Hemisphere. The disease is the most common reportable vector-borne illness in the United States, with nearly 30,000 confirmed cases in 2013 (Adams et al., 2013), and recent evidence suggests that the number of cases in Canada and Europe is on the rise (Fulop & Poggensee, 2008; Koffi et al., 2012). Furthermore, the geographic reach of *B. burgdorferi* is expanding, as spirochetes have been identified more recently in Asia and Australia (Mayne, 2011; Stanek & Reiter, 2011).

B. burgdorferi is transmitted to humans, domestic animals, and wildlife hosts by *Ixodes* ticks in the larval and nymphal stages (Anderson, 1988; Kurtenbach et al., 2006; LoGiudice, Ostfeld, Schmidt, & Keesing, 2003). Throughout the three-stage life cycle of the tick — larva, nymph, and adult — *B. burgdorferi* undergoes differential expression of various outer surface lipoproteins (Osps), each with varying levels of homology across *B. burgdorferi* isolates. OspA, expressed by spirochetes in the tick midgut, is highly conserved in North American strains of the spirochete (Probert, Crawford, Cadiz, & LeFebvre, 1997), and it is quite immunogenic, eliciting immune responses in numerous Lyme disease animal models (de Silva, Telford, Brunet, Barthold, & Fikrig, 1996; Fikrig, Barthold, Kantor, & Flavell, 1990, 1992; Probert et al., 1997). Indeed, an OspA vaccine had an efficacy of 79% in phase III human clinical trials, leading to an FDA-approved Lyme disease vaccine available from 1998 until 2002 (Abbott, 2006). Currently, there is no available Lyme disease vaccine for humans (Embers & Narasimhan, 2013).

It has been well established that antibodies are critical mediators in protection against Lyme disease (Fikrig, Barthold, Chen, Chang, & Flavell, 1997; McKisic & Barthold, 2000). Passive immunization with serum from acutely infected mice (Barthold, Feng, Bockenstedt, Fikrig, & Feen, 1997) or chronically infected humans (Fikrig et al., 1994) has been shown to provide protection in mice against tick-mediated Lyme challenge. Interestingly, OspA antibodies elicited by immunization with an OspA vaccine prevent *B. burgdorferi* infection by binding to and killing OspA-expressing spirochetes directly in the tick midgut, preventing migration of the bacteria to the salivary glands before transmission to the host can occur (de Silva et al., 1996; Fikrig, Telford, et al., 1992; Nowling & Philipp, 1999).

DNA monoclonal antibody (DMAb) delivery provides an alternative to vaccination or passive antibody therapy for generating rapid, antibody-based immunity against infectious diseases. DMAbs are highly optimized DNA plasmids capable of delivering genes encoding well-characterized monoclonal antibodies (mAbs) to hosts. We have previously described DMAb delivery in the context of HIV, generating Fab fragments of the human broadly neutralizing antibody VRC01 in mice after intramuscular injection of DMAb plasmids and *in vivo* electroporation (EP) (Muthumani et al., 2013). More recently, DMAb delivery in mice generated long-lived full-length human IgG neutralizing antibodies against Dengue virus that completely protected mice from virus-only and lethal antibody-dependent enhancement Dengue challenge (Flingai et al., 2015).

However, higher DMAb expression levels may be necessary for prophylactic or therapeutic benefits for a number of indications. As such, we investigated optimizations that may increase DMAb-mediated antibody concentrations *in vivo*. Previous studies have shown that improving antibody stability through targeted framework mutations and grafts can increase *in vitro* antibody production levels without perturbing antibody

functionality (Ewert, Honegger, & Pluckthun, 2003, 2004; Honegger, Malebranche, Rothlisberger, & Pluckthun, 2009; Jung & Pluckthun, 1997; Knappik et al., 2000). Use of these optimizations in vector-mediated antibody gene transfer systems has been limited, with one study showing that minor mutations to the light chain of an influenza antibody delivered with AAV could improve expression levels *in vivo* (Balazs et al., 2013). Additionally, several studies have shown that the use of the enzyme hyaluronidase before DNA plasmid injection significantly increased gene delivery to skeletal muscle cells (Long et al., 2005; McMahon, Signori, Wells, Fazio, & Wells, 2001; Mennuni et al., 2002; Molnar et al., 2004). We sought to explore the use of these approaches, as well as altering DMAb dosing, in an effort to increase DMAb expression levels *in vivo*.

Here, we assess the ability of targeted framework modifications, hyaluronidase pre-treatment of mouse muscle, and increased DMAb dosing to increase the *in vivo* production of human anti-Lyme IgG antibodies. We show that these optimizations, when used together, can increase DMAb expression levels *in vivo* between 2.4 to 6.4-fold without diminishing antibody functionality and that these improvements are long lasting in immunodeficient mice.

RESULTS

Characterization of anti-Lyme DMAbs. In collaboration with the non-profit organization MassBiologics, we obtained the gene sequences of four anti-Lyme human IgG antibodies with varying breadths of *Borrelia* neutralization capabilities, OspA affinities, and protective efficacies in mice (Table 1). We developed DMAb vectors expressing 319-44, 221-7, 212-55, and 857-2 by inserting the heavy and light chains of the human IgGs into our previously described DMAb expression construct (Flingai et al., 2015). We transfected human embryonic kidney (HEK) 293T cells with the four DMAb

vectors or an empty pVax vector vehicle, collected the supernatant 48 hours later, and compared human IgG expression levels *in vitro*. The plasmid p319-44 showed the highest secreted human IgG production levels of all four anti-Lyme DMAbs, with the most potent IgG, 221-7, expressing the lowest concentration of human IgG after transfection (Figure 23). Importantly, supernatant from all four DMAb transfections displayed binding to immobilized his-tagged OspA (hisOspA) protein.

In order to determine anti-Lyme DMAb expression levels in a relevant mouse model, C3H/HeNCrl mice were injected in the quadriceps muscle with 100 μg of p319-44, p221-7, p212-55, p857-2, or empty pVax vector vehicle. Within 48 hours, mice given p319-44 seroconverted to hisOspA, and by 1 week post-injection, 319-44 and 857-3 IgG levels reached 400-500 ng/mL. Serum concentrations of 212-55 and 221-7 were drastically lower at day 7, only reaching 50-150 ng/mL in mice (Figure 24). Nonetheless, all mice receiving anti-Lyme DMAbs seroconverted by day 7, illustrating the rapid expression kinetics of DMAb delivery *in vivo*.

Framework optimization of 319-44 DMAb. Given the modest expression levels of some anti-Lyme DMAbs, we investigated methods to increase DMAb expression *in vivo.* We focused on optimizing p319-44, as this DMAb expressed a fairly potent mAb that showed promising expression levels in mice. To improve p319-44 DMAb expression, we further optimized both the light chain and heavy chain variable region sequences with a targeted approach that focused on increasing antibody stability through significant framework region modifications (Figure 25), a process that has been shown to improve antibody production in both *in vitro* (Ewert et al., 2003, 2004; Honegger et al., 2009; Jung & Pluckthun, 1997; Knappik et al., 2000) and *in vivo* (Balazs et al., 2013) settings. First, we identified a high-expressing DMAb to act as an acceptor framework for grafting. This acceptor DMAb, which has been shown to express >5

μg/mL of human IgG in immunodeficient mice after a single dose of 100 μg DNA (data not shown), is comprised of heavy and light chains from the highly stable germline families hV_H3 and hVκ1, respectively. To create the optimized 319-44, we grafted the three CDRs and an additional 22 crucial heavy and light chain framework residues of 319-44 onto the high-expressing DMAb genes.

After confirming that these modifications did not alter the capacity of 319-44mod1 DMAb production of human IgG antibodies *in vitro* (Figure 26), we assessed whether the optimizations would increase the production levels of 319-44 by comparing human IgG concentration levels in 293T cell supernatants following transfection of cells with the wild type (319-44wt) or optimized (319-44mod1) DMAb constructs (Figure 27). 319-44wt and 319-44mod1 showed comparable binding to purified hisOspA protein, yet 319-44mod1 production levels were over two-fold higher than the wild-type DMAb. To ensure that the optimizations of 319-44mod1 did not abrogate borreliacidal capacity, we compared the two DMAbs in an *in vitro* borreliacidal assay against *B. burgdorferi*. 319-44mod1 showed a 11-fold increase in potency (measured as EC_{50}) compared to 319-44wt (Figure 28). Thus, we were able to increase DMAb expression levels and maintain wild-type functional activity through systematic engineering of framework optimizations.

Systemic expression of 319-44 DMAbs in mice. We have demonstrated recently that a single intramuscular administration of a DMAb encoding an anti-dengue virus neutralizing mAb resulted in serum levels of slightly over 1 μg/mL of mAb in mice (Flingai et al., 2015). Here, we sought to determine whether increasing DMAb dosing and distributing this dosage across multiple muscles could lead to greater human IgG expression levels *in vivo.* To assess 319-44 DMAb expression levels in mice, we injected various doses of either 319-44wt or 319-44mod1 DMAbs into the tibialis anterior (TA) or quadriceps muscles of C3H/HeNCrl mice, followed by EP. Administration of 100

μg, 200 μg, or 300 μg of 319-44wt DMAb resulted in a dose-dependent increase in antibody concentrations in serum, as mice receiving the highest dose of 319-44wt showed an average serum concentration levels of 1.2 μg/mL one week post-administration (Figure 29). The same dose of 319-44mod1 showed an even greater serum antibody concentration of 1.4 μg/mL one week post-administration, illustrating the positive effect of the amino acid optimizations on antibody concentration *in vivo*, albeit to a less drastic effect than seen *in vitro*. Using an anti-hisOspA binding ELISA, we demonstrated that the optimized antibodies, when expressed from muscle cells *in vivo*, show a slight dip in antigen binding compared to 319-44wt, but ultimately retain antigen binding to recombinant hisOspA protein (Figure 29).

Pre-treatment with hyaluronidase significantly increases 319-44 DMAb expression *in vivo*. Previous studies have demonstrated the positive effect of hyaluronidase on gene transfer in skeletal muscle (Long et al., 2005; McMahon et al., 2001; Mennuni et al., 2002; Molnar et al., 2004). To investigate whether DMAb production would be improved by pre-treatment of muscle cells with hyaluronidase, we injected C3H mice in the TA muscle, the quadriceps muscle, or both, with 0.4 U/μL of bovine hyaluronidase in a 30 μL volume 30 minutes prior to EP-mediated DMAb delivery. At 7 days post-injection, we measured human IgG concentration levels in mouse sera by anti-human IgG ELISA. Mice receiving 319-44wt DMAb without EP showed undetectable human IgG serum levels, regardless of whether the muscles were pre-treated with hyaluronidase (data not shown). However, mice that received EP-mediated DMAb delivery with hyaluronidase pre-treatment saw human IgG concentration levels increase by between 2.4 to 6.4-fold above animals that received EP-mediated DMAb delivery only (Figure 30). After a 300 μg dose of EP-mediated DMAb delivery with hyaluronidase pre-treatment in mice, the average human IgG serum

concentration of 319-44wt IgG peaked at 5.7 μg/mL, while 319-44mod1 IgG peaked at an average of 6.7 μg/mL.

To assess the effect of hyaluronidase pre-treatment on long-term DMAb expression kinetics, immunodeficient Foxn1nu/J mice received 300 μg of 319-44mod1 DMAb delivered with EP with or without hyaluronidase pre-treatment. Not only did hyaluronidase pre-treatment lead to increased 319-44mod1 expression levels by day 7 post-injection, but the increase in serum human IgG concentration was sustained over the course of 4 weeks, peaking at 5 μg/mL 14 days after DMAb administration (Figure 31).

DISCUSSION

There are many advantages to the use of DNA plasmid as a tool for antibody gene transfer. The simplicity of use, the lack of anti-vector immunity, the excellent safety profile, and the relatively inexpensive production costs compared to viral vectors make DNA plasmid-mediated antibody gene transfer an attractive alternative to viral vector approaches. However, one disadvantage is the relatively low mAb expression levels from DMAb delivery compared to AAV- or Ad-mediated antibody gene transfer. In this study, we explored three strategies to improve DMAb delivery *in vivo*: 1) antibody framework modifications to increase mAb stability; 2) DMAb dosing; and 3) pre-treatment of muscle cells with the enzyme hyaluronidase. While many of these strategies have been employed in other DNA delivery or mAb production contexts, there are still many questions to be answered regarding their use in DNA plasmid-mediated antibody gene transfer.

Antibody framework modifications led to substantial increases in human IgG production *in vitro*, with only moderate increases observed *in vivo*. This may be

explained by the fact that these stabilizing modifications have been extensively investigated in the context of *in vitro* antibody production and not in *in vivo* environments inside the host's muscle cells. Perhaps this *in vivo* mAb production environment dampens the positive effects seen *in vitro*; it would be advantageous to determine which framework modifications are responsible for mAb stability *in vivo*, and how these stabilizing modifications affect DMAb-generated human IgG concentration levels in serum. Further studies are needed in order to determine whether such modifications should be a major aspect of DMAb optimization strategies going forward. Importantly, if framework modifications are made, both antigen binding and functionality should be evaluated. In this particular study, the protective efficacy of 319-44wt and 319-44mod1 should be compared in a tick-mediated *B. burgdorferi* challenge model in mice.

While DMAb dosing had a clearly positive effect on human IgG concentration levels in mouse serum, intramuscular hyaluronidase pre-treatment amplified the effect to a drastic degree. Indeed, mice receiving 300 μg of 319-44mod1 without hyaluronidase had human IgG concentration levels less than mice receiving 100 μg of 319-44wt with hyaluronidase. These results suggest that hyaluronidase could act as a dose-sparing agent, which has the additive effect of minimizing the number of DMAb injection sites. Alternatively, hyaluronidase pre-treatment boosted human IgG concentrations to nearly 10 μg/mL in the mice also receiving the 300 μg dose of 319-44mod1, showing the combinatorial effect all three optimization strategies can have on DMAb expression *in vivo*. Optimal hyaluronidase pre-treatment time intervals and dosing should be explored in future studies. Taken together, this work shows a number of ways DMAb delivery can be enhanced for increase *in vivo* human IgG expression.

Criteria	319-44	221-7	212-55	857-2
Burgdorferi neutralization	YES (<0.4 nM)	YES (<0.4 nM)	YES (1.4 nM)	YES (2.0 nM)
Afzelii neutralization	YES (4.0 nM)	YES (0.9 nM)	NO	YES (2.0 nM)
Garinii neutralization	NO	YES (6.6 nM)	NO	YES (41.6 nM)
OspA affinity	328 nM	0.66 nM (Az 7.8; Gn 1.3)	0.43 nM	0.65 nM (Az 1.6; Gn 1 nM)
Protection (10mg/kg)	YES	YES	YES	YES
Protection (1mg/kg)	40%	60%	NA	NA

Table 1. Characterization of anti-Lyme purified antibodies used in study.

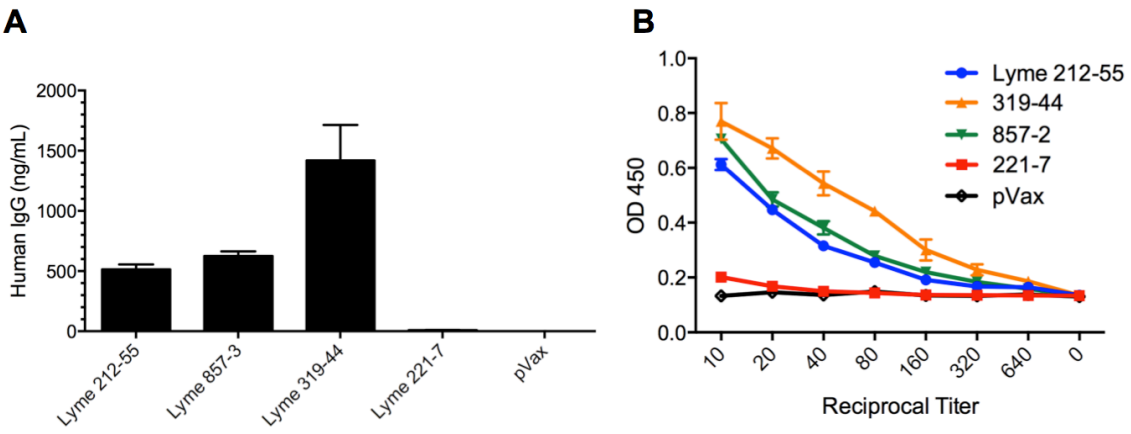


Figure 23. Comparison of various anti-B. burgdorferi DMAbs in vitro.
ELISA human IgG quantification (A) and immobilized hisOspA protein binding (B) analysis of human IgG in supernatants of HEK 293T cells transfected with either p212-55, p857-3, p319-44wt, p221-7, or pVax empty plasmids. The data displayed are the mean of duplicate values +/- standard error of the mean (SEM) and are representative of two independent experiments.

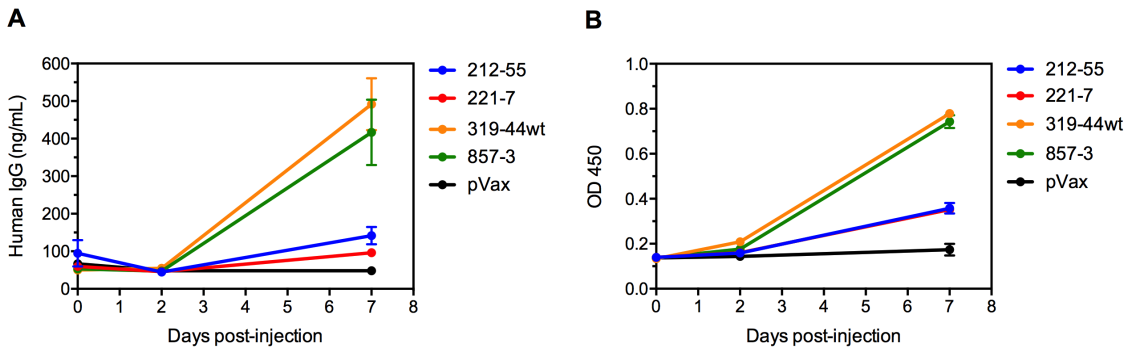


Figure 24. Expression of various anti-*B. burgdorferi* DMAbs *in vivo*.
Total human IgG in serum (A) or binding to immobilized hisOspA protein (B) was measured by ELISA after intramuscular injection of p319-44wt, p857-3, p212-55, p221-7, or pVax empty plasmids in C3H/HeNCrl mice (n = 5 mice per group, data displayed are the mean +/- SEM of each group's animals and are representative of two independent experiments).

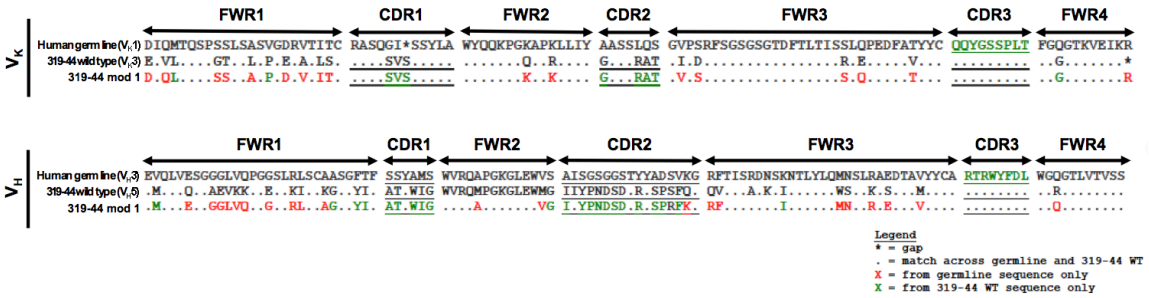


Figure 25. Design of 319-44mod1 through antibody framework optimization of 319-44wt.

Amino acid alignment of 319-44wt (319-44 wild type), 319-44mod1, and the human germ line sequences (Vκ1 for light chain, V_H3 for heavy chain) on which 319-44mod1 optimizations are based. 319-44mod1 amino acid residues identical to only 319-44wt sequences (green) or the human germ line sequence (red) are shown. Amino acids identical across all three sequences are indicated by periods. The complementarity-determining regions (CDRs) of 319-44wt and 319-44mod1 are identical; the CDR3 region of 319-44wt and 319-44mod 1 are shown in the human germ line CDR3 region above due to the uncertainty of the original human germ line CDR3 sequences.

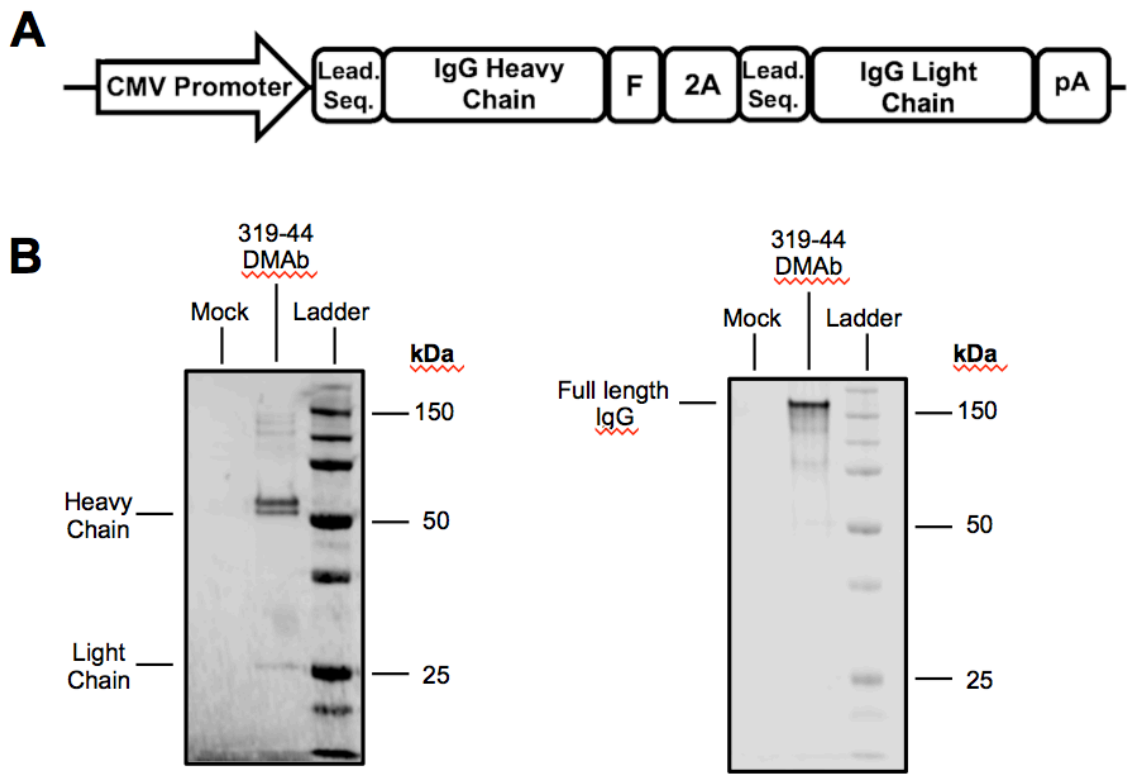


Figure 26. Design and production of 319-44mod1 DMAb.
(A) Plasmid design for single-plasmid 319-44mod1 DMAb. F, furin cleavage site; 2A, P2A peptide cleavage site; pA, polyadenylation sequence. (B) Western blot analysis of p319-44mod1 DMAb-transfected 293T supernatants containing 319-44mod1 human IgG. Antibodies were purified by Protein A spin columns and separated by SDS-PAGE under reducing (left) and non-reducing (right) conditions.

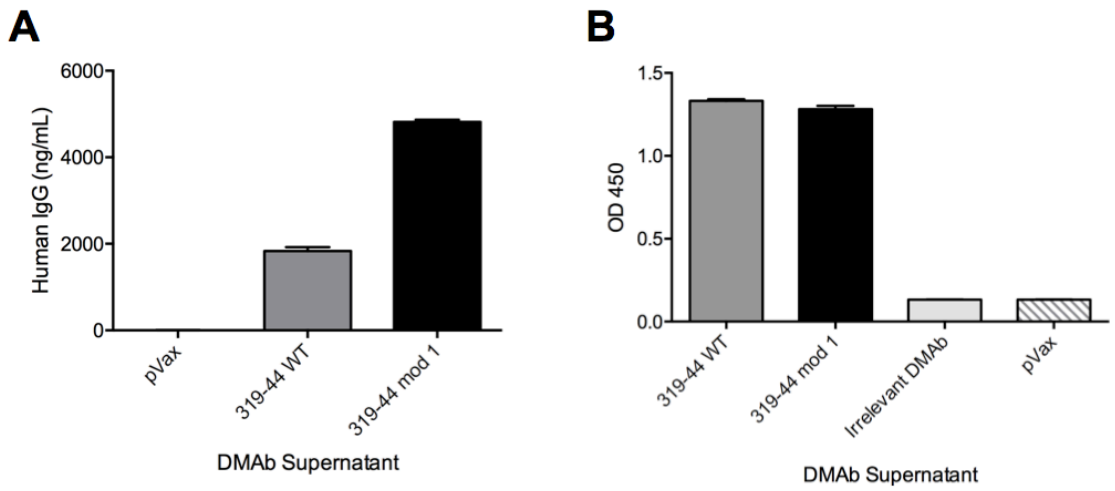
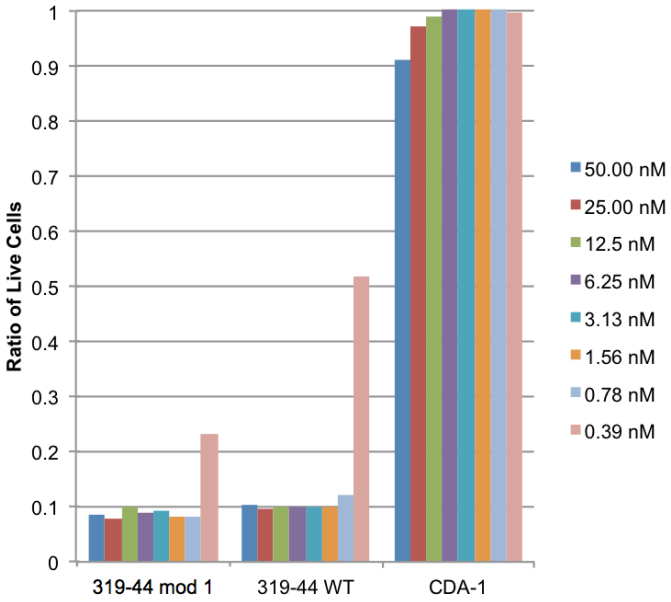


Figure 27. 319-44mod1 exhibits increased human IgG production compared to 319-44wt *in vitro.*

ELISA human IgG quantification (A) and immobilized hisOspA protein binding (B) analysis of human IgG in supernatants of HEK 293T cells transfected with either p319-44wt or p319-44mod1 DMAbs. The data displayed are the mean of duplicate values +/- standard error of the mean (SEM) and are representative of two independent experiments.



MAb	EC50 (nM)
319-44 WT	0.34
319-44 mod 1	0.03
CDA-1	>50

Figure 28. Framework modifications of 319-44mod1 have no detrimental effect on antibody borreliacidal activity *in vitro*.
Purified 319-44wt or 319-44mod1 antibodies were evaluated with a borreliacidal assay against *B. burgdorferi* bacteria (left; CDA-1 antibody = negative control); EC50 values of purified antibodies (right).

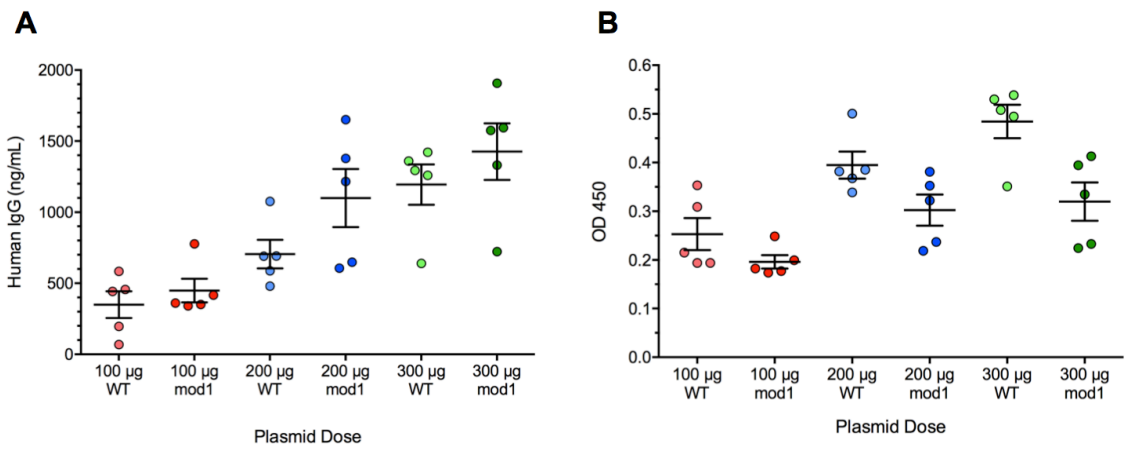


Figure 29. *In vivo* delivery of 319-44mod1 DMAb leads to slight increase in 319-44 IgG with concomitant reduction in purified hisOspA binding capacity.
Total serum-detectable levels of human IgG (A) and immobilized hisOspA protein binding (B) were measured by ELISA after various doses of 319-44wt or 319-44mod1 DMAb were injected intramuscularly in C3H/HeNCrl mice. (n = 5 mice per group, dots represent individual animals within a group, while bars represent mean +/- SEM of the group).

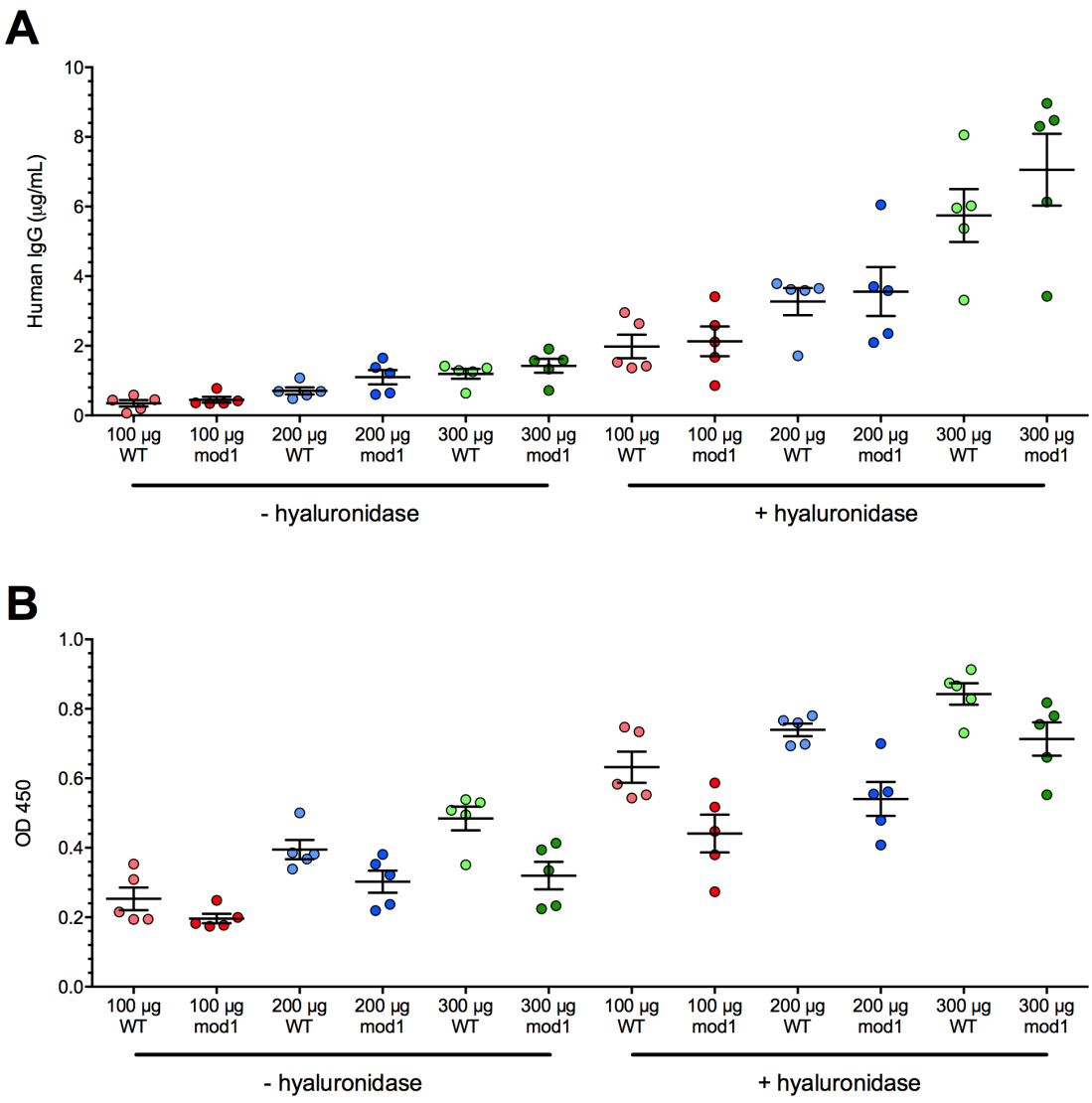


Figure 30. DMAb delivery with hyaluronidase pre-treatment increases 319-44 IgG concentration *in vivo*.
Total serum-detectable levels of human IgG (A) and immobilized hisOspA protein binding (B) were measured by ELISA after various doses of 319-44wt or 319-44mod1 DMAb were injected intramuscularly with or without hyaluronidase pretreatment in C3H/HeNCrl mice. (n = 5 mice per group, dots represent individual animals within a group, while bars represent mean +/- SEM of the group).

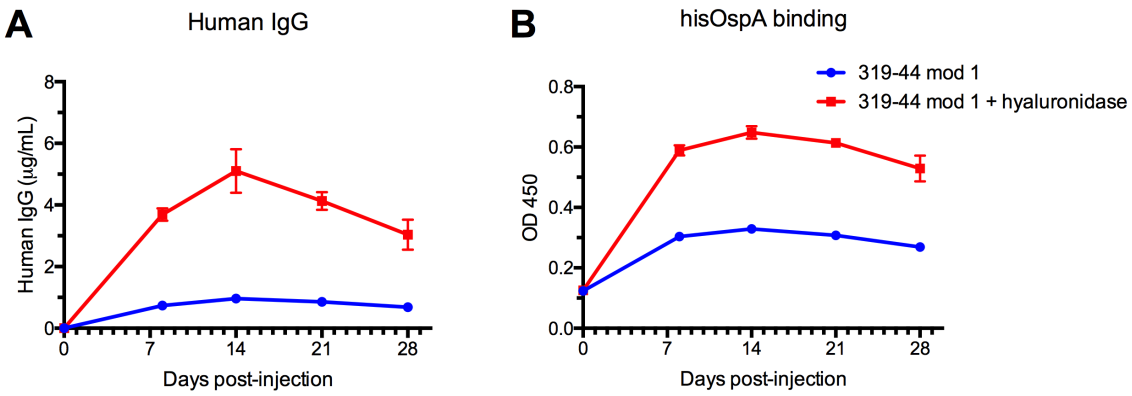


Figure 31. Hyaluronidase pre-treatment before DMAb delivery leads to long-term increase in human IgG concentration *in vivo*.
Total serum-detectable levels of human IgG (A) and immobilized hisOspA protein binding (B) were measured by ELISA after a 300 µg dose of 319-44mod1 DMAb was injected intramuscularly with or without hyaluronidase pretreatment in B6.Cg-Foxn1nu/J immunodeficient mice. (n = 5 mice per group, data displayed are the mean +/- SEM of each group's animals).

CHAPTER 5 – Discussion and Future Directions

The promise of vector-mediated antibody gene transfer has generally been countered by the field's predominant use of viral vectors, which time and again have shown the pesky trait of being targeted by pre-existing anti-vector immunity, or of eliciting an anti-vector immune response upon administration. These responses have been known to limit the clinical efficacy of viral vector gene transfer in numerous studies (Jiang et al., 2006; Manno et al., 2006; Scallan et al., 2006), even if animal models generally downplay such effects. An alternative to viral vector-mediated antibody gene transfer is DNA plasmid-mediated antibody gene transfer. Unlike viral vectors, DNA plasmids do not elicit anti-vector immunity (Klinman et al., 2000), which allows for repeated administrations of plasmid vectors when desired (Klinman et al., 2000; MacGregor et al., 2000). Furthermore, the production of large stocks of DNA is relatively inexpensive when compared to the creation of clinical grade viral vectors for gene delivery. Despite these and many other advantages (ease of use, great safety record, etc.), DNA plasmids have rarely been used as a tool to deliver monoclonal antibody genes *in vivo*. Such a dearth of research on this topic has led our lab to investigate the feasibility of DNA plasmid-mediated antibody gene transfer. Using the gene sequences of well-characterized mAbs targeting two distinct pathogens – dengue virus (subject of Chapter 3) and the *B. burgdorferi* bacteria responsible for Lyme disease (subject of Chapter 4) – we outline in this thesis the development of and optimization strategies for an effective DNA plasmid-based mAb delivery system, which we call DMAb.

This study is the first demonstration of a DNA plasmid being used to deliver full-length human IgG antibody genes for *in vivo* production (Figure 3, Figure 12). After a single intramuscular injection of a DNA plasmid cocktail encoding the heavy and light

chains of a human IgG1 antibody on two separate plasmids, biologically active antibodies were produced and properly assembled within muscle cells and secreted into the circulation within 48 hours of DNA injection (Figure 3). Seeing a need to simplify DMAb delivery and maximize the chances of the heavy and light chain genes being transcribed within the same muscle cell, we then developed a single plasmid DMAb vector in which the heavy and light chain genes of a mAb are separated on a single open reading frame by two cleavage sites used extensively in nature: the 2A peptide and a furin cleavage site (Figure 2, Figure 4). These plasmid modifications showed no detrimental effect on human IgG expression levels *in vitro*, suggesting that a single plasmid system could be feasible for further study *in vivo*. Indeed, our single-plasmid anti-DENV DMAb, DVSF-3, when injected intramuscularly with EP into mice, showed that this simplified approach could produce biologically relevant levels of human IgG in mouse serum within 1 week of DMAb administration (Figure 13). Importantly, mice injected with the modified DMAb pDVSF-3 LALA were completely protected from a lethal ADE DENV2 challenge, even though the DMAb was administered only five days prior to challenge (Figure 19). These results highlight the rapid expression kinetics of DMAb-driven ectopic human IgG expression in mice.

Similar kinetics have been observed after Ad-mediated antibody gene transfer, yet there are two characteristics that differentiate these approaches. Firstly, Ad-mediated antibody gene transfer in mice produces antibody expression levels above 100 µg/mL *in vivo*, whereas DMAb delivery in this study leads to human IgG serum concentrations between 500 ng/mL and 10 µg/mL in mice. However, these numbers deserve proper context. Studies employing Ad-mediated antibody gene transfer typically use murine mAbs, which abrogates immune responses against the transgene product in mice. In our lab, we compared DMAb-mediated human IgG expression to DMAb-mediated murine

IgG expression in mice and observed that DMAb-mediated murine IgG expression is many fold greater than DMAb-mediated human IgG expression (data not shown), suggesting that the species of the antibody plays a fundamental role in even short-term expression kinetics in animal models. Secondly, Ad-mediated antibody gene transfer, as with other viral vector systems, elicits anti-vector immune responses, whereas DMAb delivery does not. These major differences frame DMAb delivery as a promising alternative to viral vector-based antibody gene transfer that would greatly benefit from exploring multi-DMAb delivery approaches and optimizations to improve antibody expression levels *in vivo*.

Future directions: post-exposure prophylaxis or therapy

Several infectious diseases, including hepatitis A and B, rabies, and tetanus, have established post-exposure prophylaxis (PEP) protocols that are effective at preventing the development of disease or limiting the transmissibility of an infectious agent after initial exposure (Bader & McKinsey, 2013). Many of these treatments use intravenous immune globulin (IVIG) regimens alone or in combination with available vaccines. Our data suggest that DMAb delivery in mice leads to rapid production of human IgG, typically detectable in serum between 24 and 48 hours (Figure 3, Figure 12). Future studies should alter when DMAb is administered in relation to pathogen challenge to assess whether DMAbs may prove beneficial as tools for PEP. Furthermore, recent work from our lab has demonstrated the protective “immediate and persistent” efficacy of DMAb and vaccine co-administration in the context of chikungunya infection (Muthumani et al., 2016), suggesting that DMAb/DNA vaccine formulations should also be studied for their ability to replicate IVIG/vaccine regimens for PEP.

Future directions: delivery of multiple DMAbs

In Chapter 3, we used an anti-Dengue virus antibody model to explore DMAb-mediated human IgG production *in vivo*, concluding those studies with preliminary work on multi-DMAb delivery in a host (Figure 20, Figure 21, Figure 22). Our data suggest that the breadth of coverage against a pathogen can be increased through multi-site, multi-DMAb delivery, which would be a crucial need for any comprehensive antibody-based prophylactic that aims to minimize antibody escape. More studies should be devoted to optimally delivering multiple DMAbs in a simpler fashion, keeping in mind the dangers of antibody heavy chain-light chain protein rearrangements that may occur if different antibodies are being expressed in the same cell.

One future goal of these studies is to develop new antibody structures that eliminate the possibility of heavy chain-light chain protein rearrangements; such an antibody structure would allow for multiple DMAbs to be delivered into a single site. Alternatively, developing bispecific DMAbs could increase the breadth of antibody targets without the need to deliver multiple DMAb plasmids. Regardless of the method employed, clinical success of any antibody gene transfer approach will have to consider how to minimize antibody escape, no matter how broadly neutralizing a single delivered antibody may be. Antibody cocktail studies in mice and nonhuman primates have worked to address issues of antibody escape to varying degrees of success, so it is in the antibody gene transfer field's benefit to do the same.

Future directions: optimization strategies

In Chapter 4, we used an anti-Lyme disease antibody model to explore a three-tiered optimization strategy to improve DMAb expression levels *in vivo*: DMAb dosing, antibody framework modifications, and hyaluronidase pre-treatment.

DMAb dosing. Building on our previous work that suggested multiple injections of DMAb plasmids could increase the breadth of coverage against DENV and boost total human IgG expression levels in mouse serum (Figure 20, Figure 21, Figure 22), we established DMAb dosing as one tier for improving DMAb expression levels *in vivo.* The dosage studies outlined in Chapter 4 of this thesis show that there is a DMAb dose-dependent escalation in human IgG levels in mouse serum that appears to be linear in nature (Figure 29). Further studies need to be conducted to determine how much DMAb plasmid can be administered per muscle site without diminishing returns.

Antibody framework modifications. Framework modifications in the realm of antibody gene transfer is a relatively unexplored field, with the majority of studies focusing less on improving antibody stability and more on reducing immunogenicity of the transgene in large animal models. We sought to use well-established stabilizing framework modifications to improve DMAb expression levels *in vivo*, with the assumption that enhanced antibody stability *in vitro* could also enhance the longevity of antibodies *in vivo*. The framework modifications of 319-44mod1 led to greatly increased *in vitro* mAb production and a slight increase in *in vivo* mAb production, suggesting that these modifications may be a promising avenue of exploration to improve antibody concentrations in the circulation.

However, there is still much to understand about these framework modifications and how they may affect antibody half-life and functionality *in vivo*. Future studies should focus on two key areas of inquiry: 1) determining which antibody framework families

(e.g. V_H1 or Vκ3) are most stable *in vivo*, and whether this stability leads to higher antibody peak concentrations, a more durable increase in serum concentration levels, or both; and 2) assessing the antibody structure pre- and post-modifications to better understand how these modifications may affect antigen binding and functionality. An interesting observation in Chapter 4 was that 319-44mod1 appears to have reduced antigen-binding capabilities to purified hisOspA protein compared to 319-44wt, but the borreliacidal activity was unperturbed. Therefore, both affinity and functionality must be investigated concomitantly in future studies.

Hyaluronidase pre-treatment. Our data suggest that hyaluronidase pre-treatment of muscle cells can greatly enhance DMAb gene delivery and lead to increased human IgG serum concentrations in mice. However, future studies should evaluate various hyaluronidase pre-treatment regimens in order to optimize DMAb delivery. For example, the interval between hyaluronidase pre-treatment and DMAb administration with EP should be more fully explored. For studies in Chapter 4, a 30-minute interval was sufficient for the enzyme to be absorbed in the targeted muscle area, but whether that period of time is sufficient to maximize DMAb delivery is currently unknown. Balancing the length of this pre-treatment interval with the positive effects on DMAb expression is a key optimization step with relevance both in animal models and in the clinic, where one could imagine that too long of an interval may present practical logistical issues (e.g. extended doctor visits).

Hyaluronidase dosing is another aspect that must be optimized. Studies have shown that hyaluronidase pre-treatment does not increase muscle damage (Long et al., 2005; McMahon et al., 2001), but these studies have only used a single hyaluronidase dose. Furthermore, hyaluronidase has been shown to amplify the inflammatory effects of *in vivo* EP (Chiarella, De Santis, Fazio, & Signori, 2013), suggesting that future DMAb

studies using hyaluronidase should be aware of how this inflammatory milieu may exacerbate anti-transgene immune responses in immune competent animal models. Optimizing hyaluronidase dosing may help balance the potentially detrimental inflammatory response of EP + hyaluronidase with the hyaluronidase-mediated boosting of DMAb-mediated human IgG expression levels. Therefore, hyaluronidase pre-treatment requires optimizing on multiple levels, and future studies should compare various combinations of hyaluronidase dose and pre-treatment timing to improve DMAb delivery *in vivo*.

Summary and concluding thoughts.

In summary, we show that DNA plasmid-mediated antibody gene transfer is a feasible method for delivering biologically active human IgG antibodies *in vivo.* DMAb delivery of an anti-DENV neutralizing antibody was capable of completely protecting mice from a lethal ADE DENV challenge, and optimizations to DMAb delivery via antibody framework modifications, DMAb dosing, and hyaluronidase pre-treatment were able to significantly increase human IgG concentrations in mouse serum. The major focus of future studies should be on optimizing DMAb-mediated mAb cocktail delivery while increasing antibody concentration levels in serum.

As researchers continue to refine the process of mAb discovery, it is expected that more potent antibodies will be found that can be delivered by antibody gene transfer approaches. These highly potent antibodies may offset the requirement for large serum concentration levels of human IgG for prophylaxis or treatment. As DMAb technology progresses alongside this improvement in mAb discovery, the clinical feasibility of DMAb delivery will only increase.

The public health impacts of DMAb technology use in humans could be immense. With less dependence on the cold chain compared to purified protein and viral stocks, DMAbs may have a substantial impact in locations where the electrical grid and health care infrastructure lack stability. This benefit could have major importance during infectious disease outbreaks, when rapid protection is desired but health care systems are overwhelmed. From an economic standpoint, the savings in manufacturing costs (no protein or viral purification) and health care infrastructure costs (no cold chain) suggest that DMAb technology may have a favorable cost-effectiveness profile compared to other mAb delivery and antibody gene transfer methods. Of course, these implications are only important if efficacy is achieved in non-human primates and clinical trials; the next steps of this technology, in addition to all that has been mentioned above, involve determining the feasibility of DMAbs in larger animals and using those studies to inform dosing and administration regimens in humans.

Table A1. Summary of antibody gene transfer studies targeting infectious diseases.

Disease	Vector	Antibody type	Species	Clone	Animal Model	Route	Dose	Peak average expression	Reference
HIV	rAAV8	Full-length	Macaque	VRC07	SCID mice	IM	$2.5x10^{12}$ vg	377 µg/mL	Saunders et al., 2015
			Human	VRC07				100-200 µg/mL	
			Macaque	VRC07	Rhesus macaques		$1x10^{13}$ vg	38.12 µg/mL	
			Human	VRC07				–	
	rAAV8	Full-length	Human	VRC01	Humanized mice	IM	$1x10^{11}$ GC	200 µg/mL	Balazs et al., 2012
			Human	2G12				200 µg/mL	
			Human	b12				100 µg/mL	
			Human	2F5				20 µg/mL	
			Human	4E10				20 µg/mL	
	rAAV8	Full-length	Human	VRC01	Humanized mice	IM	$1x10^{11}$ GC	100 µg/mL	Balazs et al., 2014
				VRC07				100 µg/mL	
				b12				115 µg/mL	
	rAAV2	Full-length	Human	b12	Rag-1 mice	IM	$5x10^{11}$ GC	4-5 µg/mL	Lewis et al., 2002
	rAAV8	Full-length	Human	10-1074	Humanized mice	IV	$2.5×10^{11}$ GC	200 µg/mL	Horwitz et al., 2013
			Human	3BNC117		IV	$2×10^{11}$ GC	10-20 µg/mL	
	scAAV1	Full-length	Macaque	eCD4-Ig	Rhesus macaques	IM	$2.5x10^{13}$ particles	17-77 µg/mL	Gardner et al., 2015
	scAAV1			5L7				40-175 µg/mL	Johnson et al., 2009
	scAAV1	Immunoadhesin	Macaque	4L6	Rhesus macaques	IM	$2x10^{13}$ GC	100-190 µg/mL	
	rAAV1			N4				3-10 µg/mL	
	Lentivirus	Full-length	Human	2G12	Humanized mice	*Ex vivo* transduction of human hematopoietic stem cells	100 ng p24	40 ng/mL	Joseph et al., 2010
	DNA plasmid	Fab	Human	VRC01	BALB/c mice	IM	25 µg	2-3 µg/mL	Muthumani et al., 2013

Table A1 (continued)

	rAAV8	Full-length	Human	F10 CR6261	BALB/c mice	IM	1x10^{11} GC	100-200 µg/mL 0.1-100 µg/mL	Balazs et al., 2013
	rAAV9	Immunoadhesin	Human	FI6	BALB/c mice	Intranasal	10^{11} GC	--	Limberis et al., 2013
Influenza	rAAV9	Immunoadhesin	Human	FI6	BALB/c mice Ferrets	Intranasal Intranasal	10^{11} GC 10^{12} GC	0.31-0.63 µg/mL (NL) 1.34-2.18 µg/mL (BAL)	Limberis et al., 2013
	rAd5	single domain	Camel	aHA-7	BALB/c mice	Intranasal	10^{8} PFU 10^{7} PFU 10^{6} PFU	-- -- --	Tutykhina et al., 2013
	DNA plasmid	Full-length	Murine	--	ddY mice	IM + hyaluronidase	30 µg	19 µg/mL	Yamazaki et al., 2011
Malaria	rAAV8	Full-length	Human	2A10 2C11	C57BL/6 mice	IM	1x10^{11} GC	1 mg/mL 100-500 µg/mL	Deal et al., 2014
Hepatitis C	rAAV8	Full-length	Human	AR3A AR3B AR4A	NRG mice huFNRG mice	IM	1x10^{11} GC	1-3 mg/mL	de Jong et al., 2014
Dengue	DNA plasmid	Full-length	Human	82.11 87.1	Foxn1nu/J mice AG129 mice	IM	100 µg	1 µg/mL 1 µg/mL	Flingai et al., 2015
Chikungunya	DNA plasmid	Fab Full-length	Human	--	Foxn1nu/J mice	IM	100 µg	1.6 µg/mL 1.4 µg/mL	Muthumani et al., 2016
Yersinia pestis	Ad5	Full-length	Murine	--	C57BL/6 mice	IV	10^{11} pu	1.15 mg/mL	Sofer-Podesta et al., 2009
Anthrax	Ad5 rAAVrh.10	Full-length	Murine	14B7-1H	C57BL/6 mice	IV Intrapleural	10^{11} pu 10^{11} GC	-- --	De et al., 2008
	Ad	Bispecific	Camel	VNA2-PA	C57BL/6 mice BALB/c mice	IV IV	3x10^{10} pu 3x10^{10} pu	6.2 mg/mL 419 µg/mL	Moayeri et al., 2016
RSV	Ad5 rAAVrh.10	Full-length	Murine	palivizumab (Synagis)	BALB/c mice	IV Intrapleural	5x10^{10} pu 10^{11} GC	-- --	Skarcic et al., 2008

Abbreviations: HIV, human immunodeficiency virus; RSV, respiratory syncytial virus; rAAV, recombinant adeno-associated virus; scAAV, self-complementary adeno-associated virus; Ad, adenovirus; IM, intramuscular; IV, intravenous; vg, vector genomes; GC, genome copies; PFU, plaque forming units; pu, particle units; NL, nasal lavage; BAL, bronchoalveolar lavage

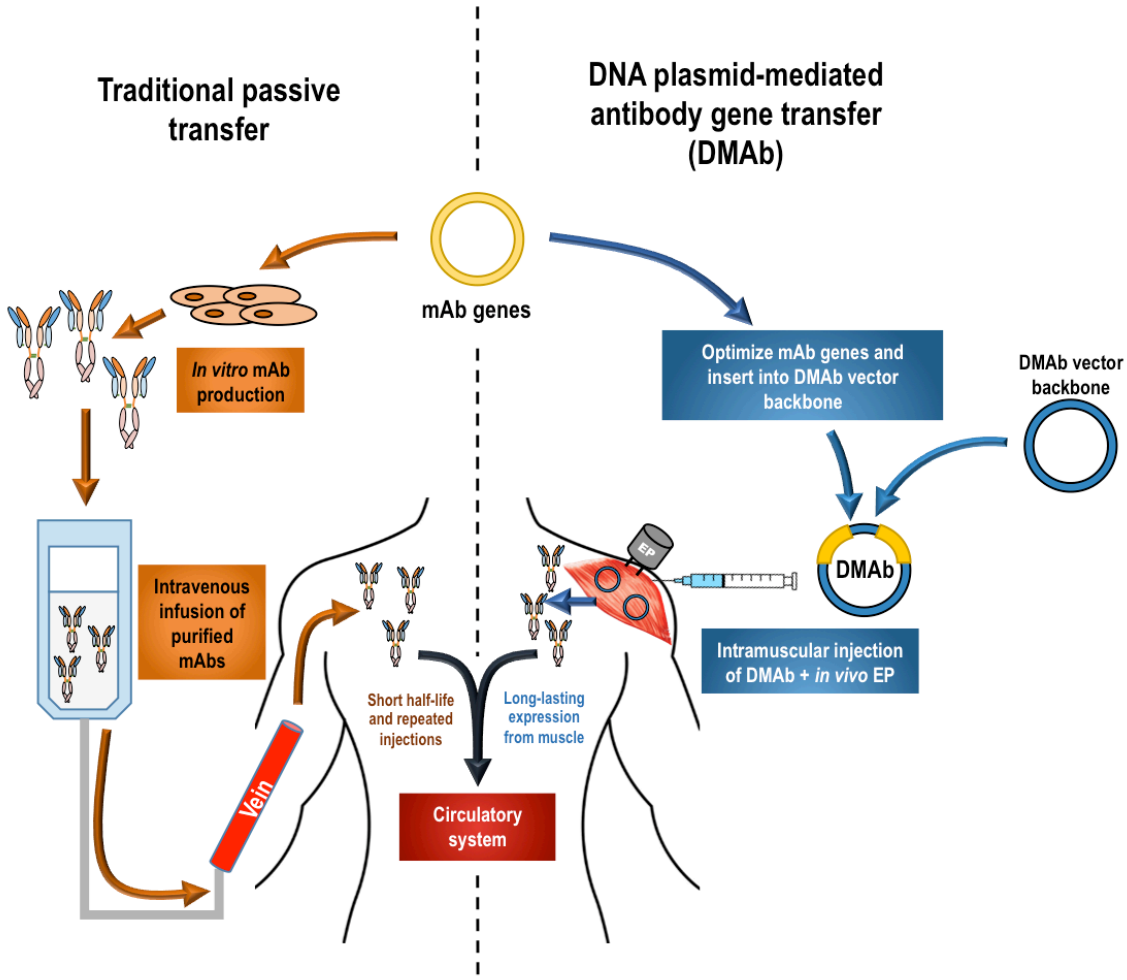


Figure A1. DNA plasmid-mediated antibody gene transfer
Traditional passive transfer requires the large-scale, expensive production of purified mAbs and the intravenous infusion of those mAbs into hosts; thi 5s process generally results in short-term duration of mAbs in the circulation, necessitating repeated infusions for clinical benefit. DNA plasmid-mediated antibody gene transfer, or DMAb delivery, involves the intramuscular injection of optimized antibody genes delivered by *in vivo* electroporation (EP). Through this process, muscle cells become antibody-producing factories that continuously secrete mAbs into the circulation.

CHAPTER 7 – Publications

The following papers have been published in support of this dissertation, starting with the most recently published work.

Rapid and long-term immunity elicited by DNA encoded antibody prophylaxis and DNA vaccination against Chikungunya virus.

Muthumani K, Block P, **Flingai S**, Muruganantham N, Chaaithanya IK, Tingey C, Wise M, Reuschel EL, Chung C, Muthumani A, Sarangan G, Srikanth P, Khan AS, Vijayachari P, Sardesai NY, Kim JJ, Ugen KE, Weiner DB. *J Infect Dis* doi:10.1093/infdis/jiw111 (2016).

Abstract

Background. Vaccination and passive antibody therapies are critical for controlling infectious diseases. Passive antibody administration has limitations including the necessity for purification and the delivery of multiple injections required for efficacy. Vaccination is associated with a lag phase before generation of immunity. Novel approaches reported here utilize the benefits of both methods for the rapid generation of effective immunity.

Methods. An antibody-based prophylaxis/therapy entailing the electroporation-mediated delivery of synthetic plasmids, encoding biologically active anti-Chikungunya virus envelope mAb (designated dMAb), was designed and evaluated for anti-viral efficacy as well as for the ability to overcome shortcomings inherent with conventional active vaccination by a novel passive immune-based strategy.

Results. One intramuscular injection of the CHIKV-dMAb produced antibodies in vivo more rapidly than active vaccination with a CHIKV-DNA vaccine. This dMAb neutralized diverse CHIKV clinical isolates and protected mice from viral challenge. Combinations of both afford rapid as well as long-lived protection.

Conclusions. We report that a DNA based dMAb strategy induces rapid protection against an emerging viral infection, which can be combined with DNA vaccination providing a uniquely both short term and long-term protection against this emerging infectious disease. These studies have implications for pathogen treatment and control strategies.

Protection against dengue disease by synthetic nucleic acid antibody prophylaxis/immunotherapy.

Flingai S, Plummer EM, Patel A, Shresta S, Mendoza JM, Broderick KE, Sardesai NY, Muthumani K, Weiner DB. *Sci Rep* **5**, 12616 (2015).

Abstract

Dengue virus (DENV) is the most important mosquito-borne viral infection in humans. In recent years, the number of cases and outbreaks has dramatically increased worldwide. While vaccines are being developed, none are currently available that provide balanced protection against all DENV serotypes. Advances in human antibody isolation have uncovered DENV neutralizing antibodies (nAbs) that are capable of preventing infection from multiple serotypes. Yet delivering monoclonal antibodies using conventional methods is impractical due to high costs. Engineering novel methods of delivering monoclonal antibodies could tip the scale in the fight against DENV. Here we

demonstrate that simple intramuscular delivery by electroporation of synthetic DNA plasmids engineered to express modified human nAbs against multiple DENV serotypes confers protection against DENV disease and prevents antibody-dependent enhancement (ADE) of disease in mice. This synthetic nucleic acid antibody prophylaxis/immunotherapy approach may have important applications in the fight against infectious disease.

Synthetic DNA vaccines: improved vaccine potency by electroporation and co-delivered genetic adjuvants.

Flingai S, Czerwonko M, Goodman J, Kudchodkar SB, Muthumani K, Weiner DB. *Front Immunol* **4**, 354 (2013).

Abstract

In recent years, DNA vaccines have undergone a number of technological advancements that have incited renewed interest and heightened promise in the field. Two such improvements are the use of genetically engineered cytokine adjuvants and plasmid delivery via in vivo electroporation (EP), the latter of which has been shown to increase antigen delivery by nearly 1000-fold compared to naked DNA plasmid delivery alone. Both strategies, either separately or in combination, have been shown to augment cellular and humoral immune responses in not only mice, but also in large animal models. These promising results, coupled with recent clinical trials that have shown enhanced immune responses in humans, highlight the bright prospects for DNA vaccines to address many human diseases.

Optimized and enhanced DNA plasmid vector based in vivo construction of a neutralizing anti-HIV-1 envelope glycoprotein Fab.

Muthumani K, **Flingai S**, Wise M, Tingey C, Ugen KE, Weiner DB. *Hum Vaccin Immunother* **9**, 2253-62 (2013).

Abstract

Monoclonal antibody preparations have demonstrated considerable clinical utility in the treatment of specific malignancies, as well as inflammatory and infectious diseases. Antibodies are conventionally delivered by passive administration, typically requiring costly large-scale laboratory development and production. Additional limitations include the necessity for repeat administrations, and the length of in vivo potency. Therefore, the development of methods to generate therapeutic antibodies and antibody like molecules in vivo, distinct from an active antigen-based immunization strategy, would have considerable clinical utility. In fact, adeno-associated viral (AAV) vector mediated delivery of immunoglobulin genes with subsequent generation of functional antibodies has recently been developed. As well, anon-viral vector mediated nucleic acid based delivery technology could permit the generation of therapeutic/prophylactic antibodies in vivo, obviating potential safety issues associated with viral vector based gene delivery. This delivery strategy has limitations as well, mainly due to very low in vivo production and expression of protein from the delivered gene. In the study reported here we have constructed an "enhanced and optimized" DNA plasmid technology to generate immunoglobulin heavy and light chains (i.e., Fab fragments) from an established neutralizing anti-HIV envelope glycoprotein monoclonal antibody (VRC01). This "enhanced" DNA (E-DNA) plasmid technology includes codon/RNA optimization, leader

sequence utilization, as well as targeted potentiation of delivery and expression of the Fab immunoglobulin genes through use of "adaptive" in vivo electroporation. The results demonstrate that delivery by this method of a single administration of the optimized Fab expressing constructs resulted in generation of Fab molecules in mouse sera possessing high antigen specific binding and HIV neutralization activity for at least 7 d after injection, against diverse HIV isolates. Importantly, this delivery strategy resulted in a rapid increase (i.e., in as little as 48 h) in Fab levels when compared with protein-based immunization. The active generation of functional Fab molecules in vivo has important conceptual and practical advantages over conventional ex vivo generation, purification and passive delivery of biologically active antibodies. Further study of this technique for the rapid generation and delivery of immunoglobulin and immunoglobulin like molecules is highly relevant and timely.

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