Development of N-glycan Specific Plant Produced Antibody Therapeutics for a Fine-tuned Immune Response

by

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ABSTRACT

Antibodies are naturally occurring proteins that protect a host during infection through direct neutralization and/or recruitment of the innate immune system. Unfortunately, in some infections, antibodies present unique hurdles that must be overcome for a safer and more efficacious antibody-based therapeutic (e.g., antibody dependent viral enhancement (ADE) and inflammatory pathology). This dissertation describes the utilization of plant expression systems to produce N-glycan specific antibody-based therapeutics for Dengue Virus (DENV) and Chikungunya Virus (CHIKV). The Fc region of an antibody interacts with Fcγ Receptors (FcγRs) on immune cells and components of the innate immune system. Each class of immune cells has a distinct action of neutralization (e.g., antibody dependent cell-mediated cytotoxicity (ADCC) and antibody dependent cell-mediated phagocytosis (ADCP)). Therefore, structural alteration of the Fc region results in novel immune pathways of protection. One approach is to modulate the N-glycosylation in the Fc region of the antibody. Of scientific significance, is the plant's capacity to express human antibodies with homogenous plant and humanized N-glycosylation (WT and GnGn, respectively). This allows to study how specific glycovariants interact with other components of the immune system to clear an infection, producing a tailor-made antibody for distinct diseases. In the first section, plant-produced glycovariants were explored for reduced interactions with specific FcγRs for the overall reduction in ADE for DENV infections. The results demonstrate a reduction in ADE of our plant-produced monoclonal antibodies in *in vitro* experiments, which led to a greater survival *in vivo* of immunodeficient mice challenged with lethal doses of DENV and a sub-lethal dose of DENV in ADE conditions. In the

second section, plant-produced glycovariants were explored for increased interaction with specific FcγRs to improve ADCC in the treatment of the highly inflammatory CHIKV. The results demonstrate an increase ADCC activity in *in vitro* experiments and a reduction in CHIKV-associated inflammation in *in vivo* mouse models. Overall, the significance of this dissertation is that it can provide a treatment for DENV and CHIKV; but equally importantly, give insight to the role of N-glycosylation in antibody effector functions, which has a broader implication for therapeutic development for other viral infections.

DEDICATION

I dedicate this work to my loved ones….

Me gustaría agradecer, en primer lugar, a mi familia con la que no podría haber tenido la oportunidad de lograr esto. Mi madre, cuya aspiración era darnos la oportunidad de perseguir nuestros objetivos y sueños. Enseñándonos a ser tan persistentes hasta el punto de la terquedad. Mi padre, la definición de trabajo duro, ingenio y “dad jokes”. Al igual que mi madre, él enfatizó "pensar con tu cabeza y no tus manos", excepto cuando "la reta, la reta". Abe, el motivo para dar un buen ejemplo y mi mejor amigo. Tan terco como tus padres. Buddy, mi otro mejor amigo y mi audiencia. Tienes el mundo a tu alcance. Rafaela, mi apoyo, mi amor, mi futuro. No puedo esperar para comenzar nuestras vidas juntos.

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PREFACE

The research in this dissertation was performed under the supervision of Dr. Qiang Chen in the School of Life Sciences and the Biodesign Institute at Arizona State University between August 2014 and May 2019. The contributions of our collaborators are referenced in the figures they have kindly generated.

The goal of this dissertation is to make apparent the untapped potential of plant expression systems for monoclonal antibody (mAb) production for greater efficacy and safety. Throughout the presented research two major questions are put into focus: (1) homogeneous N-glycosylation of mAb is an essential aspect that has to be addressed in the current production of mAb, and (2) dissection of disease-specific antibody effector functions are required in the context of treatment. Herein, is described the utilization of a plant expression systems for production of homogeneous N-glycosylated mAbs. The interaction between the GnGn N-glycan structure and the Fcγ Receptors (FcγRs) is investigated for the elucidation of unique antibody effector functions in Dengue Virus (DENV) and Chikungunya Virus (CHIKV) infections. The work presented in this dissertation explores our plant-produced mAbs for reduced and increased interactions with FcγR, which is important to address antibody-dependent enhancement of DENV infections and reduction in inflammation of CHIKV pathology, respectively.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

In 430 B.C., Thucydides experienced a deadly plague and observed survivors were able to care for the sick without the risk of becoming ill. This inspired the concept of immunity as the means of protection against diseases (Cohn, 2012). In 1000 AD, the Chinese took it a step forward and pre-expose people to smallpox by drying smallpox scabs and blowing them through the nostrils of immunologically naïve individuals, a process called variolation (Parrino & Graham, 2006). Without knowing the minute details, this inoculation led to the development of an immunological memory that protected individuals against smallpox infection. Unfortunately, due to the inconsistent preparation and application, the efficacy varied with undesired consequences such as death and disfigurements. As a result, it's prevalence was limited to China, India, Turkey, and parts of Africa (Lahariya, 2014).

In the mid-1700s, Lady Mary Wortley traveled to Istanbul and witnessed variolation first hand. After losing a brother to smallpox and surviving smallpox, Lady Mary Wortley was fascinated by the technique. She writes, ". . . I am going to tell you a thing that I am sure will make you wish yourself here. The small-pox, so fatal, and so general amongst us, is here entirely harmless by the invention of ingrafting. . . I am patriot enough to take pains to bring this useful invention into fashion in England; and I should not fail to write to some of our doctors very particularly about it, if I knew any one of them that I thought had virtue enough to destroy such a considerable branch of

their revenue for the good of mankind. . .. Perhaps, if I live to return, I may, however, have courage to war with them" (Silverstein, 2009). As a firm believer in variolation, Lady Mary Wortley had Charles Maitland, surgeon to the Embassy, inoculate her six-year-old son. Several years later, a smallpox epidemic surged in London and opened the door for Lady Mary Wortley, Dr. Emanuele Timoni, and Dr. Jacobo Pylarini to convince the Royal Society that variolation could prevent smallpox epidemics, which led to the Royal Experiments.

Through the Royal Experiments, condemned criminals were inoculated with smallpox pus to test the efficacy of variolation (Riedel, 2005). The success of the experiment led to the spread of the technique to all parts of the world, but most importantly, spurred curiosity and innovation. Through that curiosity, Edward Jenner developed the smallpox vaccine (1798) by utilizing a distinct but similar cowpox virus to induce protection against smallpox without the risks associated with the variolation technique (Jenner, 1798). Later, Dr. Emil Von Behring and Dr. Shibasabura Kitasato (1890) transferred the serum of immunized animals and "de-toxified" the diphtheria toxin (Macnalty, 1954). Eventually, these essential experiments necessitated the question, "what is responsible for immunity?". This led the way for the discovery of the humoral and cellular immune system (initially proposed by Paul Ehrlich and Ilya Mechnikov, respectively). Although both branches of the immune system are required for optimal immunological protection, the humoral branch will be of focus.

After Dr. Emil Von Behring and Dr. Shibasabura Kitasato serum transfer experiments, Dr. Paul Enrlich experimented with the concept of inducing immunity to

specific proteins. He started by feeding a low dose of toxin (ricin) to mice and gradually increasing the dosage. Later he challenged with a lethal dose of the toxin. He demonstrated that mice injected with these toxins could develop a resistance to the high dose injections. Furthermore, when using a different toxin (abrin) injection on the ricin-immune mice, all the mice succumbed to death (Prüll, 2003). This indicated the protective component was highly specific to the ricin toxin. Later, he demonstrated that this immunity could be passed on to infants through breast milk (Silverstein, 2005). He proposed the protective agent was a protein, he called an "antikörper" or antibody, and through a "lock and key" mechanism recognized and neutralized the toxin, known as the "side-chain hypothesis." Not everything he proposed in his side-chain hypothesis was correct, but it provided an ingenious explanation for the development of antibodies and the platform to describe the phenomenon subsequently.

1.2 The Humoral Immune System

B cells function in humoral immunity by secreting antibodies and together with T cells are a component of the adaptive immune system. Each B cell contains a unique immunoglobulin (B cell receptor (BCR)) on the surface of the membrane and upon recognition with the cognate pathogen secrete immunoglobulins, also called antibodies. The initial diversity of these antibodies arises during B cell maturation. Development starts in the bone marrow of mammals where immature B cells undergo somatic recombination and central tolerance to produce a functional BCR that recognizes foreign proteins (pathogens) and not self-proteins (LeBien & Tedder, 2008). Somatic recombination is the process by which different V(D)J gene segments are recombined,

along with point mutations in the hypervariable regions (also known as the complementary determining region), to produce a unique immunoglobulin molecule (Rispens & Vidarsson, 2013). The result is a naïve B cell that has a functional BCR but only generates slightly specific IgM and IgD antibodies against pathogens. Further diversification of the antibody is accomplished by affinity maturation through somatic hypermutation during activation by cognate antigen and "help" by helper $CD4^+$ T cells (Crotty, 2015). The variable region of the BCR is maintained in the secreted antibody, while alternative splicing of the constant region of V(D)J genes generates the secreted form as well as different antibody isotypes (IgG, IgE, IgA, etc.).

1.3 Antibody Structure

Antibodies are an essential component of the adaptive immune system that provides specific protection against varies pathogens. Human antibodies contain two polypeptides, the heavy chain (Hc) and the light chain (Lc), that form two distinct regions (the antigen binding fragment (Fab) and the crystallizable fragment (Fc)). The Fab region is made up of the heavy and light variable regions (V_H and V_L, respectively) and associated constant regions (C_H1 and C_L, respectively). Differences in the variable region determine the specificity of the antibody. Depending on the nature of the target epitope, the antibody could be considered a neutralizing or non-neutralizing antibody. The Fc region is more conserved and consists of the constant regions (C_H2, C_H3, and/or C_H4, depending on antibody isotype and subclass) (Actor, 2012). The Fc region is involved in recruiting the cellular immune response through the interaction between the Fc region of an antibody and the Fc receptors (FcRs) on immune cells. This interaction mediates

antibody dependent cell-mediated cytotoxicity (ADCC), antibody dependent cell-mediated phagocytosis (ADCP), and/or the complement system (CDC), and antibody recycling by the neonatal Fc receptor (FcRn) (Gómez Román, Murray, & Weiner, 2014; Lindorfer, Köhl, & Taylor, 2014; S. S. Weber & Oxenius, 2014).

The five isotypes are IgM, IgD, IgG, IgE, and IgA. IgM is a low-affinity antibody that is initially secreted to control an infection. When coupled together with the J protein, IgM could multimerize into pentamers to recruit and activate the complement cascade pathway. Secreted IgD is involved in activating basophils and mast cells for the release of antimicrobial and vasodilators (e.g., cathelicidin, heparin, and histamines), which are typically associated with an allergic reaction (K. Chen & Cerutti, 2011). IgG has four subclasses involved in neutralization, opsonization, complement activation, and activation of effector cells (e.g., NK cells through FcγRIIIa binding). The subclasses are IgG1, IgG2, IgG3, and IgG4, in order of decreasing abundance. IgG3 binds strongest to all the FcγRs followed by IgG1 and then IgG2/IgG4 (Bruhns et al., 2009; D. Jung, Giallourakis, Mostoslavsky, & Alt, 2006). Although IgG3 is the most potent, in Caucasians, it has been demonstrated to be highly unstable due to the elongated hinge region (~7-day half-life) (Einarsdottir et al., 2014; Irani et al., 2015). Alternatively, non-Caucasians carry a specific allotype that binds stronger to the neonatal receptor, involved in recycling IgGs (Stapleton et al., 2011). IgG1 is a potent antibody that makes up 60% of the IgGs in circulation with an average half-life of 21 days. IgG2 and IgG4 response to T-independent antigen such as carbohydrates and constant allergens, respectively (Vidarsson, Dekkers, & Rispens, 2014). IgE binds to the FcεR on basophils and mast

cells to activate an allergic response. Finally, IgA is involved in mucosal immunity through neutralization and can also associate with the J protein to dimerize to increase avidity. Although there are multiple antibody isotypes and subclasses, IgG1 is the most frequently used antibody for commercial production.

1.4 Antibody Fc region-Fc Receptor (FcR) Interaction

Alteration to the Fc region is of great importance for the modulation of the cellular immune system and half-life in circulation. The activity of a specific effector cell largely depends on the differential expression of the FcRs. The immunoreceptor tyrosine-based activation motif (ITAM) containing FcγRs include the high-affinity FcγRI, and the lower affinity FcγRIIa, FcγRIIc, FcγRIIIa, and FcγRIIIb. While the immunoreceptor tyrosine-based inhibitory motif (ITIM) is only associated with FcγRIIb. These receptors are differentially expressed by different innate and adaptive immune cells and are responsible for mediating the cellular immune response. For example, human natural killer (NK) cells, involved in ADCC, almost exclusively express FcγRIIIa, while monocytes and macrophages transiently express FcγRI, FcγRIIa, FcγRIIb, and FcγRIIIa depending on the cytokine signaling at the particular moment. Therefore, different antibody isotypes interact differently with FcRs and preferentially activate specific immune effector cells. (Bournazos, Wang, & Ravetch, 2016; Nimmerjahn & Ravetch, 2008). Activation of these cells would increase the level of ADCC and ADCP, respectively. Further studies are required to elucidate specific effector functions for individual FcγRs and/or what threshold levels are needed to be reached to activate specific immunological activity.

1.4.1 Expression of FcγRs Across Species

Animal models are widely utilized to explore the efficacy of candidate therapeutics to recapitulate specific mechanisms in human pathology and disease. Unfortunately, every model has a caveat for human application, yet these models provide sufficient correlation between therapeutic efficacy and safety for the proper continuation of investigations. Mouse models are extensively utilized to study the interaction between FcγRs and IgG for antibody effector functions; while non-human primate models (rhesus and cynomolgus macaque (mac)) are utilized in the latter stages of pre-clinical research even though little information is known about the macFcγRs and human (h) IgG interaction (Moldt & Hessell, 2014). For murine models, all of the hIgG antibody subclasses can bind all of the mouse FcγRs with a similar pattern as the human FcγR equivalents (Table 1, human/mouse IgG and FcγR structural ortholog equivalency described in Table 2a and b, respectively) (Dekkers et al., 2017). This provides an initial *in vivo* model to investigate the therapeutic efficacy of therapeutics.

Human IgG	Mouse receptors (K_D strength)
IgG1	FcγRI>FcγRIV>FcγRIIb>FcγRIII
IgG2	FcγRIIb>FcγRIII
IgG3	FcγRI>FcγRIV>FcγRIIb>FcγRIII
IgG4	FcγRI>FcγRIIb>FcγRIII>FcγRIV

Table 1. Summary of Binding Patterns Between Human IgGs and Murine FcγRs. Table 1 summarizes the K_D strength between IgG1, IgG2, IgG3, and IgG4 to murine FcγRI, FcγRIIb, FcγRIII, and FcγRIV. Data was summarized from Dekkers et al. (2017).

A

Human IgG	Mouse IgG
IgG1	IgG2a
IgG2	IgG3
IgG3	---------
IgG4	IgG1

B

Human FcγR	Mouse FcγR
FcγRI	FcγRI
FcγRIIa	FcγRIII
FcγRIIb	FcγRIIb
FcγRIIIc	---------
FcγRIIIa	FcγRIV
FcγRIIIb	--------

Table 2. Equivalent Orthologs of Human and Mouse IgGs and FcγRs. The IgG and FcγR orthologs between humans and mice are summarized in Table 2a and b, respectively. Human and mouse IgGs share 95% homology with orthology consisting of FcγR recruitment and level of antibody effector function. Note that IgG3 does not have a mouse equivalent because it's high potency. The tables were generated based on data from Dekkers et al. (2017); Hussain et al. (1995); Moldt et al. (2014); and https://www.invivogen.com/review-antibody-generation.

The direct translation of the results is often convoluted by differences in binding of human IgG to murine FcγRs, and differential expression patterns of FcγRs observed in the murine innate immune cells (Table 3). For example, human NK cells exclusively express FcγRIIIa (FcγRIV, murine (m) ortholog); however murine NK cells lack the expression of mFcγRIV and highly express mFcγRIII (hFcγRIIa). Therefore, proper clarification of mechanism of efficacy (e.g., antibody effector function and responsible cell population) requires additional investigation through humanized murine models (expressing human FcγRs) or evolutionarily related rhesus and cynomolgus macaques. Overall, there are considerable differences among humans and mice in the context of FcγRs binding and cellular expression, and proper characterization of the model is required to make accurate conclusions. Together, *in vitro* and *in vivo* experiments can be

used to decipher specific immunological pathways of protection and specific cell populations involved in the most optimal therapeutic outcome.

	Human						Mouse			
FcγR	FcγRIa	FcγRIIa	FcγRIIb	FcγRIIc	FcγRIIIa	FcγRIIIb	FcγRI	FcγRIIb	FcγRIII	FcγRIV
Signaling Pathway	ITAM FcR-γ	ITAM	ITIM	ITAM	ITAM FcR-γ		ITAM	ITIM	ITAM	ITAM
B cell			+					+		
Dendritic cell	+	+			+		+/-	+	+	
Macrophage/ monocyte	+	+	+		+		+/-	+	+	+
NK cell				+	+				+	
Neutrophil	+	+	+			+		+	+	+
Mast cell			+		+			+	+	

Table 3. FcγR Expression Profile of Human and Murine Innate Immune Cells. Table 3 summarizes the presence (+) and absence (- or blank) of FcγRs on human and murine B cells, dendritic cells, macrophages/monocytes, NK cells, neutrophils, and mast cells. Data was summarized from Bruhns & Jönsson, 2015; Loisel et al., 2007; and Moldt & Hessell, 2014.

1.4.2 Antibody Dependent Cell-mediated Phagocytosis

Phagocytic cells (e.g., monocytes, macrophages, neutrophils, dendritic cells (DCs)) contribute to the innate immunity by actively uptaking and subsequent digesting or processing of the particles circulating in the body. In the context of pathogen infiltration, phagocytosis provides a mechanism for the clearance of pathogens and, more importantly, antigen presentation. Complement and antibody (ADCP) opsonization of pathogens increases ligand-receptor interaction sites and facilitates phagocytosis. In ADCP, phagocytes are constantly probing their environment for IgG-opsonized particles. The succeeding FcγR-activation signals results in an “all or nothing” response with the number of IgG interactions directly correlating to the magnitude of the signal (Y. Zhang,

Hoppe, & Swanson, 2010). Phagocytosis of the IgG marked pathogen results in the maturation of the phagosome into a phagolysosome where the content is subsequently degraded (Kinchen & Ravichandran, 2008). It has been observed that ligation to FcγRIIa results in faster maturation into phagolysosomes (Trivedi et al., 2006), supporting the hypothesis that specific FcγRs activate specific pathways within the effector cell. Along with activation of the ADCP, FcγR ligation also activates the NADPH complex and the production of superoxide anion (O_2^-) (Anderson et al., 1986; C Pfefferkorn & W Fanger, 1989; Larsen et al., 2000).

Phagocytic cells differentially express both the activating and inhibitory FcγRs which are continuously regulated by cytokine signals. Monocytes are mononuclear phagocytic cells and precursors to tissue-resident macrophages and myeloid-lineage DCs (Monie, 2017). The human monocytes mainly express FcγRI and FcγRIIa, while the murine counterpart expresses the activating FcγRI, FcγRIIb, FcγRIII, and FcγRIV (Biburger et al., 2011; Pan, Darby, Indik, & Schreiber, 1999). Macrophages and DCs express all the FcγRs yet have the highest abundance of FcγRI (Berger et al., 1994; Biburger et al., 2011; Nimmerjahn & Ravetch, 2008). The outcome of FcγR-mediated phagocytosis differs greatly between macrophages and DCs. Macrophages function in killing the ingested pathogen while DCs process the pathogen and display them to T cells on both class I and II major histocompatibility complexes (Rafiq, Bergtold, & Clynes, 2002; Regnault et al., 1999). Besides killing of the pathogen, macrophages secrete proinflammatory cytokines which are tightly regulated by the inhibitory FcγRIIb. Neutrophils are the most abundant phagocytic immune cell and quickly respond to the

site of inflammation. Human neutrophils express FcγRIIa and FcγRIIIb but upon interferon-γ (IFN-γ) stimulation also express FcγRI (FcγRIIb, FcγRIII, FcγRIV in mice) (Biburger et al., 2011). Ligation to FcγRIIa mediates phagocytosis while binding to FcγRI and FcγRIIIb resulted in an increased influx of calcium (Ca^{+}) necessary for synergistic enhancement of phagocytosis (Marois, Paré, Vaillancourt, Rollet-Labelle, & Naccache, 2011; Petroni, Shen, & Guyre, 1988). Together, all the FcγRs are involved in activation of the phagocytes with the FcγRIIa demonstrating a predominant role in ADCP for monocytes, macrophages, and neutrophils.

1.4.3 Antibody Dependent Cell-mediated Cytotoxicity

$CD8^{+}$ T cells are an important part of the immune system by providing cell-mediated killing of cancerous cells, bacterium, as well as virally-infected cells. The innate immune system also performs cytotoxic killing of these cells with the help of the humoral response (ADCC). Effector cells consist of mononuclear and polymorphonuclear leukocytes that express FcγRs (e.g., NK cells, macrophages, γδ T cells, neutrophils, basophils, eosinophils). The NK cells are the predominant effector cells when studying ADCC activity. FcγIIIa is almost exclusively expressed on NK cells and is often considered the predominant ADCC receptor; although other receptors have been implicated in the activation of ADCC (FcγRI, FcγRII, and FcγRIIIb). There are two documented mechanisms of target cell killing by NK cells: the perforin/granzyme cell death pathway and the FAS ligand (FAS-L) pathway (Gómez Román et al., 2014).

Activation of the NK cells begins with the binding of a cognate antigen with an antibody, which results in a conformational change in the structure of the Fc region. This

conformational change increases the affinity to the ADCC-associated FcγRs (Kato, Fridman, Arata, & Sautès-Fridman, 2000). Downstream signaling by the ligation of the Fc-FcγR results in an increase in intracellular calcium for exocytosis of cytotoxic granules (perforin/granzyme cell death pathway). These granules contain a macromolecular complex composed of perforin, granzyme B, and other molecules necessary for the function and stability of the main components (Smyth et al., 2001). Granzyme B interacts with the mannose 6-phosphate receptor (MPR) and induced endocytosis of the complex. Within the endosome, perforin forms pores to disrupt the integrity of the endosomal lipid bilayer and release granzyme B into the cytosol. Additionally, perforin forms pores on the surface of the membrane disturbing ionic gradient homeostasis, which results in cell lysis. Granzyme B can activate caspases (direct pathway) or cleave the Bid molecule (indirect pathway) to initiate the caspase cascade resulting in DNA fragmentation and apoptosis(Trapani & Smyth, 2002). The second mechanism involved in NK cell killing is the FAS-L pathway. Upon Fc-FcγR cross-linking, there is transcriptional upregulation of the FAS-L. Binding of the FAS-L to the FAS receptor activates the death domains leading to the caspase-dependent apoptosis cascade (Wallach et al., 1999). Together, ADCC activity halts the spread of cancerous cells and pathogens throughout the individual but can also cause unwanted cytotoxicity.

1.4.4 Complement Dependent Cytotoxicity

The complement cascade is part of the innate immune system and involves the concerted sequential addition of proteins that tag and destroy pathogens. There are three initiations (antibody-antigen complex (classical pathway), mannose-binding lectin

pathway, and alternative spontaneous pathway) that converge at the C3 activation step and share the same terminal formation of the membrane attack complex (MAC). Specifically, the classical pathway for complement cascade initiation is mediated by C1q binding to a pathogen opsonized with IgG antibodies. Generation of the MAC results in lysis of the tagged pathogen by forming a pore that leads to osmotic swelling and cell lysis (Zhou, Hu, & Qin, 2008). More importantly, is the opsonization of the pathogen by the byproducts (e.g., C1q, C3b, iC3b, and C4b) to tag for disposal and further presentation to the adaptive immune system. In contrast, C3a, C4a, and C5a mediate a pro-inflammatory response with a strong chemotaxis function and activation of mast cell and basophil degranulation (Klos et al., 2009). Similar to all of the immune function, CDC activity should be modulated to achieve optimal efficacy and prevent immunopathogenesis.

1.5 Monoclonal Antibody Industry

Monoclonal antibodies (mAbs) bind one epitope and are produced from a single B-lymphocyte clone. The first mAb, Orthoclone OKT3 (muromonab-CD3), was generated in 1975 and licensed in 1986. This IgG2a murine antibody was used to prevent host versus graft kidney transplant rejection by inhibiting the activation of cytotoxic and helper T lymphocytes by binding to CD3. The success of Orthoclone was limited due to the human anti-mouse antibody (HAMA) response (Sgro, 1995). In response to the immunogenicity caused by non-human sequences, the development of chimeric (14%), humanized (32%), and human (54%) versions have been developed in the latter FDA approved mAbs. Currently, the monoclonal antibody market is valued over $100 billion

with an estimated growth to the $137-200 range by 2022 (Ecker, Jones, & Levine, 2014; Grilo & Mantalaris, 2019). As of 2017, there are 57 mAb and 11 biosimilars in the market with the majority targeting different types of cancers as checkpoint inhibitors (e.g., atezolizumab, avelumab, cetuximab, etc.) as well as autoimmunity, infectious diseases, transplantation, and other diseases (Jing Li & Zhu, 2010). To improve the mAb field a proper understanding of the discovery, production, and optimization process need to be discussed.

1.5.1 Discovery of Antibodies

There are multiple approaches to the discovery of efficacious therapeutic antibodies. The first generated mAb was produced by using the hybridoma technique on murine B-lymphocytes. Generation of the hybridomas involved immunizing the mice with a specific antigen and isolating the respective B-lymphocytes from the spleen. The B-lymphocytes are then fused with an immortal myeloma cell line lacking a specific enzyme (hypoxanthine-guanine-phosphoribosyltransferase (HGPRT)) (J. K. H. Liu, 2014). Successful fusion between the two cells will compensate for the HGPRT mutant myeloma cell line when cultured *in vitro* in selective medium. The hybridomas are then diluted in wells until they produce a single and specific antibody. The positive clone's genes are sequenced and cloned into different expression platforms (e.g., CHO, NS0, Sp2/0, *E. coli*, HEK293, PER.C6, etc.). An alternative approach for antibody discovery is to use phage display (e.g., the discovery of Humira) (Nixon, Sexton, & Ladner, 2014). This involves isolating the mRNA from human B-lymphocytes and converting it into cDNA. The V_H and V_L gene segments get amplified by PCR and cloned into a vector as

part of the PIII protein of a bacteriophage. The new bacteriophage is produced and screened against the target antigen. Positive bacteriophages are then used to infect *E. coli* to amplify the plasmid and subsequently to sequence the variable regions. Like the hybridoma approach, the genes are cloned into specific cell lines for overexpression and downstream processing. Recently, the utilization of different animal models has provided alternative antibody formats that could potentially increase the applicability (e.g., camelid single-domain antibodies) (Arbabi-Ghahroudi, 2017). The field of mAb discovery is broad with the production of mAb more established.

1.5.2 Monoclonal Antibody Cell-based Production Systems

Production of mAb can take multiple platforms but is dominated by the mammalian cell culture system. Initially, hybridomas were used as the culture system for mAb production, but due to the instability of the clones and HAMA response, the field moved toward recombinant cell lines. The cell lines that are producing approved mAbs for human therapy include CHO, NS0, and Sp2/0 (Foster, Catzel, Atwa, Zarka, & Mahler, 2003; F. Li, Vijayasankaran, Shen, Kiss, & Amanullah, 2010). The scale-up production processes include batch, fed-batch, and perfusion processes in specialized bioreactors. In the batch method, the cells are grown to a specific density and stimulated in the same initial base medium. The fed-batch method involves supplementing the nutrients that are depleted. And finally, the perfusion method circulates the nutrients and waste while harvesting the product, therefore maintaining the initial culture conditions constant in the bioreactor. The perfusion method allows maximizing the protein yield of a particular batch. This also demands the use of engineered cell lines to limit apoptosis and

increase overall expression (e.g., inhibiting P53, overexpressing human telomerase reverse transcriptase (hTERT), chaperone proteins, and SRP14) (Crea, Sarti, Falciani, & Al-Rubeai, 2006; T. Hu, Miller, Ridder, & Aardema, 1999; Le Fourn, Girod, Buceta, Regamey, & Mermod, 2014; Xiao, Shiloach, & Betenbaugh, 2014). Together, the mAb production system is capable of producing the biologic with product titers reaching between 1-10 g/L in batch and fed-batch processes, respectively in CHO cells; 0.1-0.2 g/L in batch cultures and from 0.1-0.8 g/L in fed-batches in murine cell lines (NS0 and Sp2/0); and 0.5 g/L in batch, 8 g/L in fed-batch, and 27 g/L in perfusion in PER.C6 cells (Kunert & Reinhart, 2016). Unfortunately, the lack of homogenous glycosylation limits product consistency and overall safety/efficacy. Additionally, the high cost of the facilities, bioreactors, and culture media demand the need to look for alternative production systems.

Other production systems include the use of prokaryotic (e.g., *E. coli*) and lower eukaryotic (e.g., yeast, plants, etc.) cell lines. These expression systems have overcome several impediments (e.g., cytoplasmic reductive conditions, non-human N-glycosylation (will be discussed later), etc.) that have allowed them to contribute to the mAb production field. Initially, *E. coli* was used to produce antibody fractions (e.g., scFv and Fab) due to the lack of glycosylation system. With the discovery of an N-linked glycosylation system in a helical gram-negative bacterium, *Campylobacter jejuni*, Wacker et al. have successfully transferred the N-glycosylation system into *E. Coli* (Fisher et al., 2011; Lee & Jeong, 2015; Valderrama-Rincon et al., 2012; Wacker et al., 2002). Unfortunately, it lacks the proper human glycoforms prevents its application for full-length mAb

production. The other approach has been to produce aglycosylated mAb equivalents with Fc mutations that keep the binding to the FcRs (e.g., E382V/M428I mutations for increased FcγRI binding, S298G/T299A to FcγRIIA^{131R}, FcγRIIA^{131H}, and FcγRIIB) (S. T. Jung et al., 2013; S. T. Jung et al., 2010; Robinson et al., 2015; Sazinsky et al., 2008). The lack of N-glycosylation and added mutations alter the stability and half-life of the antibody. As for the lower eukaryotic yeast (specifically *Pichia pastoris*), they have an established N-glycosylation pathway. Due to the high level of hypermannosylated *N*-glycans, efforts to humanize the N-glycosylation pathway of *P. pastoris* have resulted in production of mAb with homogenous humanized N-glycosylation with superior ADCC activity (N. Zhang et al., 2011) as well as complex sialylation (Hamilton et al., 2006; Hamilton & Gerngross, 2007). Overall, great strides have been made to develop alternative platforms for mAb production.

1.5.3 Aspects of Optimization

The advancements of the sequencing technology have allowed continued discovery and optimization of antibody structures for greater safety, stability, and efficacy in therapeutic use. Furthermore, the conventional full antibody format, in some cases, has been altered to increase penetration, cytotoxicity, and/or specificity (e.g., Fab, single chain variable fragments (scFv), diabodies, toxin-conjugated mAbs, bifunctional mAbs) (Spiess, Zhai, & Carter, 2015). Several targets that are required for improving antibody efficacy include decreasing the overall immunogenicity, optimizing the antigen-binding affinity, effector functions, and regulating the pharmacokinetics.

Reducing immunogenicity is carried out by reducing human T cell antigens. As previously mentioned, removing or reducing the number of non-human regions in the constant framework region or engrafting the CDR onto human framework regions reduces immunogenic responses. The best approach would be to generate human equivalents, but even then the allotypic difference could cause an immunogenic response in a population of individuals (Webster et al., 2016). This can also apply to the humanization of the N-glycosylation pathway in the production system. For example, production of cetuximab with galactose-alpha-1,3-galactose was highly immunogenic (Harding, Stickler, Razo, & DuBridge, 2010). Overall, having a product with reduced immunogenic domains and homogenous glycosylation increases the half-life and safety of the therapeutic.

Antigen binding affinity can be improved by utilizing phage display systems and sequencing the clone with the optimal affinity (D. Hu et al., 2015). For some circumstances having a lower affinity might be required for increased efficacy. For example, utilization of a bifunctional mAb, with a low-affinity arm, might be useful to target neutralizing viral epitopes that are only exposed in the acidic environment within endosomes. The low-affinity arm will be instrumental in initially accompanying virus into the endosome, and when exposure of a neutralizing epitope occurs, the higher affinity arm binds and prevent the infection.

Important for Fc-FcγR affinity is the structure of the Fc region. Alteration of the hinge and proximal CH2 amino acid sequence near the Fc region results in increased or decreased binding to FcγRs. Multiple groups have performed amino acid backbone

mutations and N-glycan modifications to control the level of antibody effector functions. For the enhancing mutations, they can improve the affinity over 100-fold and 50-fold from the WT mAb for ADCC and CDC, respectively (X. Wang, Mathieu, & Brezski, 2018). For certain diseases, lowering the antibody effector function might result in a better therapeutic outcome. Such as in the case of the highly inflammatory Chikungunya Virus infections, hemorrhagic fever causing Dengue Virus, and even transplant situations. These mutations have resulted in the intended 100-fold decrease in binding, but even then, some level of antibody effector function might be necessary to have optimal therapeutic efficacy. Alternatively, precise control of N-glycosylation can have detrimental effects on the antibody's half-life and efficacy (will be discussed later).

A majority determinant of the pharmacokinetics of mAb is the interaction between the Fc region of mAb and the FcRn. The FcRn is an endosomal membrane protein that binds to the C_H2-C_H3 hinge region on IgGs and prevents lysosomal degradation by redirecting the endosome in a recycling manner (Martins, Kennedy, Santos, Barrias, & Sarmento, 2016). Also, the FcRn is capable of transcytosis in specific cells (e.g., blood-brain barrier (BBB), placental membrane, intestinal tract, etc.). Increasing the affinity to the FcRn increases the half-life of mAbs and lower the therapeutic dosage or frequency of administration. An alternative to increasing the half-life of mAb and antibody fragments is through PEGylation (Banerjee, Aher, Patil, & Khandare, 2012; Knop, Hoogenboom, Fischer, & Schubert, 2010). Although, an anti-PEGylation antibody response can occur in some patients, therefore increasing the clearance of the therapeutic (Bendele, Seely, Richey, Sennello, & Shopp, 1998;

McSweeney, Versfeld, Carpenter, & Lai, 2018). Significant efforts have been made to alter the affinity toward both the FcRs and FcRn to achieve the desired effector function and longevity of the mAb therapeutic (Shen et al., 2017; X. Wang et al., 2018). Harnessing the interaction between the Fc region and the FcRs/FcRn is vital for a safer, stable, and efficacious therapeutic.

1.6 Plant-made Biologics

Cell-based recombinant protein production platforms require a high capital investment through the need of a capital-intensive facility, bioreactors, expensive culture media, and associated downstream processing (Kelley, 2009). Plant-made biologics (PMB) have been a promising alternative recombinant protein production platform for its low-cost, speed, and overall safety (Chen & Davis, 2016). Utilized plant species range from *Nicotiana benthamiana* to dietary important plants like maize, soybeans, carrots, etc. Plants allow for an amenable production system capable of producing highly complex proteins quickly and at comparable yields (Lai, He, Engle, Diamond, & Chen, 2012; Lai et al., 2014). Significantly, plant expression systems have a lower initial capital investment that consists of greenhouses and the inexpensive mineral solution necessary to propagate the plants. It is estimated that plant-based platforms have upstream costs as low as $1.00–2.00 per kilogram of protein (Chen & Davis, 2016). However, the downstream processing cost remains equivalent to the traditional cell-based platforms. Most importantly, it is the capability of plants to generate homogenous N-glycosylation which allows product consistency at the N-glycosylation level, which is necessary for a safer and more efficacious product. Additionally, the plant growth conditions limit the

potential contamination of human pathogens in the production process (e.g., human viruses, prions, etc.), which also increases overall safety. The diverse plant structures (e.g., seeds, tuber, leaves) allow for innovative uses, such as therapeutic biologic storage in seeds, edible vaccines, and large-scale expression platforms (Kusnadi, Nikolov, & Howard, 1997). Although the use of plant expression systems is relatively novel, there have been some success stories demonstrating its speed, safety, and therapeutic efficacy.

The speed of the plant expression system is far superior to those of existing systems. Medicago, a company based in Canada, produces influenza hemagglutinin (HA) virus-like particles (VLPs)-based vaccines. To control an influenza pandemic, it is necessary to develop a vaccine in the shortest timeframe to prevent the spread of the virus from person to person. In response to the A/H1N1 flu pandemic, Medicago utilized plant expression systems to generate their influenza VLP which provided a durable cross-reactive T-cell response to the HA antigen (Landry et al., 2014). From the time they received the HA sequence, it took them two weeks to obtain high expression levels of the vaccine in agroinfiltrated plants. It took an additional five days to purify the vaccine. This is in comparison to the current manufacturing technologies, which take 5-6 months to establish a stable clone before actual production (D'Aoust et al., 2010). Beyond the speed of production, the vaccine efficacy was highly potent and safe for human use. The rapid turnaround time is niche plant expression system can fulfill in response to emergencies such as bioterrorism threats, emerging, and re-emerging pathogens.

One of the re-emerging pathogens was the Ebola virus in the Western countries of Africa (mainly in Guinea, Liberia, and Sierra Leone) in 2014. Highlighting the niche of

plant expression systems to produce Biobetters is the development of ZMapp™. The drug consists of a cocktail of three mAbs targeting the Ebola GP1 protein. Production in glycoengineered *N. benthamiana* by magnICON viral vector provided a homogeneous glycoform. Early experiments in rhesus macaque animal models demonstrated greater potency than the mammalian-produced counterpart. In efficacy studies, ZMapp™ was able to rescue 100% of the animals five days after lethal Ebola challenge (Qiu et al., 2014). Due to the extreme circumstances of the Ebola outbreak, the compassionate use of the drug was administered to seven individuals (including Dr. Kent Brantly and medical missionary Nancy Writebol) in which two of the other individuals died. In a clinical study conducted by the National Institute of Health (NIH) in 2016 involving 72 patients; 8 of the 36 individuals (about 22%) who received the treatment died, compared to 13 of the 35 individuals (37%) who did not receive the treatment ("A Randomized, Controlled Trial of ZMapp for Ebola Virus Infection," 2016). Unfortunately, the study was unable to enroll enough individuals needed to have statistical significance. Despite the lack of statistically significant results, ZMapp has gained some interest for the PMB field and should facilitate pathways for U.S. Food and Drug Administration (FDA), as well as other equivalent organizations, approval.

The first FDA approved plant-made therapeutic is glucocerebrosidase (taliglucerase alfa, commercially named ELELYSO™) for the enzyme replacement therapy of Gaucher's disease. Particular for this enzyme is the requirement of a specific paucimannosidic N-glycan to function efficiently (Shaaltiel et al., 2007). Production in mammalian cell culture demanded the need for further *in vitro* N-glycosylation

processing, which drove up the cost of production. Fortunately, by producing them in transgenic carrot cell cultures, the necessary N-glycosylation profile was present and achieved better and more consistent efficacy as compared to the mammalian cell equivalent. Protalix Biotherapeutics Inc. has also developed an oral-administered version which seeks to increase patient compliance by using the edible carrot cells to deliver the therapeutic (Hollak, 2012). Animal studies have demonstrated that the plant cells protected the therapeutic from the gastric acids (Shaaltiel et al., 2015). This innovative use of plant cells has opened a mechanism for FDA approval for PMBs. Unfortunately, the PMB field still needs to overcome technical and regulatory challenges to facilitate the approval of PMBs; however, there is a niche that plant expression systems can fulfill in the production of therapeutic biologics.

1.6.1 Plant Expression Systems

At the forefront of the PMB field, is the *N. benthamiana* a close relative of the commercial tobacco plant. *N. benthamiana* is part of the *Solanaceae* family, a native species to Australia, and has become instrumental in plant virology and host-pathogen interaction research as a model plant system. Its utilization in the production of therapeutic proteins is due to (1) its susceptibility to a range of infective agents (e.g. viruses, bacteria, and fungi), (2) ease of generating a seed stock with minimal gene flow contamination, and (3) rapid cultivation (about 6-weeks) for optimal protein yields (Sainsbury & Lomonossoff, 2014). Right next to the *N. benthamiana* is the gram-negative soil bacterium *Agrobacterium tumefacien*. *A. tumefacien* is a plant pathogen that causes crown gall disease by transferring virulence genes from the Ti plasmid to plants

cells (Gordon & Christie, 2014; Pitzschke, 2013). Plant biologist have engineered the Ti plasmid to deliver genes of interest (GOI) to get expression or, in some instances, integration of the transgene into the plant genome. There are two methods for recombinant protein expression: (1) to generate a stable genetic plant line or (2) by utilizing plant viral vectors for transient expression.

Stable transgenic plant lines are generated by incorporating the GOI into either the nuclear or chloroplast genome. For proteins that require N-glycosylation, it is preferred the GOI cassette is cloned into the nuclear genome to allow the utilization of the secretory pathway. These stable plant lines are usually generated by biolistic or *Agrobacterium*-mediated transformation (Q. Chen & Lai, 2015). The benefit of the stable plant line is the flexibility of planting the amount of target therapeutic protein required. For example, in the case of a bioterrorism event, generating more of the therapeutic protein requires seeding more plants. The disadvantage is stable plant transformation is a time-consuming and laborious process that requires stringent regulation to prevent gene flow contamination to native plants.

Transient expression involves the production of the recombinant protein without the integration of the gene into the plant genome. This accomplished by the utilization of *Agrobacterium*-based transcriptional binary vectors or the use of an engineered viral vector containing the GOI transgene. Depending on the gene expression system that is utilized, production of the recombinant protein has a peak yield around days 5-8 after *A. tumefaciens* infiltration (Q. Chen, He, Phoolcharoen, & Mason, 2011; Leuzinger et al.,

2013). Due to the rapid expression and comparable protein yields of the transient plant system, it is the preferred choice for many PMB production platforms.

1.6.2 Plant Viral Vectors

Plant viral vectors have achieved incredible success in expressing high protein yields in a short timeframe (Chen & Davis 2016). Viruses are known pathogens to utilize the translational machinery of the host for replication of the self-viral proteins. The first generation of plant viral vectors utilized the entire virus including its genome. This involved having the GOI in the same open reading frame as part of a fusion protein or with a strong sub-genomic promoter upstream (Hefferon, 2017). The disadvantages of utilizing a full replicating virus is controlling its spread to other plants raising some biosafety concerns. Additionally, due to the expression of the GOI and the plant viral proteins, the overall yield of the target protein was very low, therefore, limiting the scalability of the technology. The second generation of plant viral vectors, coined deconstructed viral vectors, removed the plant viral proteins genes that were not necessary for efficient expression of the GOI. This involved *A. tumefaciens* delivery of the deconstructed vector into plant cells. This improved the overall yield of the target protein and overcame the safety concerns regarding the unintentional spread of the virus.

There are several successful plant viral vectors that utilized unique mechanisms for recombinant protein production. These include but are not limited to the Cowpea mosaic virus (CPMV), cauliflower mosaic virus (CaMV), tobacco mosaic virus (TMV), potato virus X (PVX), and the geminivirus yellow bean dwarf virus (BeYDV) (Peyret &

Lomonossoff, 2015). Through plant viral vector engineering these viruses have achieved a great yield in short timeframes of multimeric proteins. For example, magnICON ™ has utilized two non-competing viral vectors to produce different vaccines and antibody products (Yuri Gleba, Klimyuk, & Marillonnet, 2007). This system involves *A. tumefaciens* delivery of multiple constructs that encompass two different viral genomes (TMV and PVX). When the tDNA has infiltrated the plant cell, the magnICON system generates a high number of RNA replicons, which get amplified by cell-to-cell spread, and become translated at a high expression level (0.5–1 mg/g leaf fresh weight (LFW)) (Giritch et al., 2006; Y. Gleba, Klimyuk, & Marillonnet, 2005; Marillonnet et al., 2004). Alternatively, the geminivirus BeYDV viral vector utilizes the virally-encoded Rep protein to amplify the potentially multiple DNA replicons, through rolling circle replication, to achieve high expression (Diamos & Mason, 2018). The benefit of the Geminiviral system is the need for only one *A. tumefaciens* strain to deliver multiple GOI replicons in the plants and the shorter timeframe (3-4 days) (Lai et al., 2012). Together, these deconstructed viral vector systems have provided an array of tools to produce PMBs.

1.7 Glycosylation

Glycosylation is an enzymatic co-and post-translational modification that adds sugar residues onto proteins. For many proteins, the presence of glycosylation is necessary for correct folding, assembly, stability, and even to function properly as exemplified by lack of an N-linked glycan in the Asn297 resulting in a lack of Fc effector function (Alsenaidy et al., 2013; Hristodorov et al., 2013; Latypov, Hogan, Lau, Gadgil,

& Liu, 2012). The reaction is catalyzed by several enzymes called glycotransferases and glycosidases, which reside in the endoplasmic reticulum (ER) and Golgi apparatus. Since this is an enzymatic reaction, the abundance of these glycoenzymes in the ER and the Golgi of the cell will dictate the overall profile of glycans on the proteins. There is multiple type of glycosylation (e.g. *N*-glycosylation, *O*-glycosylation, *C*-glycosylation, and *S*-glycosylation), which generally are classified based on which atom and amino acids the sugar residues attach (Brazier-Hicks et al., 2009; Nagashima, von Schaewen, & Koiwa, 2018; Oman, Boettcher, Wang, Okalibe, & van der Donk, 2011; Steen, Rudd, Dwek, & Opdenakker, 1998). For example, N-linked glycosylation occurs on the amino group of asparagine residues following a consensus sequence of Asn-X-Ser/Thr; where X is any amino acid other than proline (Aebi, 2013). O-linked glycosylation attaches oligosaccharides onto the oxygen atom on superficial serine and threonine amino acids after translation (Vik et al., 2009). Although humans perform both of the above examples of glycosylation, N-glycosylation will be the primary focus in this dissertation.

1.7.1 *N*-glycosylation Pathways in Mammals and Plants

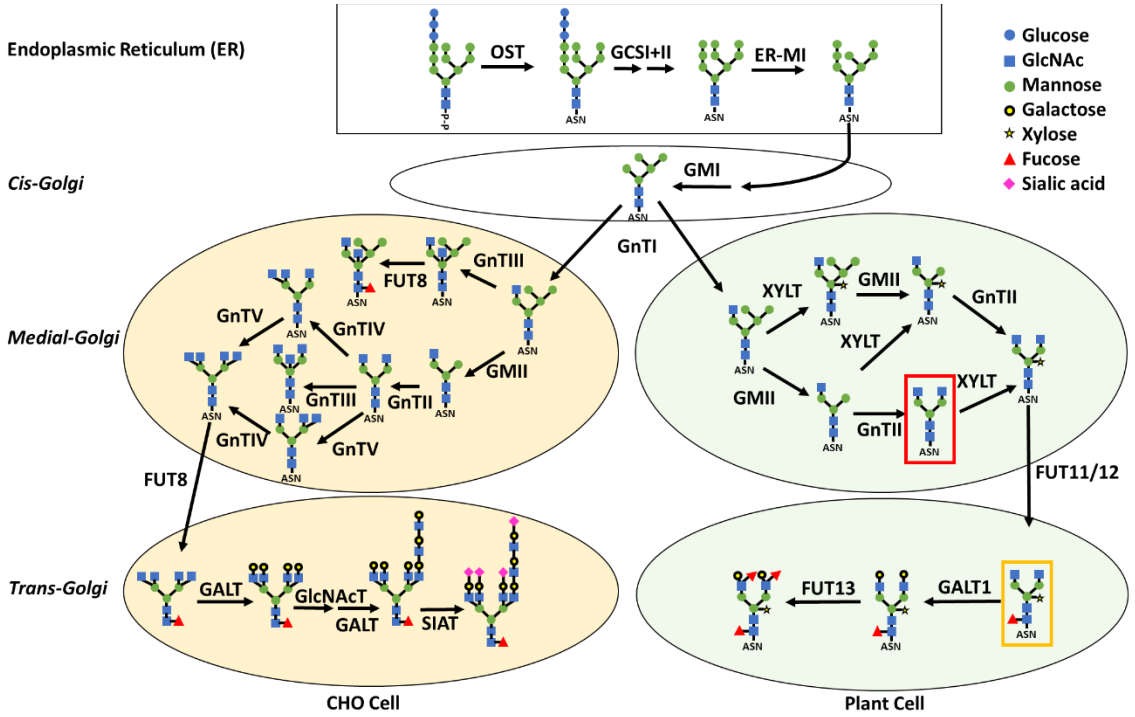

Figure 1. General Schematic of CHO Cell and Plant Cell N-glycosylation Pathway. Glycoenzymes involved in N-glycan processing in the ER and Golgi apparatus: oligosaccaryltransferase (OST), glucosidase-I and -II (GCSI+II), ER mannosidase-I (ER-MI), mannosidase-I (GMI), acetylglucosaminyltransferase-I (GnTI), mannosidase-II (GMII), acetylglucosaminyltransferase-II (GnTII), acetylglucosaminyltransferase-III (GnTIII), acetylglucosaminyltransferase-IV (GnTIV), acetylglucosaminyltransferase-V (GnTV), Fucosyltransferase 8 (FUT8), β1,4-galactosyltransferase (GALT), acetylglucosaminyltransferase (GlcNAcT), α2,3- or α2,6- sialyltransferase (SIAT), β1,2-xylosyltransfersae (XYLT), α1,3-fucosyltransferases (FUT11/12), β1,3-galactosyltransferase (GALT1), and α1,4-fucosyltransferase (FUT13). The WT and GnGn N-glycosylation structures are boxed yellow and red, respectively.

N-glycosylation, in eukaryotes, begins when proteins enter the secretory pathway in the ER (Fig. 1). A multimeric enzyme complex called oligosaccharyltransferase (OST) screens for conformation accessible N-glycosylation sites to attach a pre-assemble GlcNAc2Man9Glc3 oligosaccharide. Successful transfer results in additional glycoenzymes (e.g., glucosidase-I (GCSI), glucosidase-II (GCSII), ER-mannosidase-I

(ER-MI), and Golgi α-mannosidase-I (GMI)) trimming off glucose and mannose residues, respectively, to generate GlcNAc2Man5 in the ER and subsequent *Cis* side of the Golgi apparatus. N-acetylglucosaminyltransferase-I (GnTI) then recognizes and transfers a GlcNAc onto the α1,3 mannose residue. Further processing by Golgi α-mannosidase-II (GMII) and N-acetylglucosaminyltransferase-II (GnTII), in the *medial*-Golgi, trim additional two mannose and attach the second GlcNAc residue on the α1,6 mannose residue. To this point, these enzymatic reactions are conserved in both mammalian and plant cells, resulting in the classical biantennary GnGn glycan structure. However, substantial and complex structures arise in the *medial*- and *trans*-Golgi (Rayon, Lerouge, & Faye, 1998; Q. Wang, Yin, Chung, & Betenbaugh, 2017).

Generation of complex mammalian N-glycans is characterized by being multi-antennary (e.g., complex bi-, tri-, and tetra-antennary structures), fucosylated, galactosylated, and sialylated. The following N-glycan structures do not follow a linear enzymatic pathway and are highly dependent on the abundance of the glycoenzymes (Q. Wang et al., 2017). As a result, the final glycan structure is highly heterogeneous within the same protein, and therefore consist of multiple glycoforms. In the *medial*- and *trans*-Golgi, N-acetylglucosaminyltransferase-IV and V (GnTIV and GnTV, respectively) attach GlcNAc onto the outermost mannose residues making it tri- or tetra-antennary. Finally, the potential addition of fucose (in the core and outermost GlcNAc), galactose, and sialic acid by α1,6 fucosyltransferase (FucT), β1,4-galactosyltransferase (GALT), and α2,3- or α2,6- sialyltransferase (SIAT), respectively, results in the final complex multi-antennary glycan structures.

Alternatively, typical plant N-glycans consist of β1,2-linked xylose and core α1,3-linked fucose residues. These residues are added by β1,2-xylosyltransferase (XYLT) and α1,3-fucosyltransferases (FUT11/12), respectively. Plant α1,3 fucose and β1,2 xylose has been linked to being involved in IgE binding and therefore associated with causing an allergic response (Fitchette et al., 2003; van Ree et al., 2000). The final step involves β1,3-galactosyltransferase (GALT1) and α1,4-fucosyltransferase (FUT13) each transferring two galactose and fucose residues onto the GlcNAc to generate a Lewis antigen (type A)-like structure, which the human humoral system targets with IgM antibodies to clear. This limits the use of plant-made pharmaceuticals for human therapeutic application.

1.7.2 Glycoengineering

The low repertoire of glycoenzymes in plants allows for the ease of manipulating the N-glycosylation pathway for plant expression system. This restricted number of glycoenzymes is reflected in the homogenous profile of N-glycans of plant-produced mAb, unlike the heterogeneity of the mammalian cell production system (Schähs et al., 2007; Strasser et al., 2008). Efforts have been made to glycoengineer the pathways of several recombinant protein production platforms to have homogeneous N-glycan profiles. This dissertation will focus on the glycoengineering efforts done in plant expression systems.

Wild-type (WT) *N. benthamiana* contain XYLT, FUT11/12, GalT1, FUT13 which generate several immunogenic N-glycans on glycoproteins. This was a limitation

that raised the concern of adverse consequences of PMBs. These WT plants also lack the external machinery required to transport specific sugars to the Golgi (e.g., sialic acid). This provided Dr. Herta Steinkellner's group an opportunity to start with a relatively clean slate and build a glycosylation pathway. This involved removing or suppressing the undesired glycoenzymes (e.g., XYLT, FUT11/12) and overexpressing the mammalian glycoenzymes. Initially, the fucose and xylose transferases were suppressed by utilizing siRNA. This glycoengineered plant line produced glycoproteins with the GnGn structure, common in both mammals and plants. Currently, there are stable GnGn plants that utilized the TALENs gene editing system to knockout XYLT and FUT11/12 (Jin Li et al., 2016). This demonstrated the amenability of plants to be glycoengineered without any phenotypic change.

The subsequent addition of mammalian glycoenzymes into the plant N-glycosylation pathway required the clever use of the N-terminal CTS region to target the C-terminal enzymatic domain in the correct Golgi compartment. This provided a systematic approach to generate a proper mammalian N-glycosylation pathway. Currently, there are different glycoengineered plant lines that cover the spectrum of the mammalian glycoforms, such as the mammalian-like core-α1,6-fucosylation which is transiently expressing FUT8 (Forthal et al., 2010); generating bisecting GlcNAc residues involve overexpressing GnTIII in the *trans*-Golgi while branching requires GnTIV and GnTV in the *medial*-Golgi (Castilho et al., 2011; Castilho et al., 2013); galactosylation requires controlled expression of GalT in the *trans*-Golgi (Schneider et al., 2015; Strasser et al., 2009); sialylation requires the combined and tightly regulated interplay between 8

enzymes to produce the sialic acid conjugates and transfer mechanism to the *trans*-Golgi (Castilho & Steinkellner, 2012). Not only was the initial concern of immunogenic N-glycosylation addressed but the expansion of the mammalian N-glycosylation profile was accomplished.

1.7.3 Antibody N-glycosylation

N-glycosylation of antibodies determines its level of stability and effector function. In humans, IgG antibodies are all biantennary with 36 different possible types of N-glycosylation. 70% of antibodies are asymmetrically glycosylated, which means there are 72 different combinations of IgG1 Fc regions (Jennewein & Alter, 2017). All of these combinations have distinct binding to their cognate receptors (FcγRI, FcγRIIa, FcγRIIb, FcγRIIIa, FcRn, C1q, mannose-lectin, and c-type lectins) and therefore require experimentation to uncover resulting effector functions. IgG1 antibodies are post-translationally modified in the Asn297 of the Fc region. As previously mentioned, the Fc region of the antibody is involved in a range of antibody effector function such as ADCC, ADCP, CDC and IgG recycling. The next sections will describe several characteristics of aglycosylation, fucosylation, galactosylation, and sialylation in IgG antibodies which will provide a foundation on the potential functions of the described mAbs in this dissertation. A section for terminal mannose was excluded due to its quick clearance *in vivo* (Dirk, Alexandra, Andreas, & Arjen Schots and Herta, 2013; Y. D. Liu & Flynn, 2016).

1.7.3.1 Aglycosylation

The Fc N-glycan is crucial for the stability and effector function of an antibody. Aglycosylation of IgG1 results in the elimination of Fc binding to all the FcγRs and a tenfold decrease in binding with FcγRIa compared to the WT (Baruah et al., 2012; Nose & Wigzell, 1983; Sazinsky et al., 2008). As previously mentioned, FcR binding can be restored in aglycosylated antibodies by specific mutations (E382V/M428I mutations for increased FcγRI binding, S298G/T299A to FcγRIIA^{131R}, FcγRIIA^{131H}, and FcγRIIB).

1.7.3.2 Core Fucosylation

Defucosylation of the Fc region results in an increased affinity to FcγRIIIa by about 100-fold (Ferrara, Stuart, Sondermann, Brünker, & Umaña, 2006). Antibodies with α1,6 fucose demonstrate a reduced affinity to FcγRIIIa and therefore a reduction in ADCC (Ferrara et al., 2011; Satoh, Iida, & Shitara, 2006). This is corroborated by the crystal structure of an Fc-FcγRIIIa complex of a fucosylated and defucosylated Fc region. It is demonstrated that fucosylation sterically destabilizes the carbohydrate-carbohydrate interactions between the Fc and the Asn162 of FcγRIIIa N-glycans, therefore hindering binding (Shinkawa et al., 2003; Sondermann, Huber, Oosthuizen, & Jacob, 2000). Alternatively, a GlcNAc-bisected antibody is unable to be fucosylated due to the steric hindrance caused to FucT. This, in turn, results in an upregulation of GnTIII, which increases the amount of bisected GlcNAc antibodies (Kurimoto et al., 2014). An antibody with a bisecting GlcNAc also demonstrates a higher affinity to FcγRIIIa due to the lack of fucosylation. (Davies et al., 2001).

1.7.3.3 Terminal Galactosylation

The role of galactosylation in IgGs is not clearly defined with several contradicting studies. For example, it has been demonstrated that agalactosylation allows structure relaxation of the glycans which results in increased recruitment of the mannose-binding lectin, a complement system activating pathway (Garred et al., 2000; Malhotra et al., 1995; Raju, 2008). Alternatively, it has been demonstrated that the increased amount of terminal galactose residues on the Fc region also increase CDC activity by effective C1q binding (Duncan & Winter, 1988; Hodoniczky, Zheng, & James, 2005; L. Liu, 2015). Hypergalactosylation has been shown to increase ADCC activity with enhanced binding to FcγRIIIa and decrease the inflammatory state by binding to FcγRIIb and Dectin-1, which triggers an inhibitory signaling cascade (Karsten et al., 2012; Thomann, Reckermann, Reusch, Prasser, & Tejada, 2016). Overall, specific Fc-galactosylated glycan structures will require further studies to associate a specific function.

1.7.3.4 Terminal Sialylation

Terminal sialylation has been associated with an anti-inflammatory effect. Sialylated IgGs have been shown to have reduced binding to FcγRIIIa and FcγRIIb (Kaneko, Nimmerjahn, & Ravetch, 2006; Scallon, Tam, McCarthy, Cai, & Raju, 2007). Furthermore, depending on the level of sialylation in the Fc region could potentially decrease the antigen binding of some antibodies (Scallon et al., 2007). The exact mechanism is unknown but it is hypothesized that the bulky sialic acid residues alter the structure of the Fc region which prevent binding to classical FcRs and enhance binding to

c-type lectins (Massoud et al., 2014), DC-SIGN (Anthony, Kobayashi, Wermeling, & Ravetch, 2011; Sondermann, Pincetic, Maamary, Lammens, & Ravetch, 2013), Siglec/CD22 (Séïté et al., 2010), etc. Further investigation is required to uncover the anti-inflammatory mechanism.

1.8 Description of Dissertation

Antibodies are naturally occurring proteins that protect a host during the course of infection through direct neutralization and/or recruitment of the innate immune system. Unfortunately, in some infections, antibodies present unique hurdles that must be overcome for a safer and more efficacious antibody-based therapeutic. Plants have a unique capacity to express human antibodies with uniformed N-glycosylation. This unique feature of plant expression systems allows us to study how specific glycovariants interact with other components of the immune system to clear an infection; therefore, producing a tailor-made antibody for distinct diseases. This dissertation describes the utilization of plants to produce antibody-based therapeutics for Dengue Virus (DENV) and Chikungunya Virus (CHIKV). In the first section, our plant-produced glycovariants were explored for reduced interaction with Fc receptors and subsequent reduction of antibody-dependent enhancement for DENV infections. In the second section, our plant-produced glycovariants were explored for increased interaction with specific Fc receptors for increased antibody effector functions (e.g., ADCC) and an overall reduction in inflammation of CHIKV pathology. Overall, the significance of my dissertation is that it can possibly provide treatment for DENV and CHIKV; but equally importantly, give

some insight into the role of glycosylation in antibody effector functions, which has a broader implication for therapeutic development of other viral infections.

1.8.1 Arboviruses

Arboviruses are viruses transmitted by arthropod vectors such as mosquitos, ticks, and sand flies. Their distribution is seen throughout the *Bunyaviridae, Flaviviridae, Reoviridae,* and *Togaviridae* families with more than 100 known to cause human disease (Beckham & Tyler, 2015). For DENV and CHIKV, the dispersal is strongly associated with the movement of the highly urbanized mosquito vectors (*Aedes (Ae.) aegypti* and *Ae. alopictus*). This is of particular danger because the two viruses initially present similar febrile symptoms yet require vastly different supportive treatments (e.g., Nonsteroidal anti-inflammatory drugs (NSAIDs) for the self-limiting CHIKV which can exacerbate DENV infection) (Mardekian & Roberts, 2015). Both species of mosquitoes can co-circulate and co-infect both viruses making them a target to control associated morbidity and mortality in the endemic areas (Carrillo-Hernández, Ruiz-Saenz, Villamizar, Gómez-Rangel, & Martínez-Gutierrez, 2018). Initially, the *A. agypti* originated from Africa in the 15th century. Subsequent globalization of commercial trade and travel have been deemed responsible for its introduction into Asia and the Americas (Brown et al., 2014). The rapid urbanization and increased population density in Asia and Latin America have created a niche these mosquito vectors have been able to fill expertly. Similarly, *Ae. alopictus* mosquito has taken advantage of the trade of used tires to disperse its eggs into new territories (Reiter, 1998). Furthermore, the high adaptability of the *Ae. alopictus* mosquito has seen it spread to more temperate zones, such as the

outbreak of CHIKV in La Réunion in 2005 and north-eastern Italy in 2007 (Reinhold, Lazzari, & Lahondère, 2018; Rezza et al., 2007; Schuffenecker et al., 2006).

Vector control involving chemical or biological targeting of breeding sites has failed to stop disease transmission (Simmons, Farrar, van Vinh Chau, & Wills, 2012). Current approaches include the release of "genetically sterile" male mosquitoes to provide competition to wild-type male mosquitos for breeding opportunities. Although this approach has demonstrated great potential at controlling and eliminating the mosquitos, ethical considerations have been brought up about the cost of removing them from the ecosystem (Harris et al., 2012; James et al., 2018; Winskill et al., 2015). Nevertheless, the development of plant-based mAb therapy will provide a treatment option for those infected by DENV or CHIKV.

1.8.1.1 Dengue Virus

DENV is estimated to infect more than 390 million people annually over 100 countries in the tropical and subtropical regions of the world (Bhatt et al., 2013). DENV belongs to the *Flaviviridae* family which includes other human disease-causing viruses (e.g., Zika Virus, Yellow fever virus, West Nile Virus, etc.). There are four different serotypes of DENV that infect humans and a fifth that is maintained in the sylvatic cycle with related but distinct compositions (Mustafa, Rasotgi, Jain, & Gupta, 2015; Normile, 2013; Venkatachalam & Subramaniyan, 2014). Like other Flaviviruses, DENV has a positive sense RNA genome (10.7 kb) with one open reading frame (ORF) surrounded with a 5' and 3' untranslated regions (UTRs). Translation of the ORF generates one

polypeptide which is then processed by the host- and viral-derived proteases into seven non-structural (NS) and three structural proteins.

The mature DENV virion has an icosahedral structure composed of the capsid protein (CP), pr-membrane (prM/ M) protein and the envelope (E) protein. The NS proteins function in replication of the viral genome, initial processing of the viral polypeptide, and antagonism to immune response suppression (Thiemmeca et al., 2016). During translation of the virion, the CP, which encapsulates the positive-sense RNA genome to form the nucleocapsid, is translated in the cytosol and targeted to the ER through the hydrophobic signal peptide contained in the C-terminal end. The associated envelope is derived from the lipid bilayer of ER with 180 copies of the prM and E protein anchored in the membrane, also by the hydrophobic region in the C-terminal end (Rodenhuis-Zybert, Wilschut, & Smit, 2010). Within the secretory pathway, the immature virion undergoes furin cleavage of the prM to generate pr and M protein. The prM protein prevents the newly formed virus from re-infecting the cell by capping the E protein fusion loop (Kostyuchenko, Zhang, Tan, Ng, & Lok, 2013). The E protein monomers consist of an arrangement of three beta-barrels that make up three distinct domains (EDI, EDII, and EDIII). EDI makes up the structural backbone that connects the fusion peptide containing EDII to the immunoglobulin-like EDIII, which binds to initial receptors for receptor-mediated endocytosis. The majority of the neutralizing antibodies target the E protein (Wahala & Silva, 2011). The fusion loop is highly conserved between members of the Flavivirus family while the EDIII is distinct and highly neutralizing (Beltramello et al., 2010). Antibodies targeting the highly conserved fusion loop on EDII

are broadly neutralizing but lack the potency seen with EDIII antibodies. Normal infection initiates by attachment to various glycoproteins (e.g., heparin sulfate, DC-SIGN, mannose receptors) and receptor-mediated endocytosis by EDIII (Hidari & Suzuki, 2011). The non-activated E protein exists as a homodimer, with the fusion loop inaccessible, but once exposed to the acidic condition trimerizes and initiates class II fusion with the endosomal membrane (Klein, Choi, & Harrison, 2013; Modis, Ogata, Clements, & C Harrison, 2004). The viral genome is deposited within the host cell for viral replication and generation of the infectious virions.

Infection with DENV has varying severities that range from asymptomatic to Dengue hemorrhagic fever (DHF). Mild Dengue Fever (DF) presents itself with high fevers, headaches, myalgia, arthralgia, nausea, lymphadenopathy, and a rash; while, DHF often presents with additional abdominal pain with persistent bloody vomit, plasma leakage, hepatomegaly and eventually Dengue shock syndrome (DSS) (Malavige, Fernando, Fernando, & Seneviratne, 2004; Whitehorn & Simmons, 2011). Without proper intravenous fluid replacement therapy, individuals have a 20% mortality rate but with proper treatment can reduce the mortality rate to 1% (Bäck & Lundkvist, 2013). Normally, survivors of viral infections develop long-lasting immunity that protects them from a subsequent or related infection. Unfortunately, DENV survivors develop an immunological memory that provides life-lasting lasting protection against homotypic DENV infections but only 3-4 months for a heterotypic infection (Beltramello et al., 2010; Bäck & Lundkvist, 2013). Notably, beyond common factors (e.g. age and immunocompromised status), the severity of DENV disease, through an antibody-

dependent enhancement (ADE) manner, increases with previous exposure to a heterotypic DENV infection or structurally similar viruses like Zika virus (Christofferson & Kawiecki, 2016; Fowler et al., 2018; George et al., 2017; Guzman, x, a, & Kouri, 2003; Hermanns et al., 2018; M. Li et al., 2018).

ADE occurs in (1) the presence of sub-neutralizing antibodies or sub-neutralizing concentrations of antibodies that facilitate FcγR-mediated endocytosis in normally uninfected cells (extrinsic ADE) or (2) by indirectly compromising the innate immune response with immunomodulatory signals (intrinsic ADE) (Wan et al., 2013). For example, primary infection with DENV-1 is efficiently cleared from the individual. This results in the development of a humoral and cellular immunological memory that can protect the individual against a DENV-1 homotypic infection with specific neutralizing antibodies. The high concentration of anti-DENV-1 antibodies during the convalescence phase can also protect against a heterotypic infection, even if the antibodies are sub-neutralizing. The problem arises when the concentration of the sub-neutralizing antibodies decreases. These antibodies recognize and bind to the heterotypic DENV but fail to neutralize the virus fully. Instead, the antibodies facilitate FcγR-mediated endocytosis and expand the range of cells able to be infected (e.g., monocytes, macrophages, dendritic cells) (J Balsitis et al., 2009). This leads to increased viral uptake, replication, and consequently a higher viral load. Similarly, Fc-FcγR ligation to monocytes or macrophages results in the increased innate cell susceptibility for viral infection by reducing antiviral pathways (NOS2 expression, IFN, RIG-1 and MDA-5 signaling) as well as increasing immunosuppressive cytokines (IL-6 and IL-10)

(Halstead, Mahalingam, Marovich, Ubol, & Mosser, 2010; Huang et al., 2016). Together, the increased viral load is met with inflated cytokine production and cytotoxic T cell response, which is suspected of increasing vascular permeability and tissue damage leading to plasma leakage (Kurane et al., 1991; Mangada & Rothman, 2005).

Currently, the only approved vaccine available is the chimeric tetravalent live attenuated vaccine Dengvaxia™. After approval in over 19 countries, Sanofi Pasteur, a multinational pharmaceutical company, has encountered some controversy regarding its safety with an analysis demonstrating a potential enhancement of severe DENV symptoms in seronegative individuals (Aguiar & Stollenwerk, 2018; Fatima & Syed, 2018). Although this result was not statistically significant, the Philippine government temporarily stopped the continuation of the vaccination program. Beyond the political debate, DENV vaccines and therapeutics must ensure safety for human application. Along with Dengvaxia™, there are five other DENV vaccines are currently going through Phase III clinical trials. In Chapters 2, I will describe our efforts to develop a plant-based mAb therapeutic that addresses the ADE obstacle in *in vitro* as well as *in vivo* mouse models.

1.8.1.2 Chikungunya Virus

CHIKV is estimated to cause over 1 million infections per year in over 40 countries. CHIKV is an *Alphavirus* from the *Togaviridae* family which includes other arthritis-causing viruses (e.g., Ross River virus, Barmah Forest virus, o'nyong-nyong virus, and Sindbis virus) (Ganesan, Duan, & Reid, 2017). Initially discovered in Tanzania in 1952, CHIKV has spread to different geographical locations and has adapted specific

genotypes (e.g., Asian, West African, and East Central South African) (Sam et al., 2012). CHIKV is a positive sense RNA icosahedral enveloped virus with an approximate 11.8 kb genome. A 5' and 3' UTR surrounds two ORFs. The ORF near the 5'end codes for four non-structural proteins (NS-1, 2, 3, 4) required for RNA genome replication, protein modification, and immune antagonism (Sun et al., 2013). While the ORF nearest to the 3' end codes for five structural proteins (nucleocapsid (C), two envelope glycoproteins (E1 and E2), and two cleavage products (E3 and 6K)) that make up the virion. Translation of the structural proteins results in one polypeptide that is initially self-cleaved by the autocleavage domain of the capsid protein. The capsid assembles in the cytoplasm with the positive RNA genome. The envelope protein polypeptide is targeted to the ER where the 6K protein is cleaved off by signalase, resulting in E3-E2 and E1 proteins. After further processing, in the ER and Golgi, E3-E2 gets cleaved by furin in the *trans*-Golgi. Finally, E1 and E2 decorate the cell membrane before budding with the nucleocapsid (Solignat, Gay, Higgs, Briant, & Devaux, 2009). The E1-E2 heterodimers are arranged into 80 trimeric spikes with a single CHIKV virion containing 240 copies of the envelope proteins on its surface.

During a CHIKV infection, domain B of E2 functions in initial attachment to cell-surface glycosaminoglycans with domain A speculated to binding to prohibitin and phosphatidylserine-mediated virus entry-enhancing receptors to trigger receptor-mediated clathrin-dependent endocytosis (Solignat et al., 2009; van Duijl-Richter, Hoornweg, Rodenhuis-Zybert, & Smit, 2015; C. Weber et al., 2017). The low pH within the endosome leads to disassociation of domain B of the E2 protein from the domain II of the

E1, exposing the E1 fusion loop. Subsequently, trimerization of the E1 proteins further exposes the fusion peptides for viral to host membrane type II fusion (Kuo et al., 2012). Upon fusion, the nucleocapsid, along with the RNA genome, is deposited in the cytoplasm for viral replication (Hong, Perera, & Kuhn, 2006; Perera, Owen, Tellinghuisen, Gorbalenya, & Kuhn, 2001). Throughout a CHIKV infection, the E2 protein is the major target of antibodies and contains neutralizing epitopes. Specific anti-CHIKV E2 antibodies that are targeted around residue W64 have been shown to prevent entry and budding from the host cell (Jin et al., 2015; Kam et al., 2012). Late in the humoral response, the main target is the E2EP3 region of the E2 protein which has been shown to efficiently neutralize CHIKV in non-human primates (Kam et al., 2014).

CHIKV infection, although not lethal, is a cytopathic disease that targets stromal cells (e.g., fibroblasts, epithelial cells, and lymphoid tissues) causing immense acute damage and long-term inflammation in the skin, muscles, and joints (Tang, 2012). Infected patients develop acute symptoms within 2 to 12 days and with the viremia decreasing after 7-8 days (convalescent phase). Lingering muscle and joint pain can persist for months (Couderc & Lecuit, 2015; Galán-Huerta, Rivas-Estilla, Fernández-Salas, Farfan-Ale, & Ramos-Jiménez, 2015). The increased release of self-proteins caused by cytopathic damage is thought to increase the chances of developing chronic autoimmune diseases, such as rheumatoid arthritis (present in 10-20% of the infected individuals). It is hypothesized that there is a persistence of CHIKV replication during and after the convalescent phase. This is evident by the detection of CHIKV RNA in the joint tissue of C57BL/6J WT mice after 16 weeks of post-infection (Hawman et al.,

2013). Additionally, it has been observed that a high dose CHIKV inoculation in cynomolgus macaques lead to a persistence of viral replication after 90 days in the spleen, lymph nodes, liver, and muscle tissue, with macrophages suspected of being the reservoir (Labadie et al., 2010). Alternatively, it is also hypothesized that amino acid sequences in CHIKV E1 protein trigger immunopathology. CHIKV E1 has several conformational and linear epitopes that mimic human proteins (e.g., HLA-B27 and C3 component of the complement system) (Reddy, Desai, Krishna, & Vasanthapuram, 2017). Subsequently, these peptides were injected into C57BL/6J mice and resulted in the induction of inflammation similar to CHIKV alone. This molecular biomimicry of CHIKV has been attributed to potentially cause the chronic autoimmune manifestations (Reddy et al., 2017).

Normally, the short-term duration of the CHIKV infection is initially attributed to viral control by type I interferon (IFN) pathways. Infected stromal cells produce type-1 IFN that act on other non-hematopoietic cells to prevent the further spread of CHIKV. It is hypothesized that pattern recognition receptors (PRR), on hematopoietic cells, are engaged by phagocytosis of the CHIKV pathogen-associated molecular markers (PAMPs) after infection or cytopathic damage of tissue but not direct interaction with the virus (Schwartz & Albert, 2010). This allows the viral titers, during the acute phase, to reach levels up to 10^8 viral particles per ml of blood before controlling the infection by the innate immune system and further eliminating the CHIKV infection by both humoral and cellular branches of the adaptive immune system. During the acute infection phase, high levels of pro-inflammatory cytokines and chemokines (IFN-α/β, IL-6, IFN-γ, TNF-

α, CCL2 (MIP-1α), CCL3 (MIP-1β), and CCL4 (MCP-1)) are released in cynomolgus macaque, further increasing inflammation seen in the pathology of the disease in, as well as in the convalescent phase (Labadie et al., 2010). In addition to the innate immune system regulating the inflammatory response, the humoral response also plays a role in the chronic cases of arthritic symptoms. In a normal CHIKV infection, the overwhelming antibody response against CHIKV is the highly pro-inflammatory IgG3 type (Kam et al., 2012). The IgG3 Fc region strongly binds to all the FcγRs as well as recruiting the C1q complement pathway (Vidarsson et al., 2014). Although CHIKV infection is self-limiting, the resultant damage leads to prolonged and even chronic arthralgia and myalgia.

Currently, there are no approved treatments or licensed vaccines against CHIKV. CHIKV associated arthralgia/myalgia pain and inflammation are managed through NSAIDs. The use of aspirin and corticosteroids is not recommended due to the indirect exacerbation of the symptoms and disease (risk of bleeding and immunosuppression, respectively) (Tharmarajah, Mahalingam, & Zaid, 2017). Careful design of the vaccines must also prevent the induction of autoimmunity while providing robust protection against CHIKV. There are some recombinant and VLP vaccines (e.g., MV-CHIK, VRC-CHKVLP059-00-VP, CHIKV-NoLS) currently in Phase I and II clinical trials showing induction of a strong immune response and protective qualities in mice and non-human primates (Tharmarajah et al., 2017). In the case of CHIKV pathology, a fine-tuned immune response is required to clear the infection with minimal immunopathology. In an effort to generate this profile of therapeutic, I will describe the characterization two plant-

made mAb glycoforms for an increased ADCC to efficiently clear a CHIKV infection with minimal inflammation in *in vitro* and *in vivo* models (Chapter 3).

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CHAPTER 2

GLYCOENGINEERED PLANT-PRODUCED ANTI-DENGUE VIRUS (DENV) MONOCLONAL ANTIBODIES REDUCES ANTIBODY-DEPENDENT ENHANCEMENT AND PROTECTS MICE FROM DENV CHALLENGE.

2.1 Abstract

Cross-recognition of heterotypic DENV serotypes by antibodies from primary infection increases the susceptibility of viral infection and mediates Fc gamma receptor (FcγR) endocytosis of sub-neutralized DENV to normally non-permissive cells. Due to the increased risk of antibody-dependent enhancement (ADE) the utilization of anti-DENV monoclonal antibody (mAb) therapies has been limited. In this study, we evaluate our broadly neutralizing plant-produced anti-DENV E60 (pE60) mAb glycovariants and backbone variants for protection against DENV infection under ADE conditions. We hypothesize that the alterations in the Fc region would provide enough disruption in FcγR binding to eliminate or reduce ADE. The pE60 mAbs were transiently expressed in WT and glycoengineered- (ΔXFT) *Nicotiana benthamiana* plant lines for the generation of two distinct glycoforms. All of the pE60 mAb variants exhibited a high N-glycan homogeneity and retained pathogen specificity as well as neutralizing capacity *in vitro*. Of greater significance, our E60 mAb GnGn glycoform protected against lethal doses of DENV and reduced ADE in immunodeficient mouse therapeutic models. The N-glycosylation of the Fc region affects the stability, structure, and effector function of an antibody, and as demonstrated by our studies, is also involved in ADE. Overall, our study demonstrated great strides to develop a safer and more efficacious therapeutic against Flaviviruses while uncovering the role of N-glycosylation in antibody function.

2.2 Introduction

Dengue Virus (DENV) is an arbovirus capable of causing deadly hemorrhagic disease and is prominent in the tropical and sub-tropical areas of the world. Alone, DENV is estimated to infect 390 million people annually around the world (Bhatt et al., 2013). DENV is normally maintained in zoonotic cycles with humans as dead-end host, yet recent evidence suggests asymptomatic or silent individuals disseminate the virus in an estimated 84% of DENV transmissions (ten Bosch et al., 2018). It is estimated that an infected human can transmit the virus within 1.5 days after initial inoculation. Furthermore, the infected individuals become a DENV reservoir for mosquitos for the length of about eight days after the presentation of DENV febrile-related symptoms (Carrington & Simmons, 2014). This is an alarming development because with the increase in international travel and the recent emergence of the *Ae. aegypti* and *Ae. Albopictus* mosquito vectors in more temperate climates, the dissemination of DENV could potentially cause an epidemic in populations where knowledge and resources are scarce (Alves et al., 2013; La Ruche et al., 2010; Rezza, 2014; Schmidt-Chanasit et al., 2010).

Cross-recognition of different DENV serotypes and Zika Virus during a secondary infection has been proven to exacerbate the pathogenesis of DENV infections (Fowler et al., 2018; George et al., 2017). Epidemiological studies of DENV have witnessed an increase in the severity of the symptom several years after initial DENV infection by a phenomenon called ADE (Beltramello et al., 2010; Bäck & Lundkvist, 2013). Briefly, during primary infection by DENV, the immune system clears the

infection normally and leaves behind an immunological memory (both cell-mediated and humoral immune response) against that specific DENV serotype. Eventually, the concentration of both neutralizing and non-neutralizing DENV serotype-specific antibodies decreases. Thereafter, an infection with a heterotypic DENV leads to opsonization of sub-neutralizing antibodies. This facilitates the binding to innate immune cells through the antibody's Fc region and the Fcγ receptors. Consequently, this leads to increased viral uptake of a presumably neutralized DENV, or alternatively, increases the susceptibility of innate immunes cells to control secondary heterotypic infection due to previous immunosuppression. As a result, the viral loads increase and an amplified cellular response, with heightened cytokine production (IFNγ, TNFα, and IL 2, 6, 8, and 10), increases vascular permeability and tissue damage witnessed in dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Guzman, x, a, & Kouri, 2003; Wan et al., 2013).

Due to the dangers of ADE, vaccination might cause more harm than good (Aguiar & Stollenwerk, 2018; Fatima & Syed, 2018). If the vaccine does not generate a strong enough or specific enough antibody response, then the vaccine could potentially behave like a first DENV infection and leave an immunological memory that enhances the viral replication and symptoms of the true primary infection. The other danger is the potential emergence of a different serotype of DENV, meaning the designing of the vaccine must generate an equally potent antibody response against all five serotypes of the virus or circulating viruses of the area (Thisyakorn & Thisyakorn, 2014). For both situations, the major problem is the dependence on the immune system to pick out the

right neutralizing epitopes and generate a strong enough response for a long-lived immunological memory. In this circumstance, the best approach is to treat DENV infection with a passive neutralizing mAb-based prophylactic to prevent disease and subsequent treatment for those who do not reside and reside in the endemic area, respectively. But even then, a neutralizing mAb-based therapeutic might potentially increase ADE of DENV (Balsitis et al., 2010).

Antibodies are a major component of the adaptive immune system and the primary adaptive defense against invading pathogens. Antibodies recognize specific epitopes on pathogens, through the Fab region, and bridge the cellular immune system as well as the complement cascade pathway through the engagement of the Fc region. The Fab region plays an important role in the specificity of the mAb. Few modifications from the WT Fab is necessary to reduce the immunogenicity and increase the affinity of the antibody to the target epitopes for commercial use (Hu et al., 2015). Alternatively, the structure of the Fc region plays a role in the solubility, stability, long circulating half-life, and most importantly the antibody effector functions (Wang, Stuczynski, Gao, & Betenbaugh, 2015). Alteration of the hinge and proximal CH2 amino acid sequences greatly affects the binding to FcγRs. The interplay between the Fc region and the FcγRs on the immune cells and/or C1q dictates the overall immune response. As a result, multiple groups have been performing amino acid backbone mutations to modulate the level of antibody effector functions (Idusogie et al., 2001; Lazar et al., 2006; Stavenhagen et al., 2007). These large structural amino acid modifications greatly alter the interaction between the Fc region and the FcγRs. In some circumstances, it might be most optimal to

fine-tune the interaction to a specific FcγR, as exemplified by FcγRIIa mediation of ADE in DENV (Moi, Lim, Takasaki, & Kurane, 2010). For this reason, N-glycosylation has become a part of the Fc region engineering by adding specific N-glycans for fine-tuned control of Fc-FcγR binding. Modification of the Fc region is important to expand the safety and efficacy of the antibody-based therapeutic.

The search for an anti-West Nile Virus (WNV) mAb led to the discovery of an IgG2a anti-DENV mAb (E60). Initially raised against WNV in mice, the initial mouse E60 IgG2a mAb has been modified, with IgG1 constant regions replaced, resulting as a human-mouse chimeric mAb (Oliphant et al., 2005). E60 mAb targets EDII fusion loop and demonstrates a strong neutralization capacity towards all four serotypes of DENV (Oliphant et al., 2006). Unfortunately, in an *in vivo* lethal dose DENV challenge in IFN α/β/γ receptor-deficient AG129 mice the mammalian cell-produced E60 (mE60) failed to show any efficacy (Fig. 2). Several groups have incorporated point mutations to abrogate FcγR binding. Specifically, the asparagine to glutamine variant at position 297 (N297Q) generates an aglycosylated mAb which eliminates FcγR binding (Balsitis et al., 2010). Additionally, the leucine to alanine double mutant in positions 234 and 235 (LALA) remains glycosylated but cannot bind to FcγR or C1q binding (Hezareh, Hessell, Jensen, van de Winkel, & Parren, 2001). Both of these mutations of the mE60 mAb eliminated ADE *in vitro* and provided therapeutic protection to AG129 mice in a lethal dose model (Balsitis et al., 2010). In the ADE model, the LALA mutant provided complete protection while N297Q mutant provides partial protection (Williams et al., 2013). Together, these results suggest that N-glycosylation may be required for greater efficacy.

Previously, He et al. (2014) had demonstrated ADE in mammalian cell-produced humanized E16 (mHu-E16) and no ADE in the plant-derived Hu-E16 against WNV (He et al., 2014). This led us to question if the lack of homogenous N-glycosylation in the Fc region of the E60 mAb therapeutic was the cause of the lack of efficacy. We hypothesized that E60 mAb with plant and plant-humanized N-glycans (WT and GnGn, respectively) would have a decreased affinity to the FcγRs, which in turn would decrease the ADE and have better therapeutic efficacy. Herein, we explored the alteration of the Fc region through glycoengineering and amino acid backbone optimization. We utilized plant expression systems with an engineered glycosylation pathway for production of N-glycan specific mAb-based therapeutics in contrast to the mammalian cells due to the increased N-glycosylation homogeneity (Popp et al., 2018; Schneider et al., 2015; Schähs et al., 2007; Strasser et al., 2008). The backbone optimized mutants (N297Q, LALA, and the human FcγRs equivalent LALAGA triple mutant in positions 234, 235, and 236) were generated and expressed in both plant lines with the overall goal to see if glycoengineering could compensate for amino acid structural alterations done to the IgG1 Fc region.

2.3 Materials and Methods

2.3.1 Molecular Design and Cloning

The humanized E60 variable regions (pCI neo-chE60hG1 and pCI neo-chE60Lc) were a gift from the biotechnology company MacroGenics^{TM}. For both of the individual genes, a plant Kozac (ACA) and an intron-less plant signal peptide were placed in the 5' end to enhance the translation and ER targeting, respectively, by PCR (primers and the

reaction combinations are listed in Table 4 and Table 5, respectively). For ease of cloning, both constructs contained the *Eco*RI and *Nhe*I restriction enzyme sites at the 5' and 3' ends, respectively, and initially cloned in TOPO 2.1 vector. E60 variable heavy and light coding sequences were initially digested with EcoRI and NheI and subsequently engrafted onto human IgG1 CH1-3 and human kappa CL, respectively, by a three-way ligation into the MagnICON plant viral expression vectors (pICH21595 (3' TMV) and pICH11599 (3' PVX), respectively) and transformed in DH5α. Later, the resultant E60 Hc construct had additional backbone optimization mutations done on the Fc region by overlapping PCR (Table 5). All fully constructed clones were then transformed into the *A. tumefaciens* strain GV3101.

Concurrently, the E60 Hc and Lc sequences were cloned into pcDNA3.1, a mammalian cell expression vector by ThermoFisher scientific and subsequently transfected into the CHO-K1 cells using ThermoFisher Scientific lipofectamine protocol. Briefly, 1 μg of each pcDNA3.1 construct was mixed with 100μL of Opti-MEM media (ThermoFisher Scientific) and 12.5 μL of lipofectamine. Following a 45 minutes incubation at room temperature (RT), the mixture was diluted with 300 μL of Opti-MEM and then mixed at 1:1 ratio of F12 media (ThermoFisher Scientific). The diluted DNA-liposome complex mixture (400 μL) was then overlaid onto CHO-K1 cells (at approximately 90 % confluence) in a 12-well plate for 5 hours in a 37 °C incubator with 5% CO_2. Following the incubation, FBS (GE Healthcare Life Sciences) is added to a 10% final concentration and left to incubate for another 48 hours. Subsequently, the cells are harvested with trypsin-EDTA (ThermoFisher Scientific) and diluted to 5-10 cell/well in a

96-welled plate. Addition of F12 media with antibiotics (250 µg zeocin/mL and 300 µg hygromycin/mL) is used to select for stable transfected cells after 21 days.

2.3.2 Transient Expression via Agroinfiltration and Purification

Six-week-old *Nicotiana Benthamiana* grown in a 25 °C, 84% humidity environment with a 16/8 hour day/night cycle was infiltrated with five MagnICON (E60 Hc, E60 Lc, 5'TMV, 5'PVX, and INT) modules in GV3101 as described in (Leuzinger et al., 2013). Briefly, each module was cultured individually in YENB media (0.75% Bacto yeast extract, 0.8% Nutrient Broth, and pH 7.5) plus the appropriate antibiotic in a 30°C shaker overnight. Once there are sufficient cells (depends on the quantity of biomass material to be agroinfiltrated), the cells are removed from the culture media by initial centrifugation and decanting of the supernatant. The cell pellet is then resuspended in MES media (10 mM MES, pH 5.5; 10 mM $MgSO_4$) and diluted to an OD_{600} ratio of 4:1 (Hc:Lc components) for a final OD_{600} concentration <1 for syringe agroinfiltration. The agroinfiltrated plants are then stored back in the plant growth room for expression.

To measure temporal expression patterns leaves were harvested and processed in extraction buffer (1 X PBS (137 mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4, 1.8 mM KH_2PO_4) pH5.2, 10 mg/ml Sodium Ascorbate, 1 mM EDTA, 2 mM PMSF) at a 1 gLFW:1.5 mL ratio at 5, 6, 7, 8 days post agroinfiltration (DPI) and measured by ELISA. For the subsequent characterization and *in vivo* experiments, the leaves were harvested on 8 DPI and additionally processed. Homogenized crude plant extract is clarified by centrifugation at 15,000 g for 30 minutes at 4°C and subsequently purified by protein A affinity chromatography (He et al., 2014).

2.3.3 Mammalian Cell Expression and Purification

Positive clones are slowly propagated by transferring to large volumes (e.g., 48-welled, 24-welled, 12-welled, and 6-welled plates) every time they become confluent. Antibody production is stimulated when replaced with serum-free media. Five days later, the culture media is clarified by centrifugation at 15,000 g for 30 minutes at 4°C and subsequently purified by protein A affinity chromatography like previously mentioned.

2.3.4 Gel Electrophoresis and Western Blot

Samples for gel electrophoresis and subsequent western blots were prepared in SDS-PAGE protein loading buffer (50mM Tris-Cl pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol) in reducing (5%, v/v, β-mercaptoethanol) or non-reducing conditions on a 12% SDS polyacrylamide gel. Subsequently, gels were stained with Coomassie blue or transfer onto PVDF membranes for Western blot analysis. HRP-conjugated goat anti-human gamma or kappa antibodies (Southern Biotech) were used to detect the E60 Hc and Lc, respectively and later detected with Pierce^{TM} ECL western blotting substrate (ThermoFisher Scientific).

2.3.5 ELISA and Yeast Binding Assay

The ELISA performed to detect temporal expression after 5, 6, 7, 8 DPI, involved coating a high-binding plate with goat anti-human gamma antibodies (Southern Biotech). This was followed by incubation of the plant extract from the different time points and detection with HRP-conjugated goat anti-human kappa antibodies. mHu-E16 mAb was used as a reference standard (Lai et al., 2010). Alternatively, the DENV-2 E protein (Creative Diagnostics) was coated on the high-binding plates for the ELISA testing for

pE60 specific DENV-2 E protein binding. Subsequently, different concentrations of the purified pE60 variants were incubated and later detected with HRP-conjugated goat anti-human gamma antibodies. mE60 (positive control) and a non-DENV specific human IgG (negative control) were used as controls. For ELISAs the plates were developed with TMB Microwell peroxidase substrate (KPL). Each sample was performed in triplicates in three individual experiments. The data was analyzed by GraphPad Prism software. For the binding ELISA, the kD was calculated by a non-linear regression analysis using a one-site binding model with P-value derived from the difference comparing the pE60 to the mE60 by an unpaired t-test (P-value <0.05 indicated statistical significance). Additionally, to supplement the binding ELISA, yeast cells displaying DENV EDI-EDII were stained with E60 mAb variants. Secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG was used for detection and analyzed by a Becton Dickinson FACSCalibur flow cytometer (as described in Oliphant et al., 2005).

2.3.6 N-glycan Mass Spectroscopy Analysis

The E60 Hc and Lc were initially separated by a 10% SDS-PAGE under reducing conditions and visualized by Coomassie blue staining. Subsequently, the Hc-containing bands were excised from the gel and eluted out with 50% acetonitrile, followed by S-alkylation and tryptic or tryptic/GluC digestion. The digested fragments are then separated on a reversed-plate column (1500.32 mm BioBasic-18, Thermo Fisher Scientific) with a 1-80% gradient. Lastly, the fragments go through liquid chromatography-electrospray ionization-mass spectroscopy (LC-ESI-MS) analysis by a quadrupole time-of-flight (Q-TOF) Ultima Global mass spectrometer. The spectra were

aggregated and deciphered to individual N-glycoforms (annotated by ProGlycAn nomenclature (www.proglycan.com).

2.3.7 Virus and Cells

DENV-2 (ATCC, VR-1584) was propagated and titered in BHK-21 cells (ATCC, CCL-10). BHK-21 and Vero cells (ATCC, CCL-81) were cultured in DMEM supplemented with 10% FBS at 37°C in incubation with 5% CO_2. K562 cells (ATCC, CCL2243) were cultured in RPMI 1640 (ThermoFisher Scientific) supplemented with 10% FBS at 37°C in incubation with 5% CO_2.

2.3.8 Antibody-Dependent Enhancement Assay

Similar to the ADE assay described in (He et al., 2014), the antibody enhancing activities of the E60 mAb variants were determined using FcγRIIa$^+$ K562 cells (ATCC, CCL2243), a human erythroleukemic cell line that is non-permissive in the absence of enhancing anti-DENV antibodies. The E60 mAb variants or the negative control (non-DENV specific human IgG) were serially diluted three-fold every time in eight total concentrations and incubated with DENV-2 (ATCC, VR-1584) for 1 hour at 37°C. After, the mAb-DENV-2 complexes are mixed with the K562 cells (MOI =1) and incubated for an additional 48 hours. The cells are then washed with PBS, fixed with 4% paraformaldehyde (Sigma), and permeabilized with 0.1% saponin (Sigma) for staining with Alexa 488 (Invitrogen)-conjugated 4G2 mAb (ATCC, HB112). Additional washes rinse non-bound 4G2 mAb before analysis for infected (4G2-positive) cells with the Navios flow cytometer (Beckman Coutler).

2.3.9 DENV-2 Neutralization Assay

The E60 mAbs variants and the negative control (non-DENV specific human antibody) were serially diluted in serum-free Opti-MEM media (Life Technologies) with 100 μL transferred onto a 96-well plate. 100 μL of 200 plaque forming units (PFUs) of DENV-2 was mixed to the E60 mAb containing wells and incubated for 1 hour at 37 °C and 5% CO_2. Before the 1-hour incubation is over, the Vero cell growth medium (DMEM, 5% FBS, 100 IU/mL Penicillin, 100 μg/mL Streptomycin, 2mM L-glutathione) was aspirated and replenished with 1 mL of fresh growth media to each well of a 6-well plate. After the incubation, 200 μL of the mAb-virus mixture was added onto the Vero cell monolayer (80% confluency) and incubated for 4 hours at 37 °C and 5% CO_2. Before the 4-hour incubation is over, the first 1% SeaPlaque low-temperature melting agarose with the growth media was prepared and kept in a 44°C water bath. Following incubation, the mAb-virus mixture was carefully aspirated, and the 1% agarose overlay was put into each well. The agarose-containing plate was allowed to solidify for 15 minutes at RT and subsequently stored in the incubator (37 °C, 5% CO_2) for five days. On the 5th day, 3 mL of the second 1% agarose overlay with 4% (v/v) concentration of Neutral Red is added into each well and placed in the incubator (37 °C, 5% CO_2) for 4-6 hours. After the time elapsed, the plaques were visible and counted. The data was analyzed by GraphPad Prism software.

2.3.10 *In Vivo* Animal Studies

To evaluate the therapeutic efficacy of the E60 mAb variants, interferon α/β and γ-receptor-deficient AG129 mice were injected a lethal dose (4×10^6 PFU) of DENV-2

D2S10 intravenously. Twenty-four hours later, the mice were injected with 50 µg of each E60 mAb variant in a final volume of 100 µL also intravenously. The mice were observed for morbidity and mortality for 21 days after infection. The ADE model involved simulating secondary heterotypic infection in AG129 mice. Briefly, 20 µg of anti-DENV-4 membrane protein antibodies were injected intravenously (Day -1) and subsequently followed by infection with a sub-lethal dose (10^5 PFU) of DENV2 D2S10 24 hours later (Day 0). Twenty-four hours after the infection, the mice were intravenously injected with 50 µg of each E60 mAb variant in a final volume of 100 µL. The mice were observed for morbidity and mortality for 21 days after infection (n=20). The data was analyzed by GraphPad Prism software.

2.4 Results

2.4.1 Structural Characterization of pE60 mAb

The coding sequences of E60 mAb variants were cloned into the 3'TMV and 3'PVX plant expression vectors (Fig. 3A-E). The E60 mAb variants (Fig. 4) were then expressed and efficiently purified from WT and GnGn *N. benthamiana*. Expression involved agroinfiltration of *A. Tumefacien* containing the MagnIcon viral vector constructs with the associated heavy and light chain mAb genes. At DPI 8 of transient expression, the E60 mAbs variants were at the highest expression level (Fig. 5) and purified by low pH precipitation and protein A chromatography. The resultant products were characterized by SDS-PAGE and Western blot analysis. The non-reduced sample in the SDS-PAGE analysis indicates a high level of purity when compared to the loading crude sample as well as minimal degradation (Fig. 5A). Together with the western blots,

the non-reduced samples corroborate that a majority of HC and LC are assembling into the expected molecular size. The reduced sample (Fig. 5B) demonstrates both HC (50 kDa) and LC (25 kDa) are present at the expected molecular size. The reduced western blot was detected with an anti-gamma secondary antibody, while the Western blot with the non-reduced samples was detected with anti-human kappa antibodies, confirming the presence of the HC and LC, respectively (Fig. 5C). For both of the western blots, Lane 1 was the negative control consisting of non-infiltrated leaves while lane 2 was the commercial human IgG1 positive control. The backbone optimized mutants also resulted in proper expression and assembly demonstrated by western blot analysis (Appendix D).

2.4.2 N-linked Glycosylation Analysis of pE60

The N-glycosylation in the C_H2 domain of an antibody determines its solubility, stability, circulating half-life, and antibody effector functions (Wang, Yin, Chung, & Betenbaugh, 2017). Thus, we utilized liquid chromatography–electrospray ionization–mass spectrometry (LC–ESI–MS) to examine the N-glycosylation of all the E60 mAb variants. As expected, the pE60 mAbs demonstrated high homogeneity (70-95%) compared to the mE60 mAb counterpart, a single predominating N-glycoform. Specifically, the WTpE60 exhibited minimal heterogeneity with the main N-glycoforms including the complex-type N-glycans that terminate with N-acetylglucosamine (GlcNAc) residues and carry the plant-specific β1,2-linked xylose and core α1,3-linked fucose. The GnGnpE60 mAbs lack the xylose and fucose residues but maintain the mammalian-like $GlcNAc_2Man_3GlcNac_2$ structure. Alternatively, the mE60 mAb

exhibited core a1,6 fucosylated structures with and without terminal b1,4-galactose (GnGnF6 and AGnF6) (Table 6).

2.4.3 pE60 Specifically Binds to DENV-2 E Protein

N-glycosylation is necessary for proper folding; therefore alteration of the N-glycans could lead to a misfolded protein (Shental-Bechor & Levy, 2008). The folding and retention of specific DENV EDII binding of WTpE60 and GnGnpE60 were examined by flow cytometric analysis of yeast cells displaying DENV-2 EDI-EDII on their surface. Incubation of yeast cells displaying EDI-EDII with WTpE60 and GnGnpE60 demonstrated a similar percentage of cells with the same fluorescent peak as compared to mE60 (Fig. 7A-C). Alternatively, both negative controls (yeasts not expressing EDI-EDII with pE60 incubation and yeast displaying EDI-EDII with negative control mAb (pGP1)) failed to show any binding with a basal level fluorescence (Fig. 7D and E). The antigen binding affinity and kinetics were further analyzed by ELISA. WTpE60 and GnGnpE60 DENV-2 E protein binding demonstrated similar binding capacities (Kd value of 13.64, 12.72 and 12.37 ng/mL, respectively) independent of the source of E60 expression (P-value=0.96 and 0.93, compared to mE60, respectively) (Fig. 8). Overall, both WTpE60 and GnGnpE60 demonstrated specific binding with similar affinity and binding kinetic as mE60.

2.4.4 Neutralization Activity of pE60 Variants Against DENV-2

The neutralization capacity of all the pE60 mAb variants was assessed by plaque reduction neutralization assay. All of the pE60 variants had a similar curve pattern and neutralization capacity (Fig. 9A) with the LALAGA mutant produced in WT plant

visually demonstrating the highest EC50 while pE60 N297Q had the lowest (Fig. 9B). Overall, regardless of the alterations done by glycoengineering or combinatory backbone optimization all of the pE60 mAb variants demonstrated similar neutralization capacities with an estimated EC50 value around 300 ng/mL.

2.4.5 pE60 Eliminates Antibody-Dependent Enhancement *In Vitro*

ADE is predicted to facilitate infection of FcγR-bearing cells when ligation occurs to the Fc region of sub-neutralizing antibodies (Halstead, Mahalingam, Marovich, Ubol, & Mosser, 2010). Since the IgG1 N-glycosylation site is important for FcγR binding, modification of the N-glycans can have an impact on ADE. Accordingly, we utilized the FcγRIIa^{+} K562 cells to investigate the impact of homogenous N-glycan on WTpE60 and GnGnpE60 on ADE activity *in vitro* compared to the N-glycan heterogenous mE60. The results demonstrated a significant level of ADE activity at concentrations below 1 µg/mL when DENV-2 was treated with mE60, as previously observed (Balsitis et al., 2010). Alternatively, both WTpE60 and GnGnpE60 exhibited no ADE activity, which was similar to that of the negative control mAb (Fig. 10). Overall, both plant N-glycoforms eliminated ADE in human K562 cells.

2.4.6 pE60 Exhibits Therapeutic Potential *In Vivo* and Under ADE

Given the previous results, we investigated if the lack of ADE in the *in vitro* assay would translate to protection in a lethal dose AG129 mice model and under ADE conditions. The results showed GnGnpE60-treated mice were completely protected against a lethal dose challenge of DENV-2 after 21 days. In contrast, the combination of backbone optimization and glycoengineering resulted in a reduced percent survival of

60% in the same amount of time (Fig. 11). The mE60-treated mice were all dead by day 6 of infection. The GnGnpE60 was then used for the enhancing model. Similar to the lethal dose experiment, GnGnpE60-treated mice had over a 90% survival after 21 days, while only 15% of mE60-treated mice survived in the same amount of time (Fig. 12). Exactly like the lethal dose experiment, the negative control PBS-treated mice died within six days after initial infection. Overall, the GnGnpE60 mAb provided protection against the DENV-2 lethal dose challenge and under ADE condition.

2.5 Discussion

The range expansion of the DENV mosquito vectors has prompt the development of efficacious anti-DENV mAb therapeutics. Unfortunately, the current mammalian cell culture system has limited its accessibility to the developing countries where DENV is endemic. This is due to the high cost associated with the production and safety concerns in the application of mAbs for DENV infections. ADE limits the use of mAb-based therapeutics due to the exacerbated pathogenesis witnessed upon secondary heterotypic infection, which coincides with the hesitancy in the application of a vaccine against DENV. Nevertheless, several anti-DENV mAb candidates have been discovered with the most potent neutralizing mAbs targeting the serotype-specific EDIII. Sadly, its application is limited by the co-circulation of multiple serotypes of DENV in the same areas, which increase the risk of severe DHF and DSS. In contrast, the mAb targeting the structurally conserved fusion loop on the EDII are broadly neutralizing with less potency. Here, we investigated the impact of plant and mammalian-like homogenous N-glycosylation of our broadly neutralizing pE60 mAb in the reduction of ADE during

secondary heterotypic DENV infection. This work describes the development of the pE60 mAb with the homogenous GnGn structure, data that demonstrates a reduction in ADE activity *in vitro*, and protection against DENV-2 under *in vivo* ADE conditions.

A prerequisite for the development of the E60 mAb is a standardized production system that consistently and robustly produces the target biologic. Plants have been seen as a promising alternative to the traditional mammalian cell culture systems due to its low-cost, rapidity, low-probability of contamination by human pathogens, high-protein output, and its highly regulated post-translational modifications (Lomonossoff & D'Aoust, 2016). Our results demonstrate the ease of producing different pE60 mAb variants in *N. benthamiana* plants with accumulation levels reaching 120 µg/g LFW within 8 DPI. This expression level is comparable to other mAbs that have not been codon-optimized for plant expression (Chen & Lai, 2014). Several groups have reported mAb expression levels of codon-optimized genes ranging from 0.5 to 4.8 mg/g LFW (Bendandi et al., 2010). The other prerequisite is a feasible way to purify the target biologic. The results clearly demonstrate the efficient purification of the E60 mAb variants with minimal degradation and proper assembly by low-pH precipitation and protein A chromatography. The quick turnaround time by the plant expression system is unmatched by the mammalian cell counterpart. As further exemplified by Medicago's production of the Influenza VLP-based vaccine (D'Aoust et al., 2010). The rapid and robust production of pE60 mAb could be of value in the case of an emergency outbreak of DENV in unprepared areas.

One of the advantages of our plant expression systems is the production of mAb with a high level of homogeneous N-glycosylation. Due to the limited repertoire of glycoenzymes in the plant genome, plant N-glycosylation pathways have been optimized to produce homogenous human N-glycans for biologic production (Castilho et al., 2011; Castilho et al., 2013; Strasser, Altmann, & Steinkellner, 2014). Herein, are the utilization of the β1,2-xylosyltransferase and α1,3-fucosyltransferases siRNA-suppressed (ΔXFT) and WT plants to produce pE60 with the GnGn and GnGnXF (WT) structure, respectively. The importance of N-glycosylation is demonstrated by the aglycosylated mAb, which decreases binding to FcγRIa by at least 2-fold and eliminates binding to other FcγRs (Jefferis & Lefranc, 2009; Lai et al., 2014). Our results demonstrate pE60 produced in the WT and ΔXFT plants had 70% and 95% homogeneity, respectively, while the mE60 was almost split between two predominant N-glycoforms (50% and 40%), which is not surprising (van Berkel et al., 2009; Zhang et al., 2016). The N-glycosylation homogeneity of our pE60 mAbs allowed us to decipher the role of specific N-glycans in DENV ADE.

Preliminarily, we investigated if there were any differences between the pE60 and mE60, in terms of antigen binding kinetics and subsequent *in vitro* neutralization of DENV. A major mechanism of anti-DENV antibody neutralization is through specific antigen engagement (Brien et al., 2013; Edeling et al., 2014). Our results demonstrate specific binding to the DENV fusion loop between EDI-EDII that was displayed on the yeast cells, while our ELISA results corroborate the previous data showing similar Kd values. Furthermore, *in vitro* neutralization of DENV-2 on Vero cells demonstrated

potent neutralization capacity with a mean EC50 of 300 ng/mL of all the pE60 including the backbone mutants. This is not surprising because for this particular experiment we specifically looked at the function of the Fab region. DENV pathogenesis involves both the Fab and Fc region (Balsitis et al., 2010). Together, this indicates that, irrespective of the expression system, specific binding and Fab mediated neutralization capacity were retained by all pE60 mAbs. Next, we wanted to examine if the specific homogenous N-glycosylation of our pE60 could abolish ADE *in vitro*. Notably, our results demonstrated that both WTpE60 and GnGnpE60 eliminated ADE activity on FcγRIIa-expressing K562 cells, while the mE60 showed significant levels of ADE activity. This suggests our plant glycovariants do not bind to the necessary FcγR to induce ADE. Alternatively, one or both of the predominant mE60 glycovariants ($AGnF_6$ and AAF_6) mediates ADE. In the literature, it is ambiguous of the effects of terminal galactosylation on FcγR binding or antibody effector function. Our results suggest terminal galactose residues are required for FcγRIIa binding. Ritamo et al. corroborated our finding by reporting an increase in binding to FcγRII and FcγRIII with increase terminal galactosylation on CHO cell expressed mAbs by surface plasmon resonance (Ritamo, Cloutier, Valmu, Néron, & Räbinä, 2014). Together, our pE60 demonstrate great potential to eliminate ADE *in vitro*.

Given the promising data with the pE60 mAb variants, we narrowed our candidates to GnGnpE60 and GnGnpE60 LALA and tested the variants for therapeutic efficacy in viral infection susceptible AG129 mice a lethal dose model. Treatment with the 50 µg of GnGnpE60 resulted in the survival of all of the mice after 21 days. This demonstrates pE60 ability to strongly protect against DENV-2 infections. Surprisingly,

the combination of backbone optimization and glycoengineering resulted in a lower survival (60%) as compared to glycovariant alone. The goal of the amino acid backbone variants was to see if combining both approaches could yield better therapeutic efficacy, yet the results demonstrate the contrary. This establishes the role of N-glycosylation in Fc-FcγR binding is a fine-tuning of the interaction and highlights the importance of homogenous N-glycosylation in mAb production. Furthermore, to control the DENV infection, some degree of antibody effector functions is required. Finally, we wanted to investigate if the GnGnpE60 mAb retained its therapeutic efficacy under ADE conditions. Treatment of mice with 50 µg strongly protected mice with 90% surviving the sub-lethal dose of DENV-2, while the mE60 treatment only had 15% survival. The results show that ADE was reduced in the GnGnE60 treated mice which led to better overall protection. It is speculated that an earlier administration of the GnGnpE60 would have saved the mice that succumbed to the DENV infection. This highlights the importance of a timely administration of the therapeutic. Together, GnGnpE60 demonstrates a great potential for the treatment of DENV with a better safety and efficacy profile than the mammalian cell counterpart.

We hypothesize there are two antibody effector pathways responsible for protection: (1) through ADCC and/or (2) a specific phagocytosis pathway (ADCP). First, DENV non-structural protein 1 (NS-1) inhibits CDC activity by binding C1s, C4, C4-binding protein, and the mannose-binding lectin; therefore CDC is inherently decreased in DENV infections (Thiemmeca et al., 2016). Secondly, the LALA mutant abolishes all binding to FcγRs and C1q, meaning there is no ADCC, ADCP, and CDC (Hezareh et al.,

2001). This eliminates CDC activity as a mechanism for *in vivo* protection against DENV. The lack of ADE of GnGnpE60 in the *in vitro* assay suggests ADCP activity is minimal or a specific pathway is utilized as highlighted in (Trivedi et al., 2006). The lack of fucosylation suggests a greater affinity to human FcγRIIIa, which in turn, would lead to a stronger ADCC response in humans (Ferrara et al., 2011; Ferrara, Stuart, Sondermann, Brünker, & Umaña, 2006). Unfortunately, the utilization of a mouse model also changes the contributing cells. For example, the orthologue for the human FcγRIIIa is the murine FcγRIV which is expressed on monocytes, macrophages, and neutrophils (Moldt & Hessell, 2014). These are cells that are involved in both ADCC and ADCP (Bournazos, DiLillo, & Ravetch, 2015; Lu, Suscovich, Fortune, & Alter, 2017). This suggests that the *in vivo* protection might have come from the increased ADCC and ADCP activity of the monocytes, macrophages, and the neutrophils.

Previous serum analysis of individuals presenting DF and DHF suggests ADCC activity is involved in the increased severity of DENV pathogenesis (García et al., 2006). However, the depletion of NK cell-mediated ADCC activity resulted in an increased DENV ADE *in vitro*. It is highly unlikely that the DENV E protein is exposed on the surface of the infected cells (Rodenhuis-Zybert, Wilschut, & Smit, 2010). Meaning ADCC might control the DENV infection through mAb-virus complex activation of cytotoxic granule release and indirectly kill DENV infected cells by proximity to the increased cytokine and viral gradient (Chan, Smyth, & Martinet, 2013). This highlights the importance of a fine-tuned response for mAb-based therapeutic development as well as demonstrates the complexity of ADE as a component to the pathogenesis of DENV.

Additional studies in non-human primate models are required to fully uncover the neutralization mechanism of GnGnpE60 mAb for DENV infections.

In summary, we were able to express and purify the E60 mAb variants from two glycoforms (WT and GnGn). Both glycovariants and backbone optimized pE60 retained its specificity and neutralization capacity *in vitro* and demonstrated *in vivo* protection against DENV under ADE conditions. These results demonstrate the importance of homogenous N-glycosylation and highlight a unique niche plant expression systems could fill in the production of pharmaceuticals. Overall, this is an example of great stride to understand the role of N-glycosylation in the context of the mAb for a safer and more efficacious therapeutic.

2.6 Figures

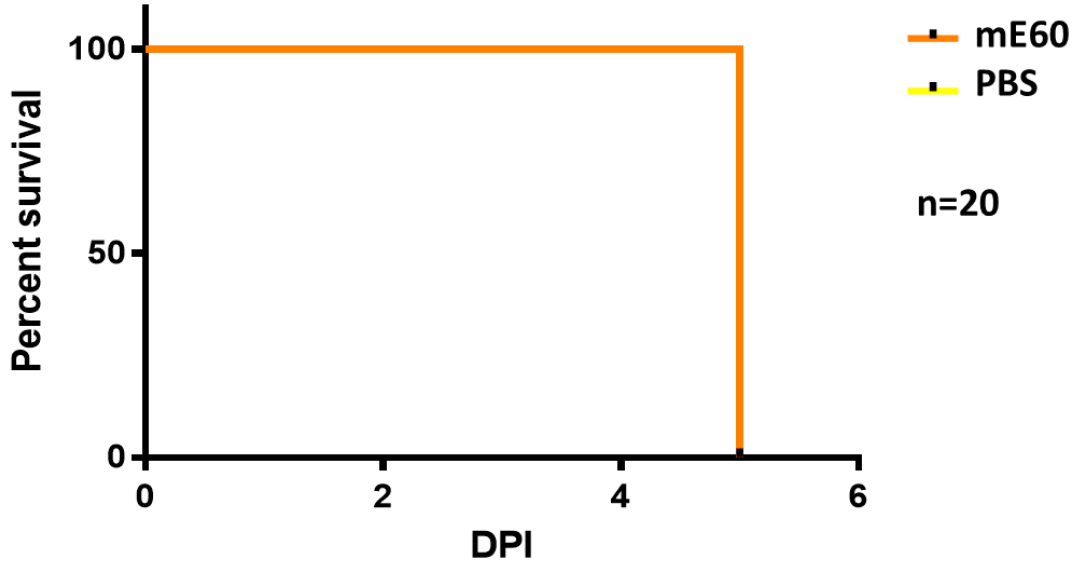

Figure 2. Lethal Dose Model of Interferon α/β/γ Receptor-deficient AG129 Mice. Mice were injected with 4×10^6 PFU of DENV-2 intravenously and 24 hours later administered either E60 mAb (orange) or PBS (yellow). The mice were observed for morbidity and mortality for 21 days after infection.

Table 4. List of Oligonucleotides Used in the Study.

Strategy Number	Primer	Sequence
1	Forward E60 VH Plant Signal Peptide Overhang	5'-TTGGTTGCAACAGCTACTGGTGTTCATTCTGAGGTCCAGGTGCAACAG-3'
2	Forward EcoRI-Kozac Sequence-Plant Signal Peptide	5'-AGAATTCACAATGGGATGGTCTTGTATCATCCTTTTCTTGGTTGCAACAGC-3'
3	Reverse NheI-E60 VH	5'-TGCTAGCTGAGGAGACTGTGAGAGTGGTGC-3'
4	Forward E60 VL Plant Signal Peptide Overhang	5'-TTGGTTGCAACAGCTACTGGTGTTCATTCTGACATCCTGATGACCCAATC-3'
5	Reverse NheI-E60 VL	5'-TGCTAGCTTTGATTTCCAGCTTGGTGCCTC-3'
6	Forward N297Q	5'-GAACAGTACCAAAGCACGTACAGGGTTGTCTCA-3'
7	Reverse N297Q	5'-GTACGTGCTTTGGTACTGTTCCTCTCTCGGCTT-3'
8	Forward L234A-L235A	5'-CCTGAAGCTGCTGGAGGACCGTCAGTCTT-3'
9	Reverse L234A-L235A	5'-TCCTCCAGCAGCTTCAGGTGCTGGGCA-3'
10	Forward L234A-L235A-G237A	5'-CCTGAAGCTGCTGGAGCACCGTCAGTCTT-3'
11	Reverse L234A-L235A-G237A	5'-TGCTCCAGCAGCTTCAGGTGCTGGGCA-3'
12	Reverse BamHI-CH3	5'-AGAGGATCCTCATTTACCCGGAGACAAGGAGAGA-3'

Table 5. Details of PCR Reactions to Generate Each Anti-DENV mAb Variants.

Reaction	PCR Primer and DNA Combination	Products
1	E60 VH+1+3	A
2	A+2+3	EcoRI-Plant Kozac-signal peptide-E60 VH-NheI
3	E60 VL+4+5	B
4	B+2+5	EcoRI-Plant Kozac-signal peptide-E60 VL-NheI
5	E60 Hc+2+7	C
6	E60 Hc+6+12	D
7	C+D+2+12	E60 Hc N297Q
8	E60 Hc+2+9	E
9	E60 Hc+8+12	F
10	E+F+2+12	E60 Hc L234A L235A
11	E60 Hc+2+11	G
12	E60 Hc+10+12	H
13	G+H+2+12	E60 Hc L234A L235A G237A

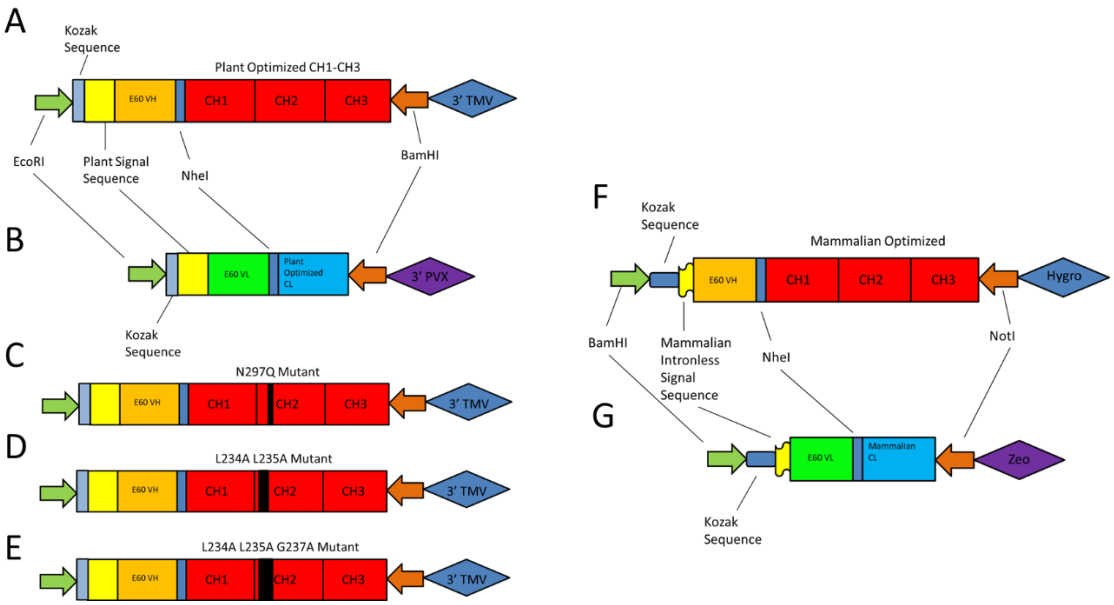

Figure 3. General Schematic of Anti-DENV mAb Variants Utilized in this Study. Plant anti-DENV mAb genes (A-E) consist of an EcoRI, a plant Kozac sequence (ACA), and an intron-less plant signal peptide in the 5' end. Between the end of the variable region and the beginning of the constant reion (CH1 and CL) a *Nhe*I restriction enzyme site was put in place to facilitate variable region switching for future constructs. The E60 variable heavy and light coding sequences were engrafted onto human IgG1 CH1-3 and human kappa CL, respectively. The BamHI is required for orientation-controlled ligation into the viral expression vectors (pICH21595 (3' TMV) and pICH11599 (3' PVX)). Backbone optimization mutations were later incorporated by overlapping PCR. Mammalian anti-DENV mAb Hc and Lc genes (F-G, respectively) were cloned into pcDNA3.1 Hygromycin and Zeocin by BamHI and NotI restriction enzyme sites. Both mammalian genes have mammalian expression elements.

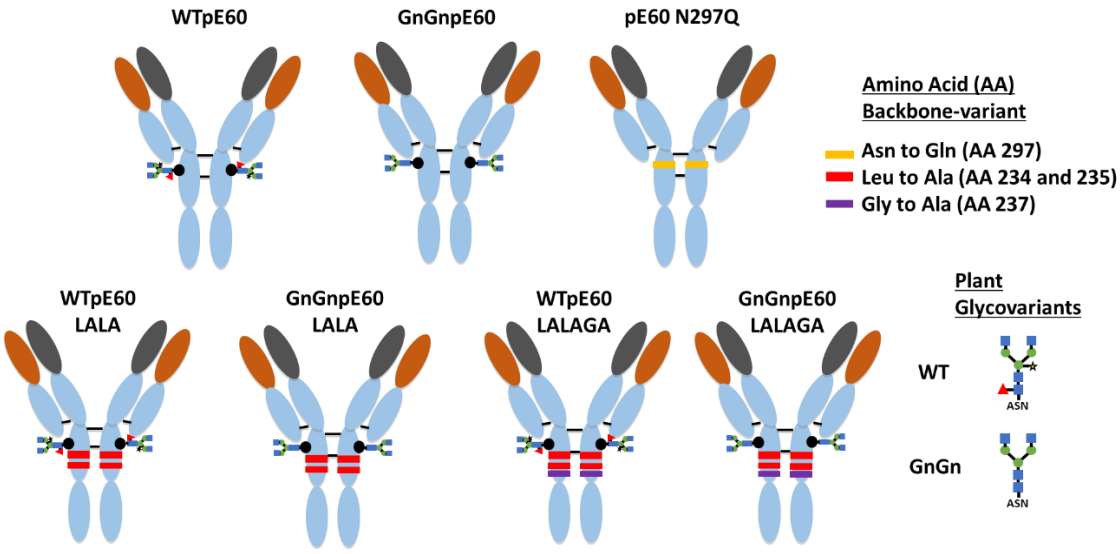

Figure 4. Constructs of pE60 Glyco- and Backbone Variants Utilized in this Study. Backbone pE60 mutants were generated by targeted mutation (asparagine to glutamine (AA 297); leucine to alanine (AA 234 and 235); and leucine to alanine (AA 234 and 235) plus glycine to alanine (AA 237)) by overlapping PCR of the plant-optimized Hc gene. The N-glycosylation was determined by transient expression in WT and ΔXF *N. benthamiana* plants.

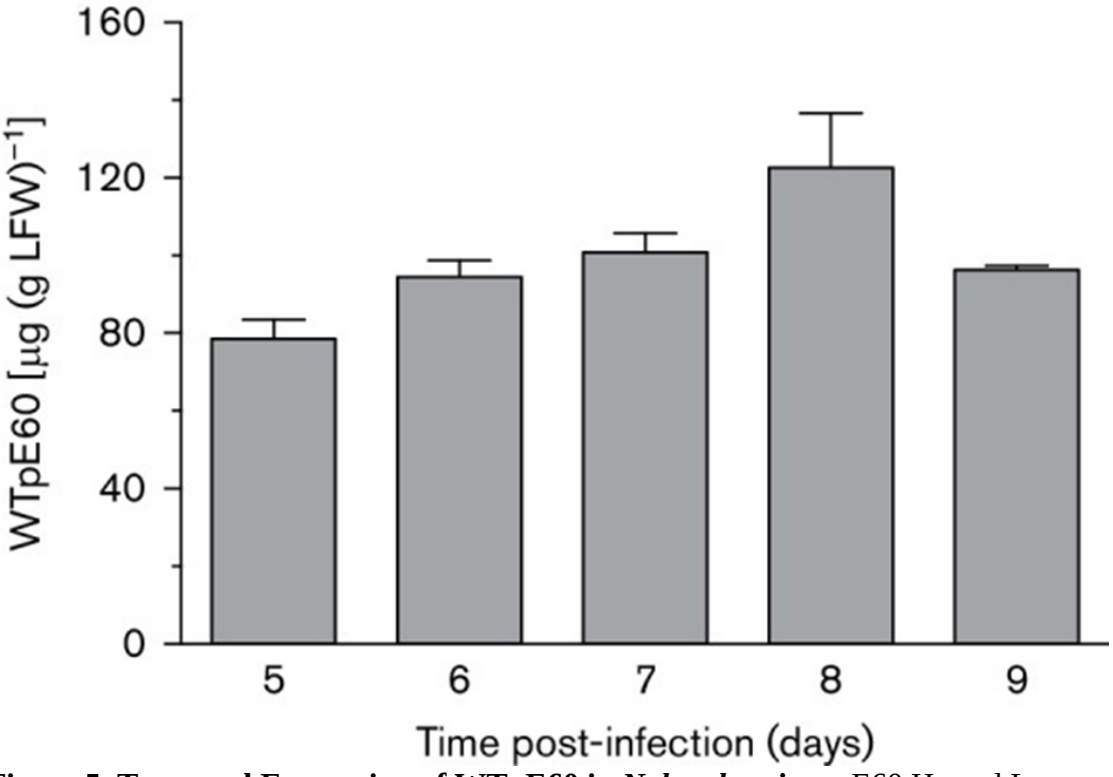

Figure 5. Temporal Expression of WTpE60 in *N. benthamiana*. E60 Hc and Lc constructs were agroinfiltrated into six-week old WT plants. Leaves were harvested at 5, 6, 7, 8, and 9 days post infiltration (DPI) and homogenized in extraction buffer. Properly assembled pE60 were analysis by ELISA. The mean ± SD were calculated from three independent infiltrations. Taken from (Dent et al., 2016).

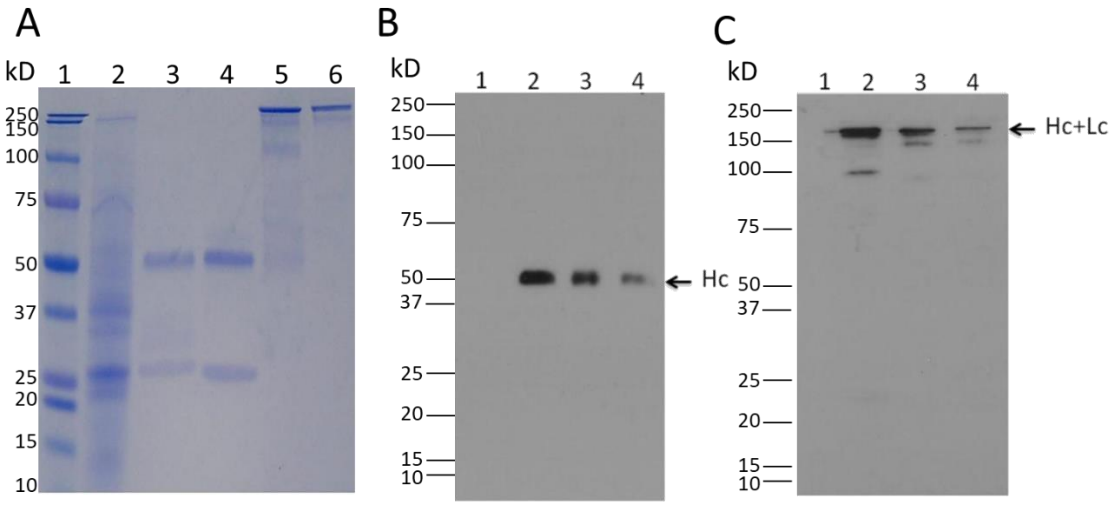

Figure 6. Structural Characterization of pE60 mAb by SDS PAGE and Western Blot. WTpE60 was harvested on DPI 8 and analyzed under reducing (lane 3 and 4 of Fig.3A) and non-reducing (lane 5 and 6 of Fig. 3A) conditions by Coomassie-stained SDS PAGE (12% gel). Lane 1 (Fig. 3A) represents the crude extract before Protein A affinity chromatography, Lanes 3 and 5 are WTpE60 mAb while lanes 4 and 6 is the mammalian produced anti-WNV mAb positive control (Fig.3 A). Concurrently, SDS PAGEs were transferred onto PVDF membrane for western blot analysis by HRP conjugated goat anti-human gamma (Fig. 3B) and kappa (Fig. 3C) antibodies under reducing (Fig. 3B) and non-reducing (Fig. 3C) conditions. Lane 1 represents a non-infiltrated plant extract as a negative control while lane 2 is the anti-WNV mAb positive control (Fig. 3B and C). Lane 3 and 4 are the WTpE60 and GnGnpE60, respectively (Fig. 3B and C). Hc, heavy chain; Lc, light chain.

Table 6. LC-ESI-MS Analysis Summary of N-glycan Profile of pE60. Numbers represent the percent accumulation of each N-glycan specie. Taken from (Dent et al., 2016)

Major N-glycan species	WTpE60 (%)	GnGnpE60 (%)	mE60 (%)	GnGnpE16 (%)
$GnGnXF_3$	70			
GnX	10			
GnGn	10	95		95
$AGnF_6$			50	
AAF_6			40	

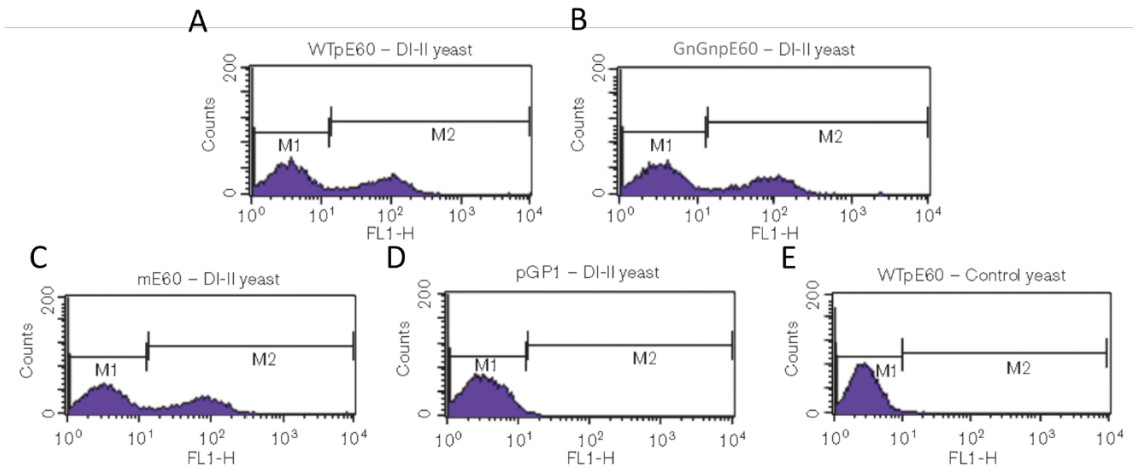

Figure 7. DENV EDI-EDII Yeast Display Binding Assay of pE60. Recombinant yeast cells were induced to display the DENV EDI-EDII (A-D) or nothing (E, control yeast) for specifc binding of pE60 mAb variants (WtpE60 (A), GnGnpE60 (B), or mE60 (C)) or pGP1 (D, anti-Ebola Virus mAb negative control). Yeast cells were labeled with fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG and analyzed by flow cytometry. M1 gate was set by the flourence peak from the WtpE60 negative control yeast. The M2 indicates different flourescence intensity from the M1 gate and specifies secondary antibody binding. The figure was taken from (Dent et al., 2016).

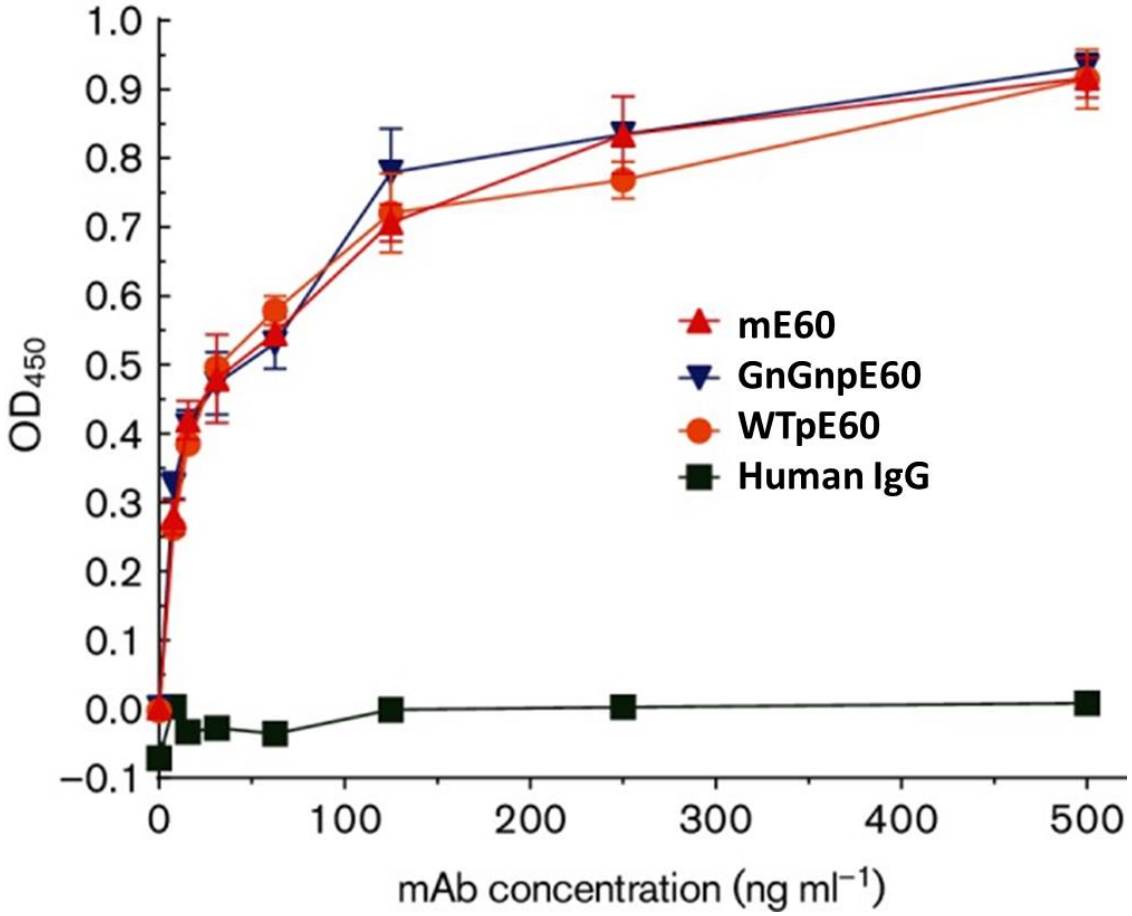

Figure 8. DENV E Protein Binding ELISA by pE60 Variants. DENV E protein was coated on microtitre plates, incubated with a serial dilution of WtpE60 (orange), GnGnpE60 (blue), mE60 (positive control, red), and a generic Hu-IgG (negative control, black), and detected with an HRP-conjugated anti-human gamma antibody. Each sample was performed in triplicates in three individual experiments with OD_{450} represented by the mean±SEM. The figure was taken from (Dent et al., 2016).

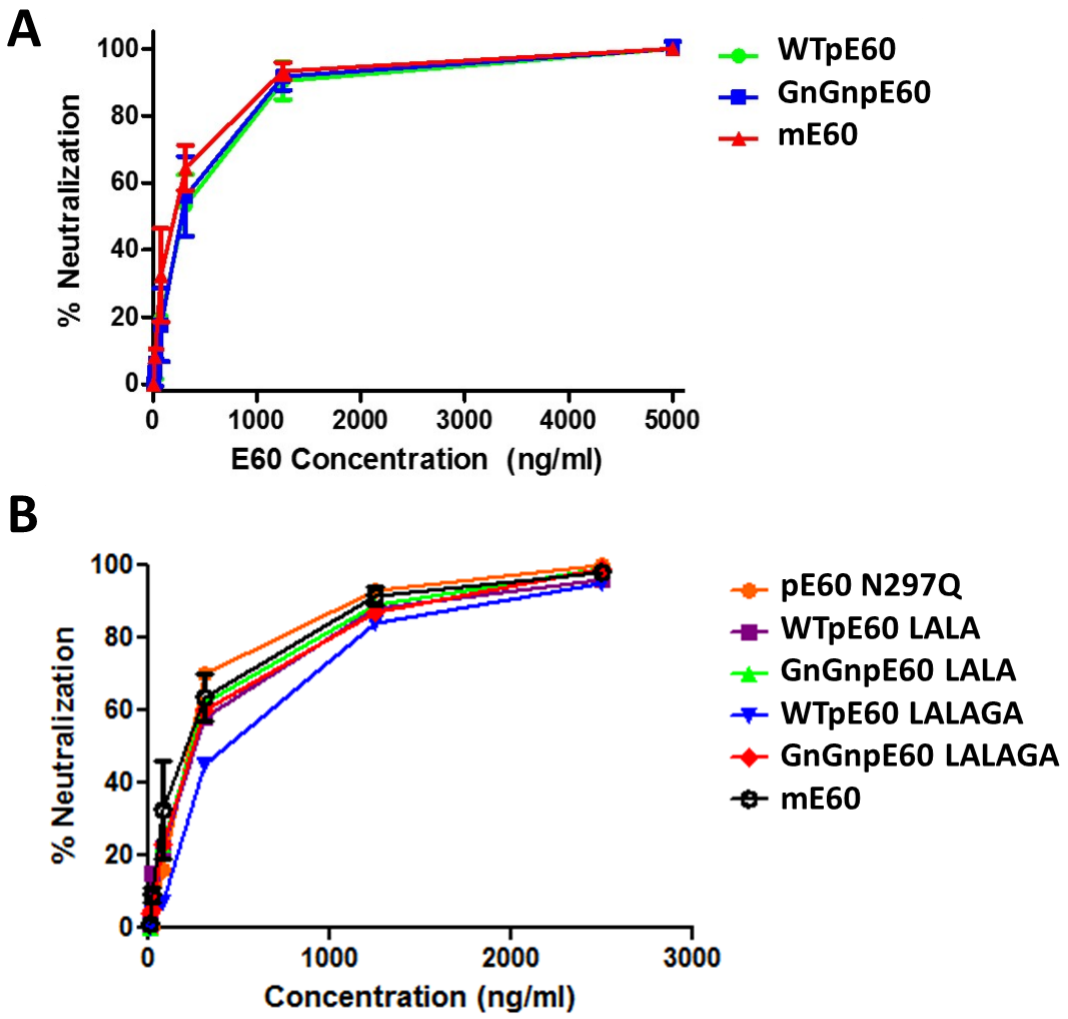

Figure 9. Plaque Reduction Neutralization Test of DENV-2 with pE60 Variants. Serial dilution of pE60 mAb variants were mixed with DENV before being incubated on top of a Vero cells monolayer for 5 days. Plaques were visually counted and curve was generated (Graphpad). For figure 6A, the experiment was conducted twice with triplicates for each sample (WT, green; GnGn, blue; mE60, red). For figure 6B, triplicate counts were averge and plotted for the pE60 N297Q (orange), LALA (WT, purple; GnGn, green), and LALAGA (WT, blue; GnGn, red) samples. Figure 6A was taken from (Dent et al., 2016).

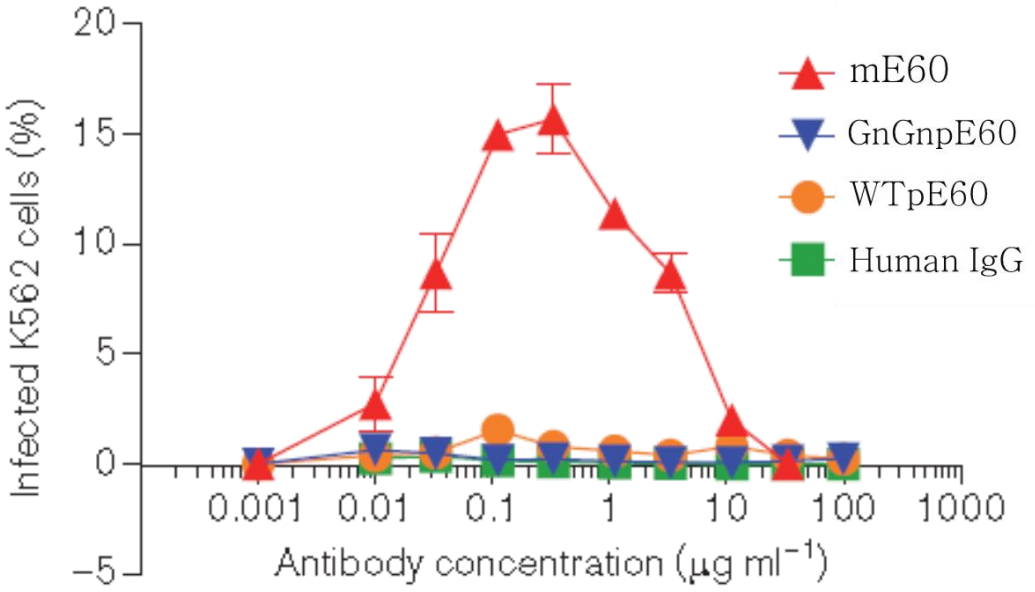

Figure 10. Antibody Dependent Enhancement Assay of pE60. Different concentrations of pE60 glycovariants (WT, orange; GnGn, blue), mE60 (positive control, red) or a human IgG (negative control, green) were mixed with DENV-2 and later incubated on FcγRIIa^{+} K562 cells. Cells were fixed and permealized for staining with Alexa 488-conjugated 4G2 mAb and analyzed by flow cytometry. Taken from (Dent et al., 2016).

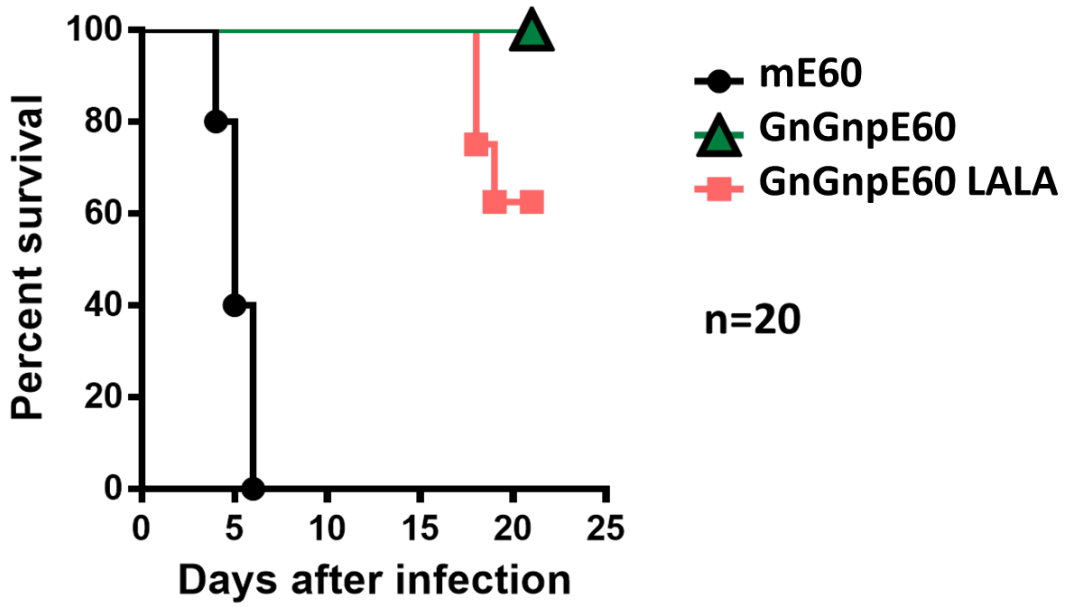

Figure 11. pE60 Treatment of AG129 Mice Following a Lethal Dose of DENV. AG129 mice were intravenously injected with 4×10^6 PFUs of DENV-2 and treated 24 hours later with mE60 (black), GnGnpE60 (green), or GnGnpE60 LALA (salmon). The mice were observed for survival for 21 days post infection. Provided by Dr. Michael S. Diamond's Lab.

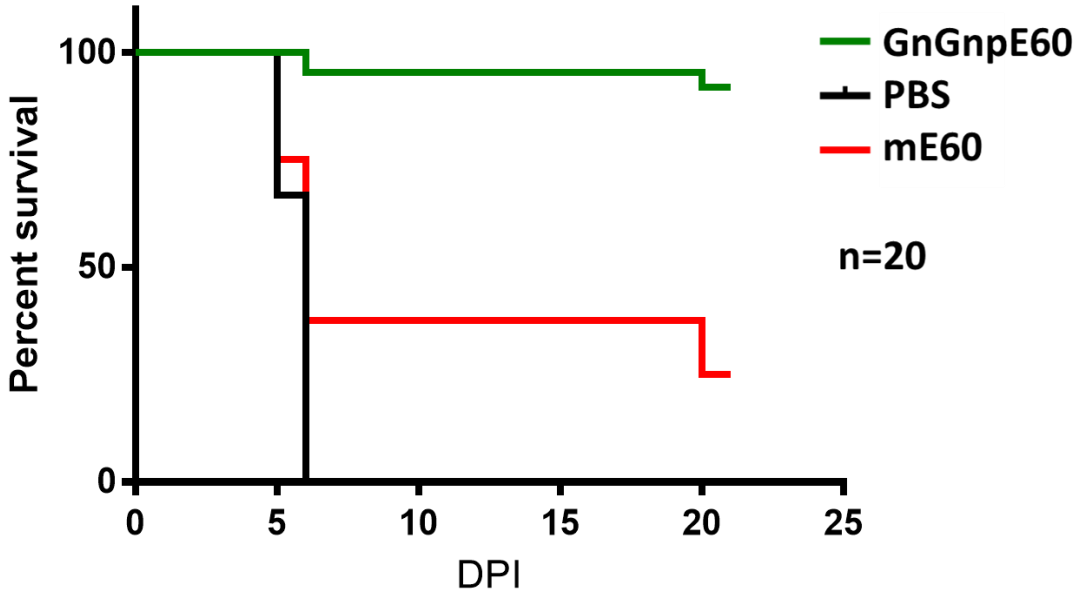

Figure 12. pE60 Treatment of AG129 Mice Following a Sub-lethal Dose of DENV Under ADE Conditions. AG129 mice were primed with anti-DENV-4 membrane protein antibodies (Day -1) and subsequently intravenously injected with 1×10^5 PFUs of DENV-2 (Day 0). 24 hours later (Day 1), mice were treated with GnGnpE60 (green), mE60 (red), or PBS (negative control, black). The mice were observed for survival for 21 days post infection. Provided by Dr. Michael S. Diamond's Lab.

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CHAPTER 3

NEUTRALIZATION AND REDUCTION OF MOUSE FOOTPAD INFLAMMATORY PATHOLOGY BY PLANT PRODUCED ANTI-CHIKUNGUNYA MONOCLONAL ANTIBODY WITH SPECIFIC N-GLYCOSYLATION

3.1 Abstract

Chikungunya Virus (CHIKV) infections are highly pathogenic and are often characterized by debilitating febrile arthralgia and myalgia symptoms. CHIKV is self-limiting, but the immune system's exaggerated response toward the infection often causes the acute and chronic inflammation; witnessed through the acute as well as convalescent phases. Thus, it is important to minimize the response of the immune system with an anti-CHIKV prophylactic. Unfortunately, molecular biomimicry of CHIKV prevents vaccines from being utilized due to the increased risk of autoimmunity. The development of monoclonal antibody (mAb)-based therapies against CHIKV envelope protein have shown high neutralization and protection against CHIKV *in vitro* with necessary Fc region engagement for full efficacy against the CHIKV infection *in vivo*. Herein, we utilized a plant expression system to develop highly homogenous glycoform samples of plant-produced CHK152 (pCHK152) and CHK166 (pCHK166) to explore the role of N-glycosylation for antibody effector function. We hypothesized the specific IgG1 glycoforms (WT and GnGn) would activate specific FcγRs, leading to selective effector cells, which in turn would reduce the overall inflammation and self-damage to host tissues while taking full advantage of the mAb neutralizing capabilities. Both of our pCHK152 and pCHK166 glycovariants retained its strong neutralization and protective

properties *in vitro.* Significantly, our pCHK152 GnGn mAb had reduced footpad swelling compared to the pCHK152 WT mAb *in vivo,* presumably indicating a controlled clearance of CHIKV by ADCC and/or ADCP activity. Overall, our study demonstrates the importance of N-glycosylation in antibody effector functions and the feasibility of utilizing plant expression systems for therapeutic development.

3.2 Introduction

Normally, inhabitants of the tropical and sub-tropical regions of the world, the *Aedes aegypti* and *albopictus* species mosquitoes have managed to extend their distribution to more temperate environments bring the morbidity-causing Chikungunya virus (CHIKV) to a naïve population (Petersen & Powers, 2016; Powers, Staples, & Breiman, 2009; Tsetsarkin, Vanlandingham, McGee, & Higgs, 2007). CHIKV is an *Alphavirus* from the *Togaviridae* family that causes highly incapacitating febrile arthralgia symptoms. Although not lethal, CHIKV infection is a cytopathic disease that targets stromal cells (such as fibroblasts and epithelial cells) and to a lesser extent monocyte-derived macrophages; therefore, causing an immense acute damage and inflammation in the skin, muscles, and joints (Hoarau et al., 2010; Schilte et al., 2013). The increased release of self-proteins caused by cytopathic damage increases the chances of developing chronic autoimmune diseases, such as rheumatoid arthritis, present in 10-20% of the infected population (Miner et al., 2015; Reddy, Desai, Krishna, & Vasanthapuram, 2017). Infected individuals develop acute symptoms within 2 to 12 days while the viremia decreases after 7-8 days (convalescent phase), although, lingering muscle and joint pain can persist for months (Couderc & Lecuit, 2015; Galán-Huerta, Rivas-Estilla, Fernández-Salas, Farfan-Ale, & Ramos-Jiménez, 2015; Tang, 2012). This persistence of the arthritic symptoms has been associated with the lingering replication of CHIKV (e.g., macrophage viral reservoirs and non-structural protein 3-derived stress granules concealment of CHIKV genome) and overwhelmingly inflammatory immune response to CHIKV (Labadie et al., 2010; Remenyi et al., 2018; Tanabe et al., 2018).

CHIKV-infected mosquitos initially introduce the virus by intradermal inoculation. Resident skin cell fibroblasts, and potentially macrophages, are the first to become infected. This leads to induction of the local innate immune response leading to the release of chemoattractants and the consequential influx of macrophages, neutrophils, DCs, basophils, and eosinophils (Couderc et al., 2008; Kupper & Fuhlbrigge, 2004; Sourisseau et al., 2007). Normally, the short-term duration of the CHIKV infection is initially attributed to viral control by type I interferon (IFN) pathways (Nair, Poddar, Shimak, & Diamond, 2017; Schilte et al., 2010). Infected stromal cells produce type-1 IFN that act on other non-hematopoietic cells to prevent the further spread of CHIKV (Schwartz & Albert, 2010). It is hypothesized that pattern recognition receptors (PRR), on hematopoietic cells, are engaged by phagocytosis of viral pathogen-associated molecular markers (PAMPs) after infection or cytopathic damage of tissue and not direct interaction with the virus (Schilte et al., 2010). This allows the viral titers, during the acute phase, to reach high levels (up to 10^8 viral particles per ml of blood) before controlling the infection by the innate immune system and further eliminating the CHIKV infection by the adaptive immune system (Schwartz & Albert, 2010). Although this immunological response rapidly clears the CHIKV infection, it also leaves behind damaged tissues as well as an immunological memory that reacts in the same manner when re-exposed to the same antigen or similar autoreactive peptides/epitopes.

Both the humoral and cell-mediated immune response is required to efficiently control and eliminate the CHIKV infection, yet both are involved in its pathogenesis. Cytotoxic $CD8^+$ T cell activity controls the infection by killing the infected cells while

the $CD4^+$ T helper cells support and orchestrate the activity of other immune cells by cytokine secretions. In the early stages of CHIKV infection, $CD8^+$ T cells have been demonstrated to help control the spread of the infection yet persist for 7-10 weeks post-infection leading to the prolonged inflammatory state. Similarly, elevated levels of $CD8^+$ T cells have been observed in individuals with rheumatoid arthritis (Miner et al., 2015; Wauquier et al., 2011). During the acute infection phase, high levels of pro-inflammatory cytokines and chemokines are released, which results in an increase the inflammation in the joints (Ganesan, Duan, & Reid, 2017). Chronic maintenance of the high inflammatory state is mediated by the $CD4^+$ helper T cells (Teo et al., 2017; Teo et al., 2013). In addition to the cellular immune system regulating the inflammatory response, the humoral response plays a role in the chronic cases of arthritic symptoms. Induction of anti-CHIKV antibodies has been demonstrated to lead to the rapid clearance of the CHIKV, and lack of has resulted in more severe symptoms and persistence of the virus (Lum et al., 2013). Primarily, the humoral response targets the neutralizing E2EP3 linear region of the CHIKV E2 protein (Kam et al., 2014; Kam et al., 2012). In Rag1 knockout mice as well as rhesus monkeys, passive administration of neutralizing anti-CHIKV monoclonal antibodies (mAbs) protected the animals from persistent viral infection and lowered the overall swelling (Broeckel et al., 2017; Hawman et al., 2013). Unfortunately, in a normal CHIKV infection, the overwhelming antibody response is of the highly pro-inflammatory IgG3 type, which strongly binds to all FcγRs as well as recruits the C1q complement pathway (Moldt & Hessell, 2014; Verma et al., 2014; Vidarsson, Dekkers, & Rispens, 2014). Additionally, it has been suggested that antibody-dependent enhancement (ADE) might also exacerbate the severity of the disease (Lum et al., 2018).

Due to the high self-damaging immunological response, it is important to minimize the role of the immune system during a CHIKV infection with a prophylactic therapy. This would reduce the inflammation and overall cytopathic damage to infected tissue while clearing the infection before damage is too extensive. Unfortunately, there is a lack of an approved prophylactic vaccine against CHIKV infections, but even then, a milder passive therapy would be preferred due to the homology between CHIKV E1 protein and human proteins (Reddy et al., 2017). It has been established that prevention and control of CHIKV infection could be accomplished by the administration of a mAb-based therapeutic. Antibodies clear infections through several mechanisms: (1) the Fab region specifically binds to epitopes that can be neutralizing if important to viral life cycle; and (2) the Fc region-Fc receptor recruitment of specific immune cells for antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC) (Michaelsen, Sandlie, Bratlie, Sandin, & Ihle, 2009; Nimmerjahn & Ravetch, 2008). Combinatory administration of two anti-CHIKV mAbs (CHK152 and CHK166), resulted in protection against CHIKV, reduced overall swelling, and production of attenuated CHIKV escape mutants (Pal et al., 2013; Pal et al., 2014). CHK152 and CHK166 target the E2 and E1 protein, respectively. CHK152 functions by stabilizing the domain B of E2, preventing structural changes of the E1-E2 heterodimer and exposure of the fusion loop of CHIKV E1 protein, while CHK166 binds proximal to the conserved fusion loop in domain II of E1 protein, therefore preventing fusion of the viral membrane to the host endosomal membrane (Pal et al., 2013; Sun et al., 2013). Furthermore, the aglycosylated and LALA mutant CHK152 and CHK166 equivalents, demonstrate that an innate immune response

is necessary to clear a CHIKV infection, but regulation of its potency/cytotoxicity is required to reduce pathology (Fox et al., 2019).

In the previous chapter, we developed GnGnpE60 mAb and demonstrated its reduction in Dengue Virus (DENV) ADE which led to better safety and therapeutic efficacy profile. Additionally, we postulated the enhanced involvement of ADCC and/or ADCP activity might lead to a more efficacious therapeutic. Unfortunately, the biology of DENV did not lend itself for investigation of ADCC activity for pE60 mAb, due to the lack of DENV envelope protein on the surface of the cell membranes. In contrast, CHIKV E1 and E2 proteins do complex on the cell membrane and allow for the investigation of ADCC. In an effort to continue to characterize the minute differences between the effector functions of our plant-produced mAbs, we transiently expressed CHK152 and CHK166 mAb in wild-type (WT) and glycoengineered (ΔXFT) *Nicotiana benthamiana* plants and tested them ADCC activity.

3.3 Methods and Material

3.3.1 Construction of Expression Vectors of Anti-CHIKV mAbs

The VH and VL coding gene sequences for CHK152 and CHK166 were taken from (Pal et al., 2013). These variable regions were synthesized by DNA2.0 after some modifications. The original gene was optimized for mammalian cell expression. Thus, we exchanged the mammalian expression elements with plant expression equivalents. Briefly, at the 5' end of the coding sequence, we added a plant Kozac sequence (ACA) to enhance translation and an ATG start codon followed by an intron-less signal peptide sequence for ER targeting. Surrounding the coding sequences, we added an *Eco*RI and

*Nhe*I enzyme restriction sites at the 5’ and 3’end, respectively, for ease of sub-cloning (Fig. 13A and B). All of these genes were plant codon optimized to the codon frequency table of *Nicotiana Tabacum* since there are more available data for this species. The synthesized variable regions were genetically fused to the corresponding DNA sequences coding for human IgG1 CH1-3 and human kappa CL by using *Eco*RI and *Nhe*I restriction digest, and subsequent three-way ligation into the TMV and PVX-based deconstructed plant viral expression vectors (pICH21595 and pICH11599, respectively) of the MagnICON system. The new constructs were then transformed into GV3101, an *Agrobacterium tumefaciens* strain, by electroporation as described in (Lai, He, Engle, Diamond, & Chen, 2012). Concurrently, the CHK152 and CHK166 were cloned into pcDNA3.1 (Thermo Fisher Scientific) and transfected into the CHO-K1 cells using ThermoFisher Scientific lipofectamine protocol as explained in the previous chapter.

3.3.2 *N. benthamiana* Transient Expression of Anti-CHIKV mAbs

WT and GnGn *N. benthamiana* plants were grown in a 25 °C, 84% humidity environment with a 16/8-hour day/night cycle. At six weeks, the *N. benthamiana* were agroinfiltrated with the HC and LC 3’ modules, their respective 5’ modules, and an integrase module in *A. tumefacians* at a 4:1 ratio (HC (3’TMV, 5’TMV and integrase constructs):LC (3’PVX and 5’PVX)) for maximized pCHK152 and pCHK166 mAb expression and assembly.

3.3.3 Anti-CHIKV mAb Extraction and Purification from Plants

Agroinfiltrated leaves were harvested at 7 DPI. Initial bulk purification involved homogenizing the leaves in a low pH extraction buffer (1x phosphate buffered saline

(PBS) pH5.2, 10 mg/ml Sodium Ascorbate, 1 mM EDTA, 2 mM PMSF) to eliminate the major plant contaminate Ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCo). After passing through both a miracloth and cheesecloth filter, the low pH crude extract was clarified by centrifugation at 14,800 x g for 30 minutes at 4 °C. Due to the change in protein composition, the supernatant results in an increased pH (around 6.0); therefore, the supernatant was re-adjusted back to pH 5.2 and stored at 4 °C for 4 hours followed by another round of centrifugation. The final supernatant was pH adjusted to 7.0, centrifuged, and passed through a 0.22-micron filter (Millipore Sigma). The clarified supernatant was then purified with a protein A affinity column in accordance with the manufacturer's instruction (Millipore Sigma) as previously describes in (Lai et al., 2010).

3.3.4 Mammalian Cell Expression and Purification of CHK152

Positive clones were select by Hygromycin and Zeocin selection (300 µg/mL and 250 µg/mL, respectively) and subsequently expanded to a large volume. By replacing the culture medium with serum-free medium, we stimulate antibody production. After five days, the culture is centrifuged at 15,000 g for 30 minutes at 4°C with the supernatant loaded onto a protein A column for affinity chromatography.

3.3.5 Gel Electrophoresis and Western Blot

Purified proteins were subjected to electrophoresis either with 10% SDS-PAGE for reduced (5 %, v/v, b-mercaptoethanol) samples or 4– 20% gradient SDS-PAGE for non-reduced samples and Coomassie blue stained gel. For Western blot gels, proteins were transferred onto PVDF membranes and later blocked with 5% milk-PBST solution. Horseradish peroxidase (HRP)-conjugated antibodies against human-kappa or gamma

(Southern Biotech) contained in a 1% milk-PBST solution were used to specifically bind the LC and HC, respectively, and later detected with Pierce™ ECL western blotting substrate (ThermoFisher Scientific).

3.3.6 N-linked Glycan Analysis

Liquid chromatography-electrospray ionization-mass spectroscopy (LC-ESI-MS) was used to determine the N-glycosylation profiles of the plant-produced anti-CHIKV mAb as previously reported (Dent et al., 2016). Initially, the HC and LC bands were separated by SDS-PAGE under reducing conditions. Coomassie stain was used to locate the target HC bands for excision from the gel. Next, the HC peptide fragments were put through S-alkylation to prevent reforming of the disulfide bonds and tryptic or tryptic/GluC digestion. The peptide fragments were eluted from the gel with 50% acetonitrile. Subsequently, separation of the peptide fragments was performed on a Reversed-Phase Column (150 x 0.32 mm BioBasic-18, Thermo Fisher Scientific) with a 1%–80% acetonitrile gradient and readily analyzed with a quadrupole time-of-flight (Q-TOF) Ultima Global mass spectrometer. The spectra were aggregated and deciphered to individual N-glycoforms (annotated by ProGlycAn nomenclature (www.proglycan.com).

3.3.7 Surface Plasmon Resonance

The binding of human FcγRs to the plant-produced anti-CHIKV mAb was analyzed by SPR using a BIAcore 3000 biosensor (GE Healthcare Life Sciences). Protein A was immobilized to the CM-5 sensor chip (~3990 RU) for the capture of the mAbs (~4225 RU). This was followed by injection of the soluble monomeric human FcγRs (FcγRI, FcγRIIa, FcγIIIa) and C1q at several concentrations to calculate the K_D value

(Appendix E). Briefly, different concentration of FcγRI and FcγRIIIa were injected at a flow rate of 40 µl/min for 135 sec with a 600 sec dissociation time. Similarly, FcγRIIa was injected at a flow rate of 45 µl/min for 30 sec with a 60 sec dissociation time. The antigen surface was regenerated by injection of glycine pH 1.5 twice between each FcγR experiments. For the C1q SPR binding analysis, the analyte was injected at a flow rate of 30 µl/min for 120 sec with a 600 sec dissociation time. For regeneration, a single round of glycine pH 2.5 was injected between experiments. All binding experiments were performed in HBS-EP buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% P20 surfactant). Sensograms were fitted and analyzed with the BIAcore Evaluation Software using State Reaction model (FcγRI and FcγRIIIa) or Steady State Affinity (for FcγRIIa and C1q).

3.3.8 CHIKV Neutralization

Plaque reduction neutralization test (PRNT) was used to determine the neutralization capacity of the plant-produced anti-CHIKV mAbs. The plant- produced anti-CHIKV mAbs variants were serially diluted in PBS, mixed with 100 plaque forming units (PFUs) of CHIKV in serum-free DMEM, and incubated for 1 hour at 37 °C and 5% CO_2 for antibody binding to cognate epitopes. After the incubation, the mAb-virus mixture was added to a 90-95% confluent monolayer of Vero cells and incubated for 1 hour at 37 °C and 5% CO_2. Following incubation, the mAb-virus mixture was carefully removed and substituted with fresh medium containing the 1% sea plaque low-melting agarose (Lonza). The agarose-containing plate was subsequently stored in the 37 °C and 5% CO_2 incubator for three days. On the third day, a second 1% agarose overlay with 4% (v/v) concentration of Neutral Red was added to stain the plaques for visualization. The

plaques were counted, and percent neutralization was calculated as [(number of CHIKV plaque per well with no mAb) - (number of CHIKV plaque per well of diluted mAb) / (number of CHIKV plaque per well with no mAb) x 100]. Neutralization experiments were conducted twice. The data was analyzed with the half maximal effective concentration (EC_{50}) calculated by GraphPad Prism software (Version 6.0). Comparisons of neutralization potency between different mAbs was performed using t-tests.

3.3.9 Antibody-Dependent Cell-mediated Cytotoxicity Assay

The ADCC activity of the plant-produced anti-CHIKV mAbs was determined by an NK cell-based killing assay (modified from Somanchi, Senyukov, Denman, & Lee, 2011). VERO cells were infected with CHIKV (MOI=0.05) for about two days. Infected and uninfected Vero cells were counted, washed, resuspended in MEM medium with 5% FBS at a concentration of 1×10^6 cell/ml. Subsequently, the cells were loaded with Calcein AM solution (1mg/ml in DMSO) at 37°C for 1 hour. After the incubation, the loaded Vero cells were sufficiently washed, to reduce background signal, and mix with the plant-produced anti-CHIKV mAbs for a final concentration of 20 μg/mL. Both the effector NK cells and Vero cells were adjusted to reach a final effector-to-target (E/T) ratio of 2.5:1 and 1.25:1 and added onto a V-bottom 96-well plate. The 96-well plate was centrifuged at 100g for 1 minute to initiate cell contact and later incubated at 37°C for 4 hours. Later, the cells were centrifuged at 100g for 5 minutes to separate the dye-containing supernatant from the cells. The supernatant was then transferred to a black clear bottom 96-well plate to be read by a fluorescent plate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Each sample was conducted in triplicates with three wells saved for the spontaneous release of Calcein

AM. The percent specific lysis was calculated by the following formula: [(test release - spontaneous release) / (maximum release - spontaneous release)] x100. Comparisons of ADCC activity between the pCHK152 mAb glycovariants and the mCHK152 mAb was performed using two-sample T-test.

3.3.10 Animal Studies

To evaluate the therapeutic efficacy of the plant-produced anti-CHIKV mAb, Five-week-old female C57BL/6J mice (purchased from the Jackson Laboratory) were subcutaneously inoculated on the ventral side of the right hind footpad with 10^5 PFUs of CHIKV in PBS as previously described (Acharya, Paul, Anderson, Huang, & Bai, 2015). For the pre-exposure therapeutic study, mice were administered 50 µg of plant-produced anti-CHIKV mAbs or PBS intraperitoneally at 24 hours before CHIKV infection. Alternatively, 50 µg of plant-produced anti-CHIKV mAbs or PBS were intraperitoneally at 12 hours after CHIKV infection for the post-exposure study. Blood sample was retro-orbitally collected at 2 dpi to measure viremia by RT-qPCR. The induced footpad swelling was measured daily for 10 days post-infection (DPI) by using a digital caliper (Electron Microscopy Science). The relative increase of the footpad swelling was calculated by the following formula: (swelling measurement on X DPI – initial measurement) / initial measurement. Comparison of swelling between pCHK152 was performed by one-way ANOVA (p-value < 0.05 was considered statistically significant).

3.3.11 Histology

Five-week-old female C57BL/6J mice followed the same infection and treatment protocol for the post-exposure study mentioned above but were euthanized at 6 DPI. The

inoculated footpad tissues were fixed overnight in 4% paraformaldehyde and subsequently decalcified in 10% EDTA for over ten days. The footpads were then dehydrated and embedded in paraffin for 10 µm sectioning with a microtome (American Optical Spencer 820). Subsequent staining with hematoxylin and eosin (H&E) highlights the nucleus and extracellular, respectively, for visualization of inflammatory pathology. A bright-field microscope (Olympus BH2) was used to acquire the images.

3.3.12 Virus and Cells

CHIKV (strain LR-OPY1, kindly provided by Dr. Robert B. Tesh at University of Texas Medical Branch) was propagated and titered in Vero cells (ATCC, CCL-81). Vero cells were cultured in DMEM supplemented with 10% FBS at 37°C in incubation with 5% CO_2. NK6918 cells were cultured in RPMI 1640 (ThermoFisher Scientific) supplemented with 10% FBS and 50 unit/ml human IL-2 at 37°C in incubation with 5% CO_2.

3.3.13 Real-Time Quantitative PCR (RT-qPCR)

Viral load in mouse blood samples was quantified using real-time RT-PCR. The primers used for CHIKV *E1* gene were the forward primer: 5'-TCCGGGAAGCTGAGATAGAA-3'; and the reverse primer: 5'-ACGCCGGGTAGTTGACTATG-3. Total viral RNA from the mouse blood samples was isolated using the RNeasy mini kit (Qiagen). Synthesis of the first-strand complementary DNA (cDNA) was accomplished by using the iSCRIPT cDNA synthesis kit (Bio-Rad). RT-qPCR of the CHIKV envelope protein 1 (CHIKV E1) and cellular β-actin RNA copy numbers were determined with the CFX96 Real-Time system (Bio-Rad) using SYBR

Green Supermix (Bio-Rad). The viral copy number was expressed as a ratio between CHIKV E1 to cellular β-actin. Comparison of viral copy numbers between different groups was performed by one-way ANOVA (P-value of < 0.05 indicated statistical significance difference).

3.4 Results

3.4.1 Anti-CHIKV mAb Expression in *N. benthamiana*.

The pCHK152 and pCHK166 mAb constructs were agroinfiltrated for transient expression in both WT and GnGn N-glycosylation specific *N. benthamiana* plant lines. After a two-step purification process, the resultant products were characterized by SDS-PAGE and Western blot analysis. The SDS-PAGE analysis demonstrates an efficient purification of both pCHK152 and pCHK166 mAbs with over 90% homogeneity (Fig. 14A and B, respectively). Western blot analysis of the reduced sample for all the plant-produced mAbs (Fig. 15A, B, D, and E) shows both HC and LC are present at the expected molecular size (50 and 25 kDa, respectively). Additionally, both pCHK152 and pCHK166 mAb in the different N-glycoforms were fully assembled with pCHK152 having minimal degradation of the HC as compared to the positive control IgG (Fig. 15A). The expression level of pCHK152 is estimated to be around 150 µg/g leaf fresh weight (LFW) while pCHK166 is below 30 µg/g LFW. These results indicate the expected expression of the HC and LC as well as efficient assembly of the pCHK152 and pCHK166 mAbs.

3.4.2 N-Linked Glycosylation Pattern of Anti-CHIKV mAbs

The N-glycosylation of an IgG mediates significant alteration to the binding to FcγRs and can vary depending on the expression system (Strasser et al., 2008). Accordingly, we examined the *N*-glycosylation of pCHK152 and pCHK166 produced in the WT and GnGn specific plants by LC–ESI–MS. Both pCHK152 and pCHK166 produced in the ΔXF plant line had homogeneous glycoforms (over 95%) (Table 7) carrying a more mammalian-like N-glycosylation without the β1,2-linked xylose and α1,3 fucose. Alternatively, pCHK152 produced in the WT plants exhibited a lower homogeneity of the *N*-acetylglucosamine residue with plant-specific β1,2-linked xylose and α1,3 fucose (about 70%), while pCHK166 was found to carry a mixture of glycoforms (e.g., $GnGnXF_3$, incomplete complex N-glycans, and oligomannosidic structures). After a complete structural characterization of both pCHK152 and pCHK166 glycovariants, we examined if the specificity and neutralization capacity was retained.

3.4.3 Neutralization Activity of Anti-CHIKV mAbs

A PRNT assay was performed to assess the neutralization potential of the pCHK152 and pCHK166 mAb glycoforms (WT and GnGn) against CHIKV. Compare to the anti-WNV mAb negative control both glycoforms of the two plant-produced anti-CHIKV mAbs demonstrated strong neutralization. Moreover, the EC_{50} of the pCHK152 WT and GnGn glycoforms were at 9.46 and 11.11 ng/ml, respectively (Fig. 16A). Likewise, pCHK166 mAbs, WT and GnGn, had an EC_{50} of 130.5 ng/mL and 390.8 ng/mL, respectively (Fig. 16B). pCHK152 had more potent neutralization activity than pCHK166 (Fig. 16**)**. When we compare the two different glycovariants of the same anti-

CHIKV mAb, there were no significant differences. Overall, our pCHK152 and pCHK166 glycovariants displayed specific recognition of CHIKV and further demonstrated potent neutralizing activity *in vitro*.

3.4.4 FcγR Binding of pCHK152

IgGs have a conserved N-glycosylation site that plays a tailor-like structural role that affects the half-life as well as the Fc-mediated functions of the molecule (Jefferis, 2012). Accordingly, the binding affinity of pCHK152 WT and GnGn glycoforms were investigated against FcγRs including FcγRI, FcγRIIa, FcγRIIIa, and C1q by SPR analysis. The GnGn pCHK152 mAb demonstrated the highest affinity to FcγRI followed by FcγRIIIa, C1q, and FcγRIIa in descending order ($K_D = 1.97 \times 10^{-10}$, 3.00×10^{-8}, 2.86×10^{-7}, and 2.08×10^{-6} respectively) (Table 8). The WT pCHK152 demonstrated diminished binding to FcγRI, FcγIIIa, and C1q ($K_D = 3.34 \times 10^{-9}$, 2.67×10^{-6}, and 1.20×10^{-6}, respectively), as well as equivalent binding to FcγRIIa ($K_D = 2.19 \times 10^{-7}$) compared to the GnGn glycoform.

3.4.5 ADCC Activity of pCHK152

One of the mechanisms to control the spread of CHIKV is through the direct killing of infected cells by $CD8^+$ T cells or ADCC of innate cells. Thus, we investigated the ADCC activity of NK cells when directed by our pCHK152 mAb glycovariants. CHIKV-infected Vero cells coated with pCHK152 mAb WT and GnGn resulted in a ~8.6% and ~14.7%, respectively, increase in cell lysis compared the mCHK152 mAb when the E/T ratio was 1.25:1 (P-value < 0.05, Fig. 17A). Likewise, the 2.5:1 E/T resulted in the same trend with the WT and GnGn glycoforms having a ~8.5% and

~11.0%, respectively, increase in cell lysis compared to the mCHK152 (P-value < 0.001, Fig. 17B). The mCHK152 had a ~22.5% and ~19.0% increase in cell lysis compared to no and unspecific human IgG treatment in the 1.25:1 and 2.5:1 E/T ratio, respectively (P-value < 0.001, Fig. 17A and B). Overall, the pCHK152 GnGn demonstrated the most ADCC activity in CHIKV infected Vero cells *in vitro*.

3.4.6 Prophylactic and Therapeutic *In Vivo* Studies

One major hallmark of CHIKV infections is the acute viral titers and increased swelling in the joints. We evaluated the prophylactic and therapeutic efficacy of pCHK152 mAbs for reduction of the inflammatory pathology during CHIKV infection *in vivo*. We administered pCHK152 glycovariants pre- and post-exposure to CHIKV inoculation of C57BL/6 mice and measure viral titers on 2 DPI and footpad swelling daily. For this mouse model, swelling occurs in a biphasic manner with swelling in the first biphasic peak (1-2 DPI) caused by viral replication and the second peak (6 DPI) was associated with infiltration of inflammatory cells (Fox & Diamond, 2016; Pal et al., 2013). The viral titers and overall footpad swelling were minimal or undetectable for all the pre-exposure studies (Fig. 18A and B, respectively). In the post-exposure studies, mice were treated with pCHK152 12h after exposure to CHIKV, following the same protocol for measuring viral titers and footpad swelling (Fig. 19A and B, respectively). The results show a difference between both glycoforms. The GnGn glycoform had a small level of viral replication compared to the undetectable viral titers of the WT glycoform (not statistically significant) (Fig. 19A). Analysis of the footpad swelling in the post-exposure study demonstrated the biphasic pattern. Specifically, all the groups

had the same level of swelling in the first biphasic peak. In the second biphasic peak, both pCHK152 glycovariants had decreased swelling compared to the PBS treated group. Specifically, the GnGn glycoform demonstrated the least amount of swelling followed by the WT glycoform (P-value < 0.01, Fig. 19C). Furthermore, post-exposure mice, from a different group, were sacrificed six days after of CHIKV infection to be sectioned and prepared for histopathological analysis. The pathohistological sections of the PBS-treated mice result in complete disruption of the epithelial and connective tissue layers with leukocyte infiltration. The pCHK152 treated mice show minimal leukocyte infiltration with the epithelial and connective tissue layer still intact (Fig. 20), which corroborates the decreased footpad swelling results at DPI 6. No difference was observed between the two glycovariants in terms of leukocyte infiltration into the tissues. Overall, pCHK152 demonstrates the potential for therapeutic treatment of CHIKV with the GnGn glycoform having reduced swelling.

3.5 Discussion

The spread of CHIKV is reaching naïve populations and causing a high level of debilitating febrile and arthritic morbidity. CHIKV infection is characterized by being highly inflammatory with an over recruitment of the immune system. The prophylactic and therapeutic administration of an anti-CHIKV mAb against the CHIKV E1 and E2 proteins have been demonstrated to protect against CHIKV infection. Additionally, the FcγR involvement and recruitment of specific innate immune cells is required for efficient clearance of the CHIKV infection (Fox et al., 2019). Here, we investigated the impact of the homogeneous N-glycosylation of our plant-produced anti-CHIK mAbs for

the reduction of the immune-caused inflammation, in the therapeutic administration context. We transiently expressed CHK152 and CHK166 in two *N. benthamiana* plant lines (WT and ΔXF) and demonstrated significant differences, in terms, of FcγR binding, ADCC activity, and an overall reduction of inflammation caused by the immune system.

The production of mAbs in plants has been promising to the traditional mammalian CHO cell expression because of its low-cost, speed, safety, and control of N-glycosylation. In this current study, we further characterized the GnGn glycoform for ADCC activity and its application on CHIKV infection. Thus, we produced pCHK152 and pCHK166 each in two glycoforms WT and GnGn and efficiently purified them by low-pH precipitation and subsequent Protein A affinity chromatography. Both mAbs were expressed and fully assembled in the two plant lines. Both glycoforms of the pCHK152 mAb resulted in a low amount of HC degradation. The recent expression of pCHK152 in the geminiviral vector (pBYR11eK2Md), by (Diamos & Mason, 2018), has resulted in an increased expression level with reduced degradation HC products (Appendix F). As expected, the N-glycosylation analysis of the pCHK152 was highly homogeneous with the predominant glycoforms constituting over 70-90% of the total N-glycan in the GnGnXF and GnGn structure, respectively. The pCHK166 had no detectable degradation, yet we could not accumulate enough protein for further studies. N-glycosylation analysis of pCHK166 in ΔXF *N. benthamiana* resulted in a high degree of uniformity with the predominant GnGn glycoform forming 95% homogeneity. In contrast, the WT pCHK166 resulted in a mixture of N-glycan with high level of oligomannosidic and partially processed structures. We believe there might be an ER

stress-related problem in the secretory pathway that prevents the accumulation of the antibody. It might be worth the effort to express it in the pBYR11eK2Md plant expression vector. Together, these results demonstrate the potential utilization of plant expression systems to produce anti-CHIKV mAbs.

Both CHK152 and CHK166 neutralize CHIKV by preventing the fusion between the viral envelope and the endosomal membrane. We examined if the neutralization profiles of both pCHK152 and pCHK166. Our results demonstrate retention of the neutralization potential of both pCHK152 and pCHK166. Specifically, pCHK152 WT and GnGn had an EC_{50} of 9.46 and 11.11 ng/ml, respectively, which is comparable to the mammalian equivalent (3 ng/ml, (Pal et al., 2013). The pCHK166 mAbs were less potent than the pCHK152 mAbs; nevertheless, both glycovariants demonstrated a strong neutralizing activity (WT=130.5 ng/mL, GnGn=390.8 ng/mL) similar to the mCHK166 (EC_{50}=154 ng/mL). Due to the difficulty of producing pCHK166 and the lower neutralization profile, we continued our study with the pCHK152 mAb glycovariants. Overall, both plant-produced anti-CHIKV mAb retained their neutralization capacity, and pCHK166 should be further developed to increase the production for further investigation.

Next, we examined pCHK152 WT and GnGn binding to FcγRs. The WT pCHK152 demonstrated a reduced binding to all of the FcγRs compared to previously reported mammalian-produced mAbs (He et al., 2014), indicating potentially minimal antibody effector function. In contrast, the GnGn pCHK152 demonstrated preferential binding to FcγRIIIa and reduced binding to the rest of the FcγRs which suggests the main

antibody effector mechanism of neutralization would be ADCC activity in human application. The increased binding to FcγRIIIa is not a surprise because it has been established that the defucosylated antibodies result in greater flexibility of the Fc region and greater carbohydrate to carbohydrate interaction, resulting in a greater affinity (Shinkawa et al., 2003; Sondermann, Huber, Oosthuizen, & Jacob, 2000). Surprisingly, the GnGn pCHK152 mAb also had a higher affinity to C1q compared to the WT pCHK152 mAb. Generally, increased galactosylation has been attributed to enhancing CDC activity (Duncan & Winter, 1988; Hodoniczky, Zheng, & James, 2005). This result requires further investigation to properly uncover the antibody effector profile of the GnGn glycan structure. We further investigated if the increased FcγRIIIa binding would translate into increased ADCC activity by an NK cell killing assay. Our results show reduced ADCC activity of the WT pCHK152 compared to the mCHK152, while the GnGn pCHK152 exhibited an increase in NK cell killing of CHIKV-infected Vero cells. Together, the results conclude that the GnGn pCHK152 mechanism of neutralization is ADCC activity.

Lastly, we sought to investigate if the promising *in vitro* results for pCHK152 GnGn could translate into a more efficacious treatment *in vivo*. Initially, we tested to see if there was a difference in efficacy when C57BL/6J mice were prophylactically administered both pCHK152 glycovariants. It resulted that the no difference was seen between the two pCHK152 glycovariants. Both the viral titers and footpad swelling were undetectable and similar to the mock infection. Since CHIKV is an acute infection, preventing the virus from getting into cells is the most effective way of neutralizing the

infection. Subsequently, we tested our pCHK152 mAb glycovariants in the context of a post-infection. We allowed the CHIKV to establish an infection and 12 hours later administered the treatment. Our results show that both the pCHK152 are able to protect against CHIKV infection but even more importantly decrease the immune system-related inflammation in the second biphasic peak compared to the negative control. Interestingly, there was a statistical significance in the swelling between the two pCHK152 mAb glycovariants with the GnGn glycoform having reduced swelling. Based on the *in vitro* results, we hypothesize that the increased ADCC activity decreases the viral load by killing the viral reservoirs, which results in a proportional decrease in the overall inflammatory state. Unfortunately, the utilization of a mouse model meant *in vitro* results have a chance of not translating over to the CHIKV infection in humans. Specifically, the structural orthologue of the human FcγRIIIs is the murine FcγRIV, which is highly expressed in monocytes, macrophages, and neutrophils, cells that are involved in both ADCC and ADCP (Moldt & Hessell, 2014). A recent study by (Fox et al., 2019), investigates the role of the Fc-FcγR recruitment of monocytes for optimal therapeutic efficacy in CHIKV infections. Together, we hypothesize that both ADCC and ADCP activity is involved in the lower inflammatory state when treated with pCHK152.

Collectively, we produced pCHK152 and pCHK166 in two glycoforms in *N. benthamiana* plant (WT and ΔXF). Both plant-produced anti-CHIKV mAbs retained strong neutralization activity, and pCHK152 demonstrated great potential for the treatment of CHIKV. Further studies in non-human primate models are required to decipher the ambiguity of the prominent mechanism of neutralization of our pCHK152

GnGn. Overall, the plant expression systems provide an alternative for mAb production with the added benefit of superior safety and enhanced efficacy.

3.6 Figures

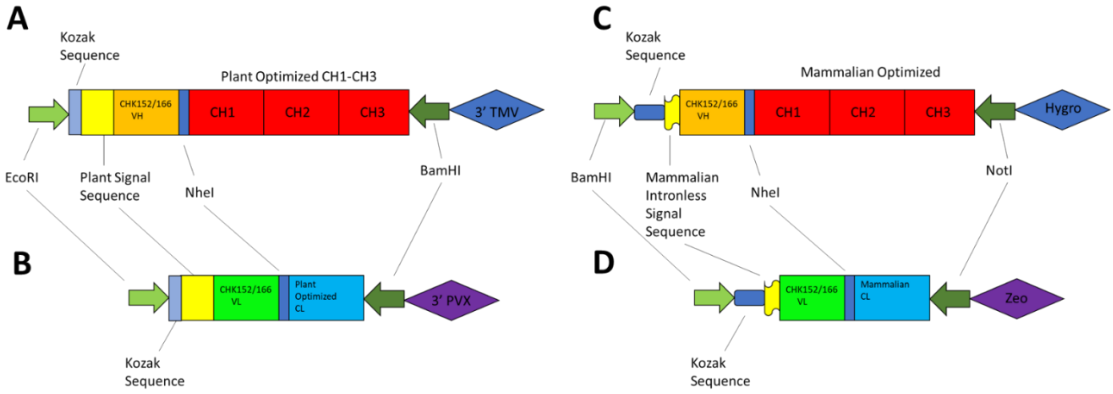

Figure 13. General Schematic of Anti-CHIKV mAbs Utilized in this Study. A plant Kozac sequence (ACA), and an intron-less plant signal peptide were put in place in the 5' end of the plant anti-CHIKV mAb genes (A-B). An EcoRI and NheI were designed in the ends of the variable region to facilitate cloning to the constant region (human IgG1 CH1-3 and human kappa CL). The full Hc and Lc were cloned into the viral expression vectors (pICH21595 (3' TMV) and pICH11599 (3' PVX)) by EcoRI and BamHI orientation mediated ligation. Mammalian anti-CHIKV mAb Hc and Lc genes (C-D, respectively) were cloned into pcDNA3.1 Hygromycin and Zeocin by BamHI and NotI restriction enzyme sites. Both mammalian genes have mammalian expression elements.

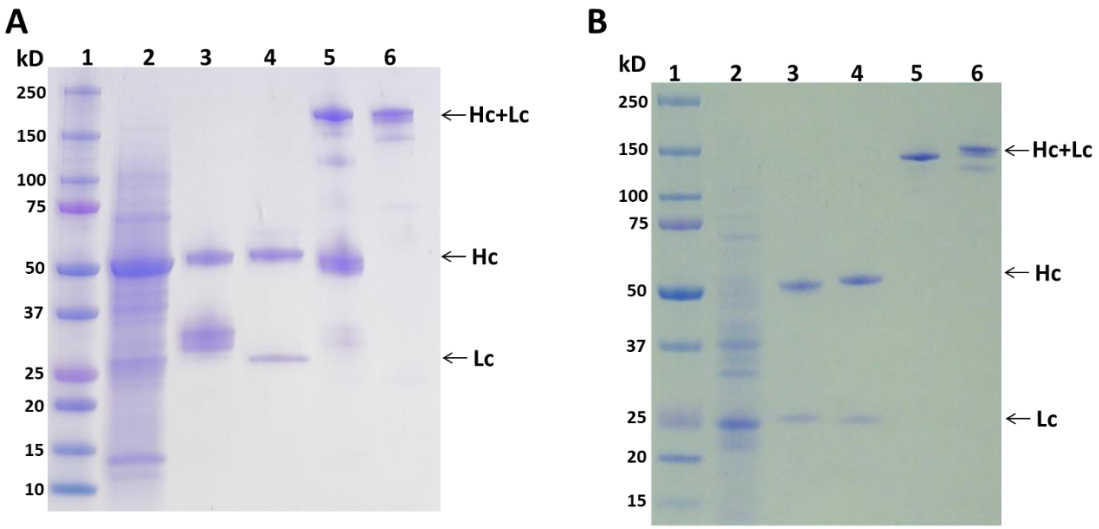

Figure 14. Purification of pCHK152 and pCHK166 from *N. benthamiana* Leaves. Both pCHK152 (Fig. 2A) and pCHK166 (Fig. 2b) were harvested at day 7 and purified by low-pH precipitation/protein A chromatagraphy and ran under reducing (3 and 4 for both Fig. 2A and B) and non-reducing (5 and 6 for both Fig. 2A and B) conditions on a 4-20% SDS PAGE gradient gel. Coomassie staining was used to visualize bands. Lane 1 is the molecular weight ladder; lane 2 is the crude load extract; lanes 3 and 5 are the plant produced anti-CHIKV mAb; and lanes 4 and 6 are the anti-WNV mAb positive control. Hc, heavy chain; Lc, light chain.

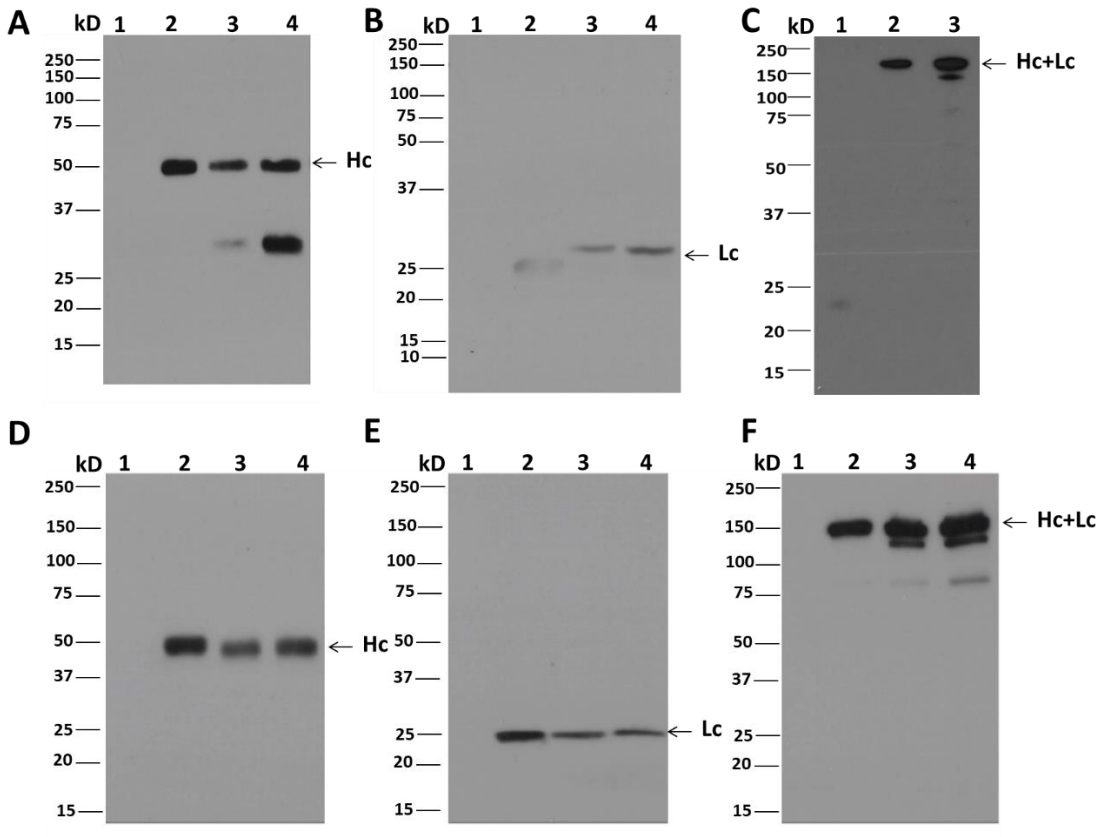

Figure 15. Western Blot Analysis of pCHK152 and pCHK166. Purified pCHK152 (A, B, and C) and pCHK166 (D, E, and F) were initially ran on an SDS PAGE and later transferred onto a PDVF memmbrane for analysis by HRP conjugated goat anti-human gamma (A, D) and kappa (B, C, E, and F) antibodies under reducing (A, B, D, and E) and non-reducing (C and F) conditions. Lane 1 represents a non-infiltrated plant extract as a negative control; lane 2 is the anti-WNV mAb positive control; lane 3 and 4 are the WT and GnGn glycoforms, respectively (A, B, D, E, and F). For Fig. 3C, lane 3 is the GnGnpCHK152. Hc, heavy chain; Lc, light chain.

Table 7. N-glycan Analysis of pCHK152 and pCHK166. The N-glycan of pCHK152 and pCHK166 were analyzed by LC-ESI-MS. The pCHK166 distribution was calculated by spectra analysing progam, while pCHK152 N-glycan distributation is a rough estimate from the spectra. Provided by Dr. Herta Stienkellner's Lab.

Major N-glycan species	WT pCHK152 (%)	GnGn pCHK152 (%)	WT pCHK166 (%)	GnGn pCHK166 (%)
$GnGnXF_3$	56		33	
GnGnX	11		15	
$GnMXF_3$	21		9	
GnGn		>95	15	>95
$Man_{(8\text{-}9)}$	11		28	

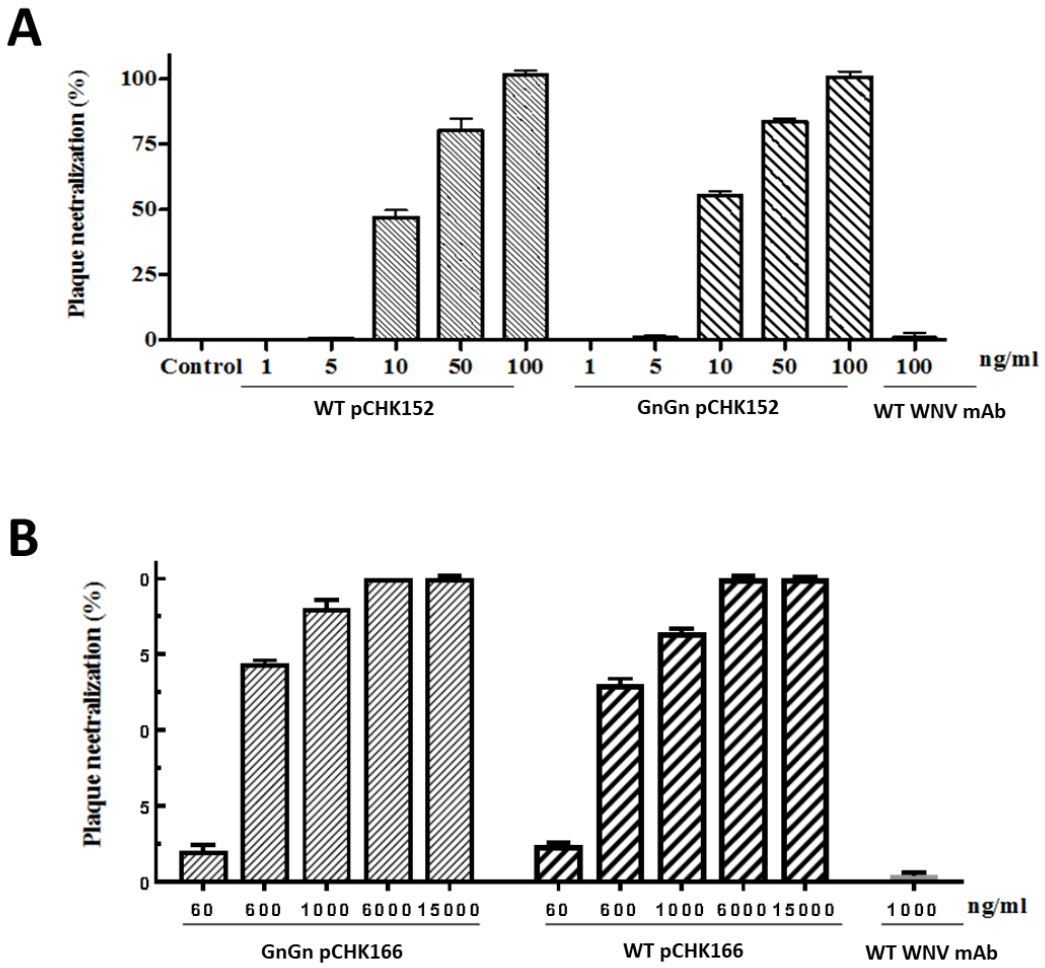

Figure 16. Neutralizaiton Activity of pCHK152 and pCHK166. A plaque reduction neutralizaiton test was utilized to determine the neutralization capacity of the pCHK152 (A) and pCHK166 (B). Plaques were counted and percent neutralization was calculated by the following formula: [(number of CHIKV plaque per well with no mAb) - (number of CHIKV plaque per well of diluted mAb) / (number of CHIKV plaque per well with no mAb) x 100]. Neutralization experiments were conducted twice with triplicates for each sample. Bars represent the Mean±SD. Provided by Dr. Fengwei Bai's Lab.

Table 8. Binding Affinity of pCHK152 Glycovariants. SPR was utilized to investigate the binding kinetics between the Fc regions of pCHK152 (WT and GnGn) against the different FcγRs and C1q. Results are summarized below. Provided by Dr. Julian Ma's Lab.

	FcγRI	FcγRIIa	FcγRIIIa	C1q
WT pCHK152	3.34×10^{-9}	2.67×10^{-6}	2.19×10^{-7}	1.20×10^{-6}
GnGn pCHK152	1.97×10^{-10}	2.08×10^{-6}	3.00×10^{-8}	2.86×10^{-7}

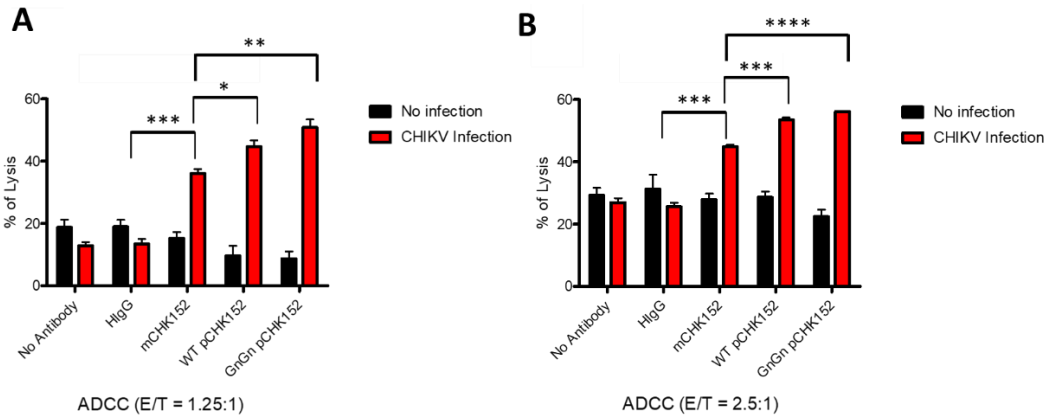

Figure 17. ADCC Activity of pCHK152 WT and GnGn Glycovariants. CHIKV-infected Vero cells were loaded with Calcein AM dye and later opsonized with HuIgG (negative control), mCHK152 (positive control), WT pCHK152, GnGn pCHK152 or no antibody (negative control). NK cells were added at a 1.25:1 and 2.5:1 effector cell to target cell ratio (A and B, respectively). Bars represent the Mean±SD and statistical significance levels were calculated by two-sample T-test. Provided by Dr. Fengwei Bai's Lab and Dr. Haiyan Sun.

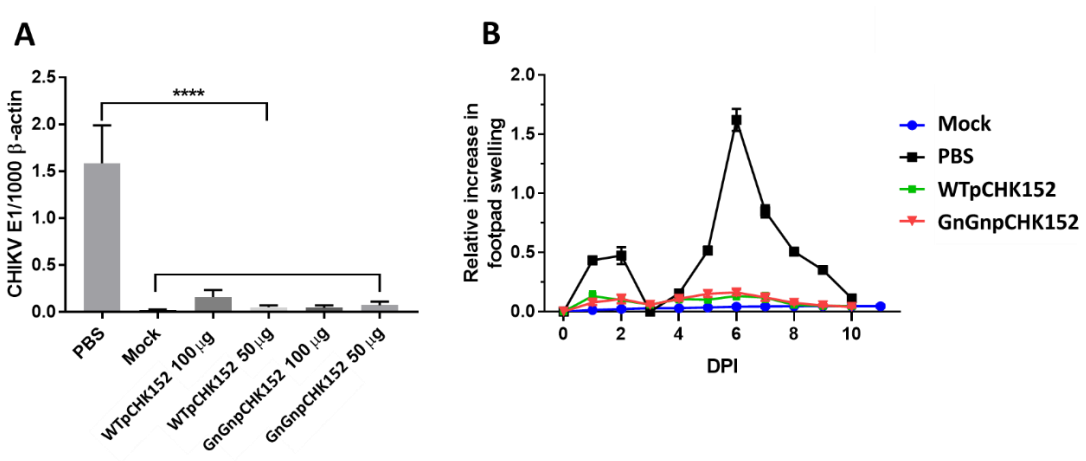

Figure 18. Pre-exposure pCHK152 Treatment of CHIKV-infected C57BL/6J Mice. The mice were intraperitoneally administered the WT pCHK152, GnGn pCHK152, PBS (negative control), or no treatment (mock) and challenged with 10^5 PFUs of CHIKV 24 hours later. Blood samples were taken at day 2 post infection for RT-qPCR analysis of viral titers (Fig. 6A). pCHK152 treatment was compared to PBS treated by a one-way ANOVA (**** indicate a p-value < 0.0001). The footpad swelling was measured daily for pathology assessment (Fig. 6B). Bars represent the Mean±SD. Provided by Dr. Fengwei Bai's Lab.

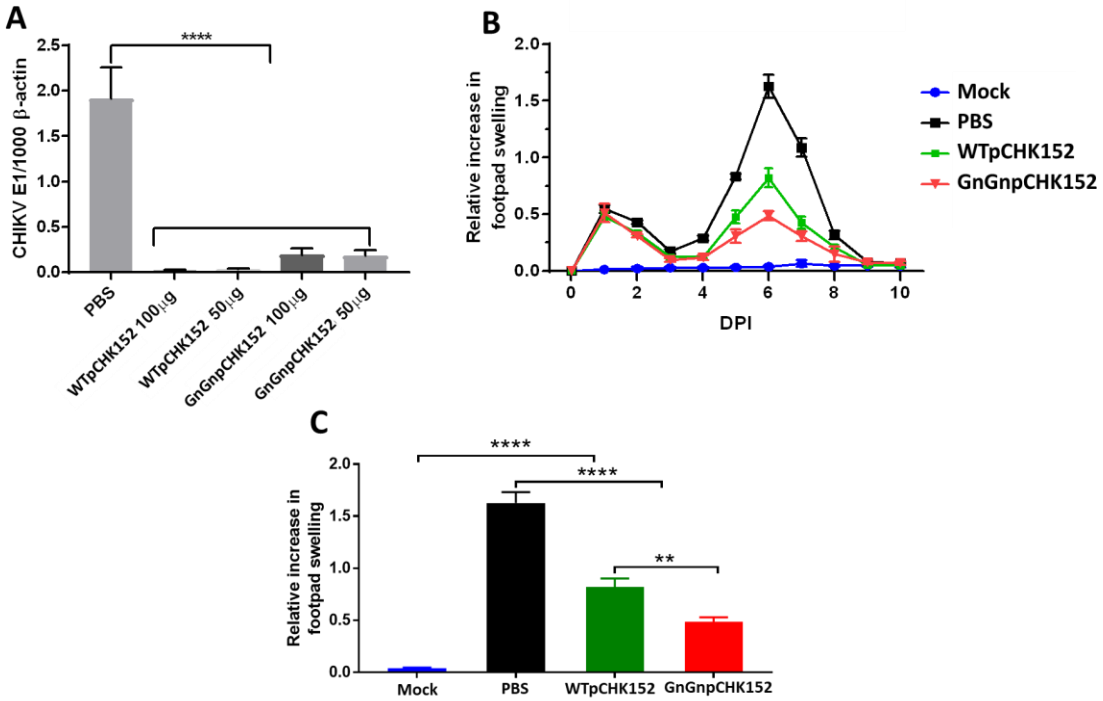

Figure 19. Post-exposure pCHK152 Treatment of CHIKV-infected C57BL/6J Mice. The mice were intraperitoneally administered 10^5 PFUs of CHIKV and treated with WT pCHK152, GnGn pCHK152, PBS (negative control), or no treatment (mock) 12 hours later. Blood samples were taken at day 2 post-infection for RT-qPCR analysis of viral titers (Fig. 7A). pCHK152 treatment was compared to PBS treated by a one-way ANOVA. The footpad swelling was measured daily for pathology assessment (Fig. 6B), bars represent the Mean±SD. A one-way ANOVA was additionally performed on the footpad analysis (Fig. 6C). **, ***, and **** indicates p-values < 0.01. < 0.001, < 0.0001, respectively. Provided by Dr. Fengwei Bai's Lab.

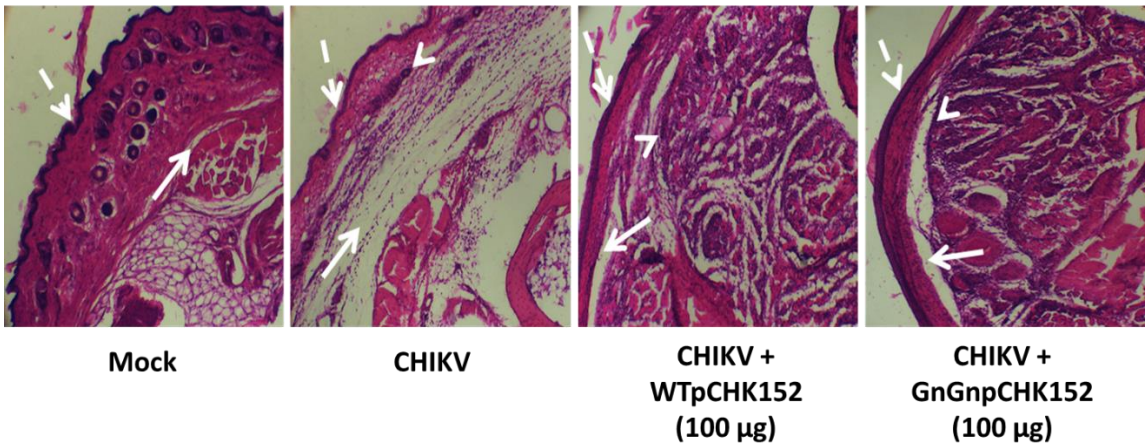

Figure 20. Histological Analysis of pCHK152-mediated Immunopathogenesis. C57BL/6J mice were euthanized at 6 DPI after a 10^5 PFU CHIKV infection and treatment with WT pCHK152, GnGn pCHK152, no treatment, or no infection (mock). Tissue samples were prepared and stained for visualization with a bright field microscope. The solid arrow points to the connective tissue layer; the dotted arrow points to the epithelial layer; and the arrowhead points to infiltrating lymphocytes. Provided by Dr. Fengwei Bai's Lab.

3.7 References

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CHAPTER 4

CONCLUSIONS

As my mentor always tells me, “science doesn’t stop” and with it medicine and technology advance for the betterment of society. One of the areas that have seen a continued increase in revenue and impact on the health of individuals is the antibody industry. Broadly, antibodies are multifaceted and a great resource in research, diagnostics, and therapy. The current production of monoclonal antibodies (mAbs) consists of a highly capital-intensive platform requiring expensive fermenters and highly regulated downstream processing that constitutes the high cost of the mAb-based product. This limits the accessibility of the treatment to necessitated populations and encourages the development of an alternative production system. Herein, the utilization of plant expression system has been described in the production of therapeutic mAbs against Dengue Virus (DENV) and Chikungunya Virus (CHIKV), pathogens that are largely endemic in lower-income populations. Along with being a low-cost alternative to mAb production, plant expression systems have an inherently high homogeneity of N-glycosylation and robust amenability for N-glycoengineering (Q. Chen, 2016; Q. a. D. Chen, KR, 2016; Tusé, Tu, & McDonald, 2014). These characteristics are of high importance for the increase of product consistency, safety, and overall efficacy in mAb-based therapeutics. This dissertation describes a great effort to overcome hurdles that have limited the utilization of mAb-based therapeutics against DENV and CHIKV. Specifically, we investigated the fine-tuned modulation of the antibody effector functions of the GnGn glycan structure in our plant-production mAbs for a reduction in antibody-dependent enhancement (ADE) in DENV and high inflammatory pathology in CHIKV.

Initially, our plant-produced glycovariants were explored for reduced interaction with Fc receptors and subsequent reduction of ADE for DENV infections. In ADE, sub-neutralizing antibodies facilitate the increase number of infected cells through FcγR-mediated endocytosis (extrinsic ADE) as well as increasing the infectious viral particle production in infected cells by altering the immune response through anti-inflammatory cytokine production (intrinsic ADE). This limits the use of mAb-based therapeutics due to the potential of exacerbating the severity upon secondary heterotypic infection. We transiently expressed several pE60 mAb amino acid backbone and glycovariants (WT and GnGn) in *N. benthamiana* plants. We observed robust expression of all the pE60 variants with a homogeneous N-glycosylation profile that allowed us to study the effects of antibody N-glycosylation on ADE. Notably, we demonstrated elimination of ADE activity on FcγRIIa-expressing K562 cells when treated with both WTpE60 and GnGnpE60 mAb in an *in vitro* assay. We narrowed our candidates to GnGnpE60 mAb and GnGnpE60 LALA mAb and compared their efficacy *in vivo* with the viral infection susceptible AG129 mice in a lethal dose model. Significantly, our GnGnpE60 mAb resulted in a greater survival compared to the GnGnpE60 LALA mAb. Subsequently, we investigated if the reduced ADE in the *in* vitro assay would translate *in vivo*. Our results show that ADE was reduced in the GnGnpE60 mAb-treated group which led to better overall protection compared to the mE60mAb-treated group. Together, we demonstrated that a reduction in ADE increases the efficacy *in vivo*, but more importantly, the activation of antibody effector functions is necessary for optimal DENV clearance. The resultant question was “which effector functions does our GnGn glycoform activate?”

In the second section, our plant-produced glycovariants were explored for increased interaction with specific FcγRs. In our GnGnpE60 mAb, we observed a reduction in ADE which suggested a decrease in binding to FcγRIIa (Moi, Lim, Takasaki, & Kurane, 2010). Furthermore, the increased protection of the GnGnpE60 mAb compared to the LALA variant, in the lethal dose model, suggested the involvement of ADCC and/or ADCP. Finally, the observation that defucosylated antibodies had increased FcγRIIIa binding led us to hypothesize that the increased ADCC activity would result in a reduction in CHIKV replication in infected cells, which would reduce overall inflammation. Likewise, we produced highly homogeneous glycovariants of two anti-CHIKV mAbs (pCHK152 and pCHK166) that retained their *in vitro* neutralization capacity. Unfortunately, the low expression of pCHK166 mAb forced us to continue the study with pCHK152 mAb glycovariants. The surface plasma resonance binding kinetic study supported our suspicion that the GnGn glycovariant had increased binding to FcγRIIIa. Furthermore, our NK cell-mediated ADCC assay resulted in an increased ADCC activity of the GnGn pCHK152 mAb compared to both WT pCHK152 and mCHK152 mAbs. Finally, we sought to investigate if the increased ADCC activity in the *in vitro* study would result in early control of viral replication and less immune-mediated inflammation *in vivo*. Our post-infection treatment of C57BL/6J mice demonstrated a statistically significant difference in the swelling between the two pCHK152 mAb glycovariants with the GnGn glycoform having reduced swelling. Indicating the GnGn pCHK152 mAb recruits the appropriate level of cellular immune intervention to control the CHIKV spread and decrease the immunopathology.

This dissertation describes the incredible potential of plant expression systems for the production of mAb with greater safety and efficacy. Scientifically, the production of homogenous N-glycosylation of mAb allows us to study how alteration of the sugar moieties affects recruitment of certain immune pathways. Furthermore, it also allows to dissect which antibody effector functions are necessary for optimal efficacy during treatment. In the context of a DENV and CHIKV infection, additional studies are required to fully comprehend the role of each FcγR on immune cells. This is a niche that the current mAb production systems have struggled to fulfill and is effortlessly performed by plant expression systems. Collectively, the significance of my dissertation is that it can possibly provide treatment for DENV and CHIKV; but equally importantly, give some insight into the role of N-glycosylation in antibody effector functions, which has a broader implication for therapeutic development of other viral infections.

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APPENDIX A

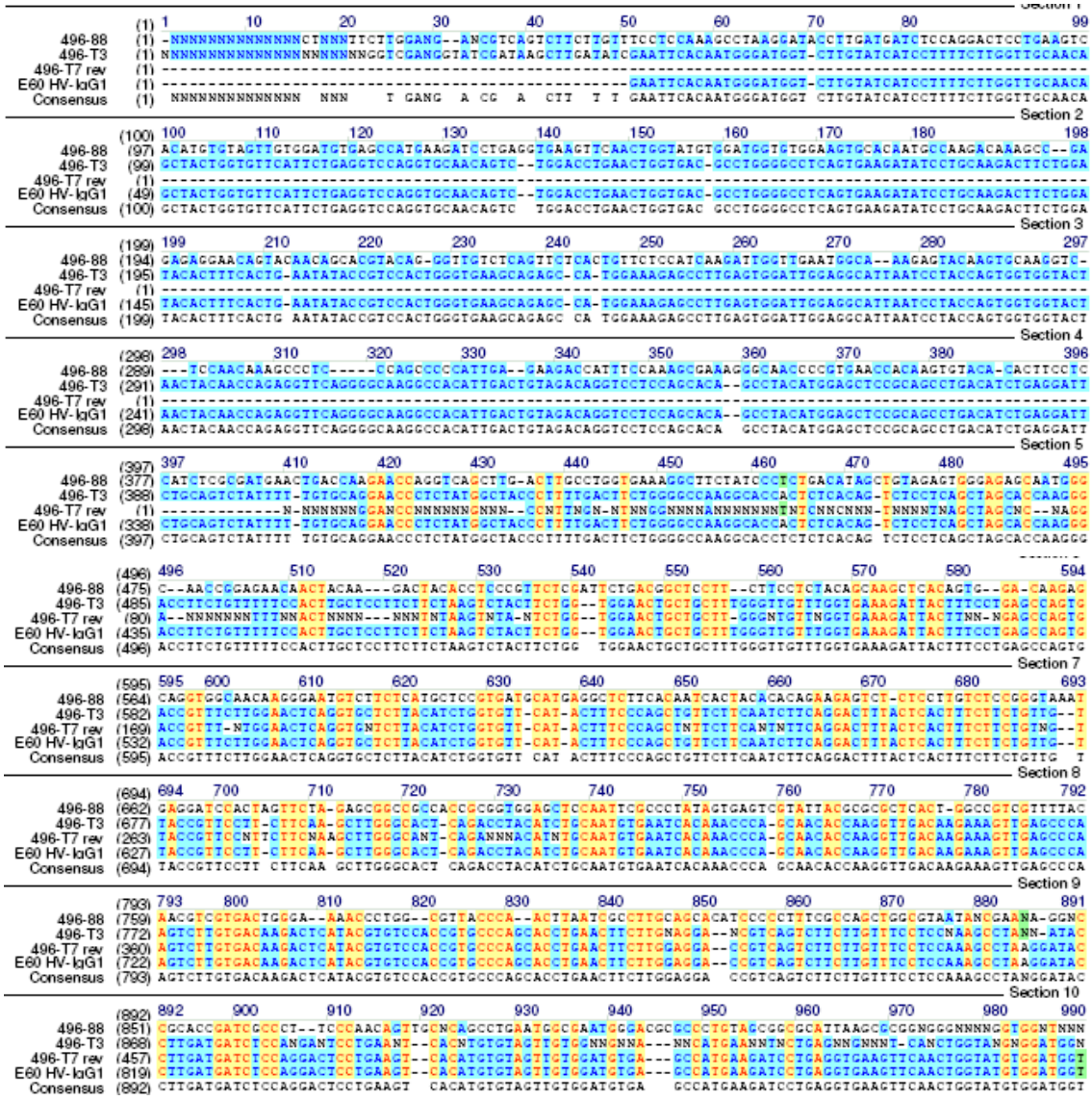
AUTHOR'S PERMISSION

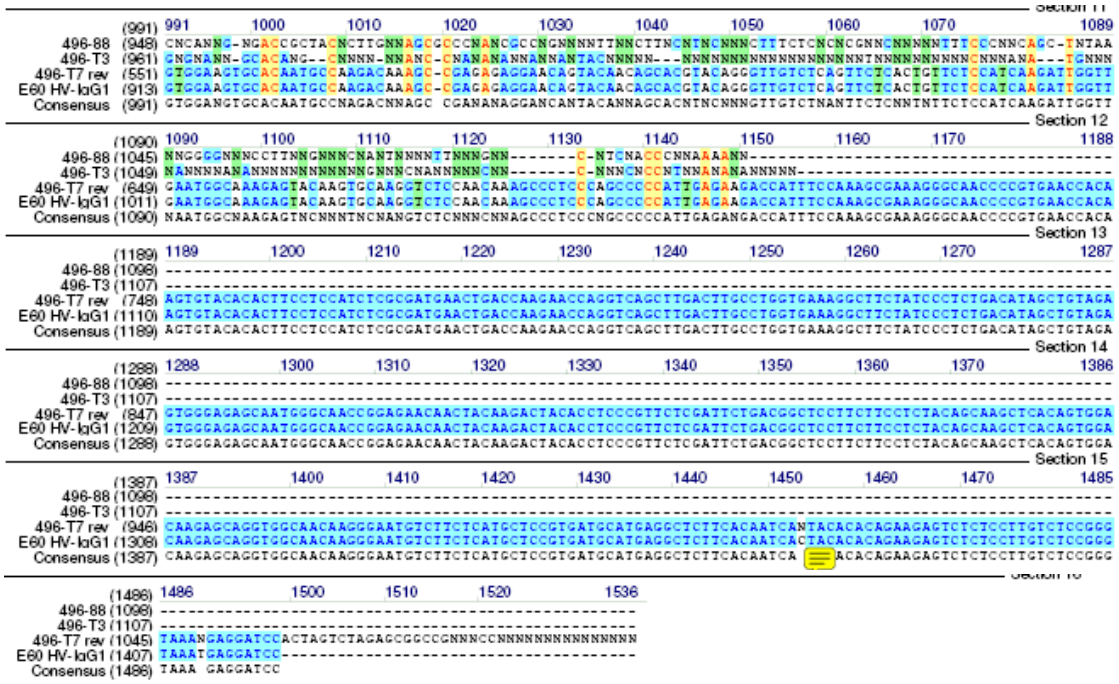
All authors have provided their permission for me to utilize their data in my dissertation, titled “Development of N-glycan Specific Plant Produced Antibody Therapeutics for a Fine-tuned Immune Response”.

APPENDIX B

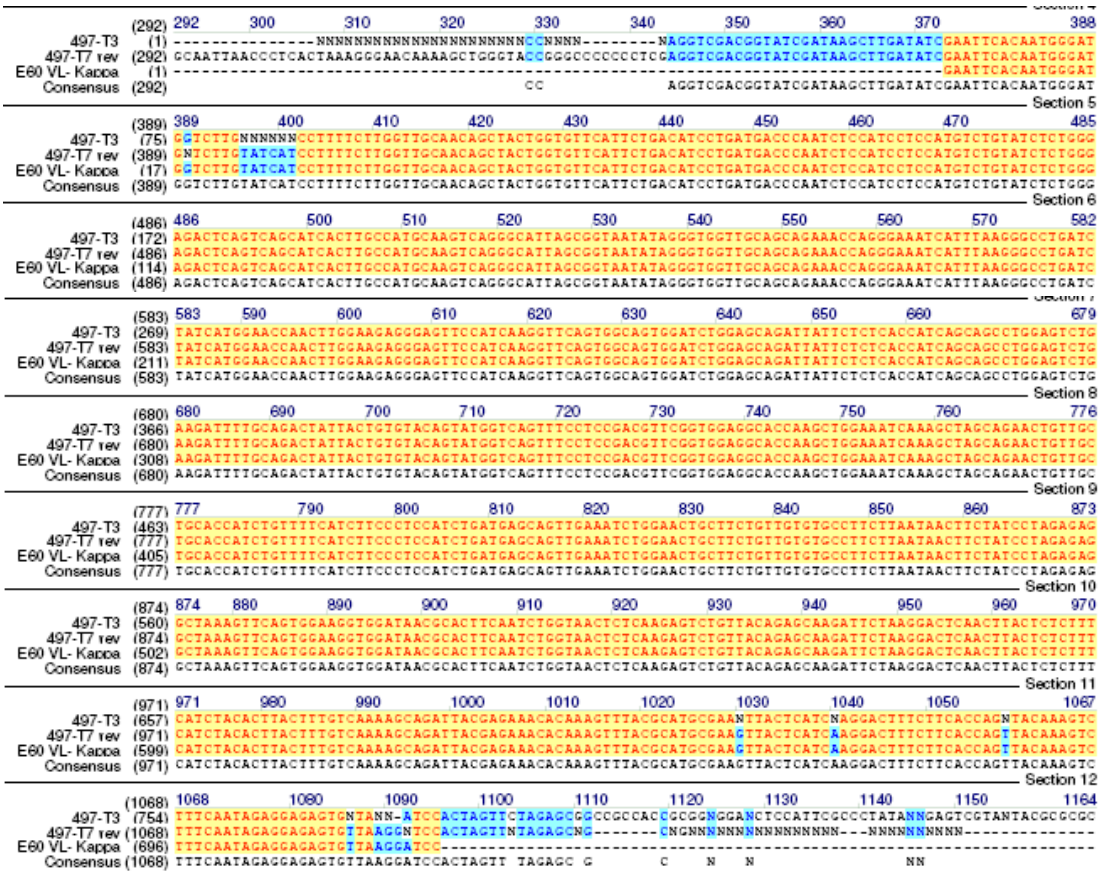
SEQUENCE CONFIRMATIONS

pE60 Hc Sequence Alignment

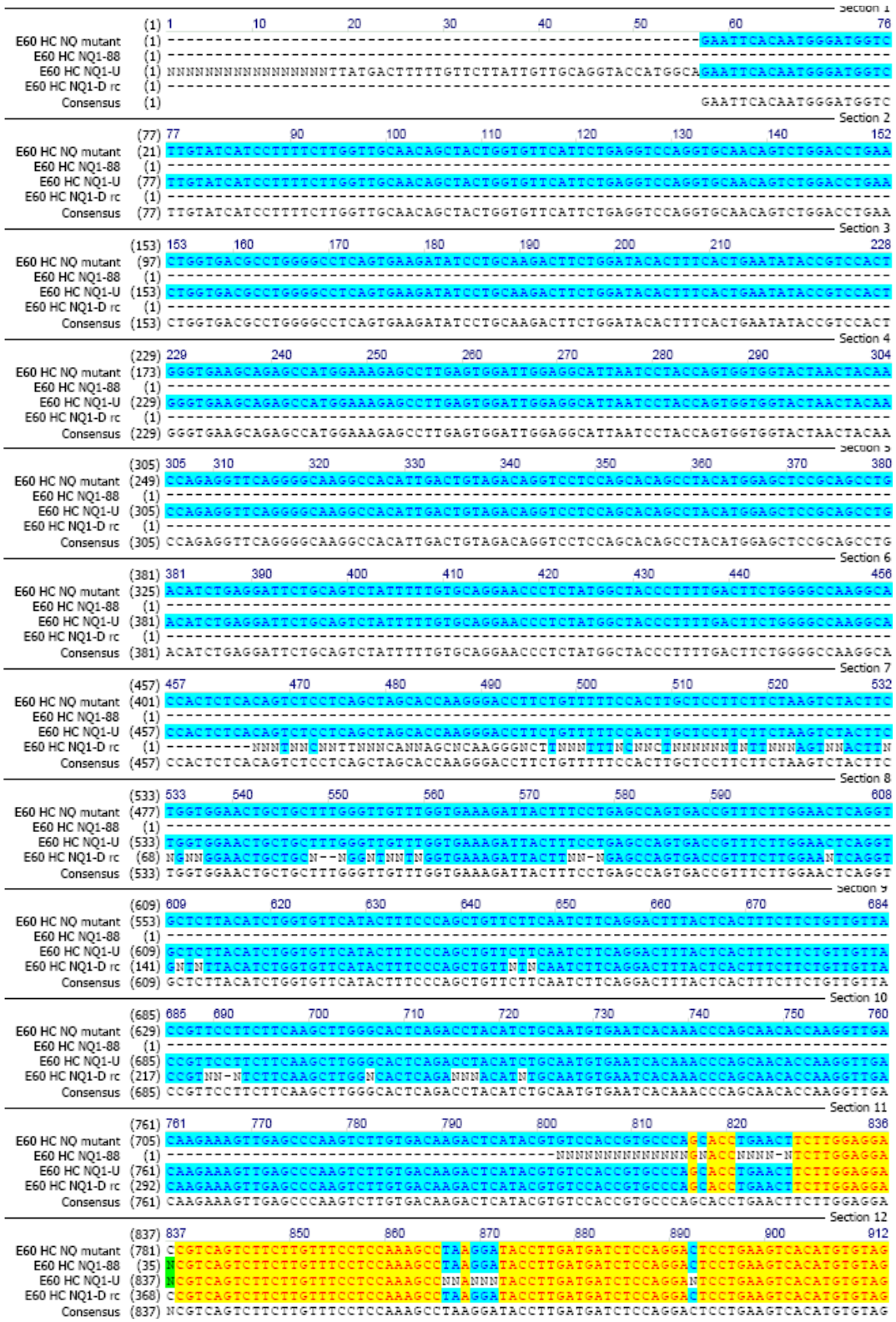


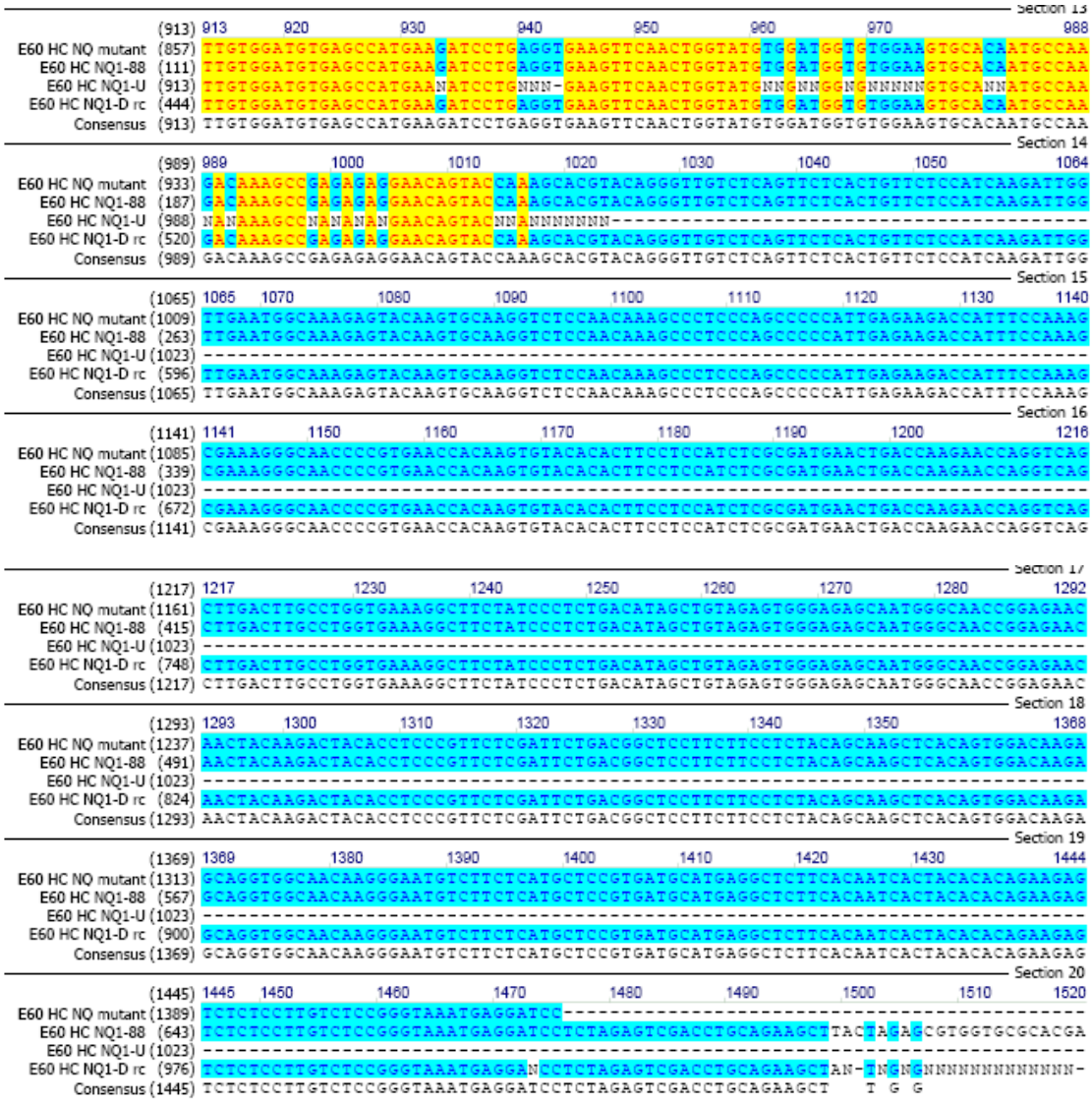


pE60 Lc Sequence Alignment

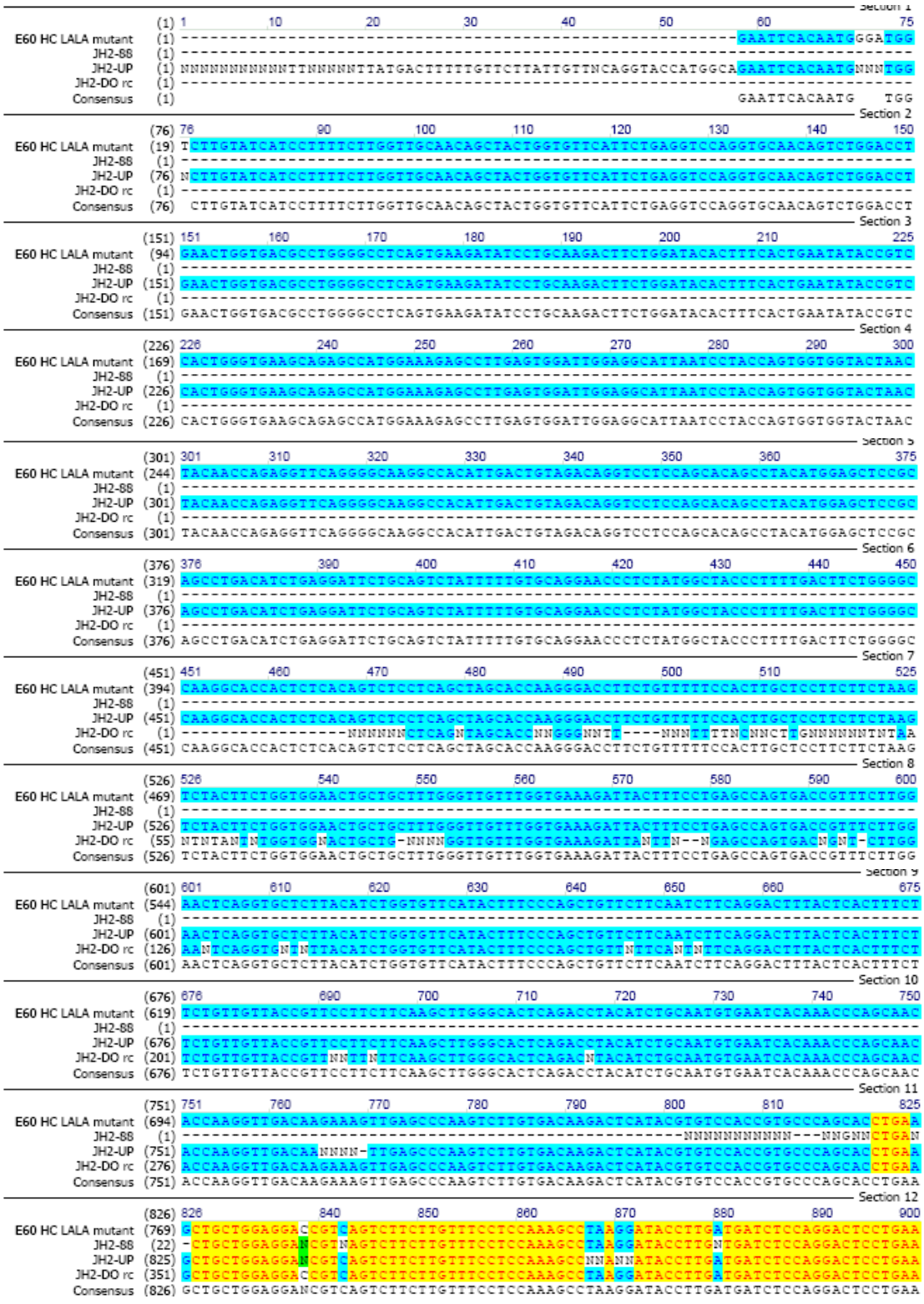


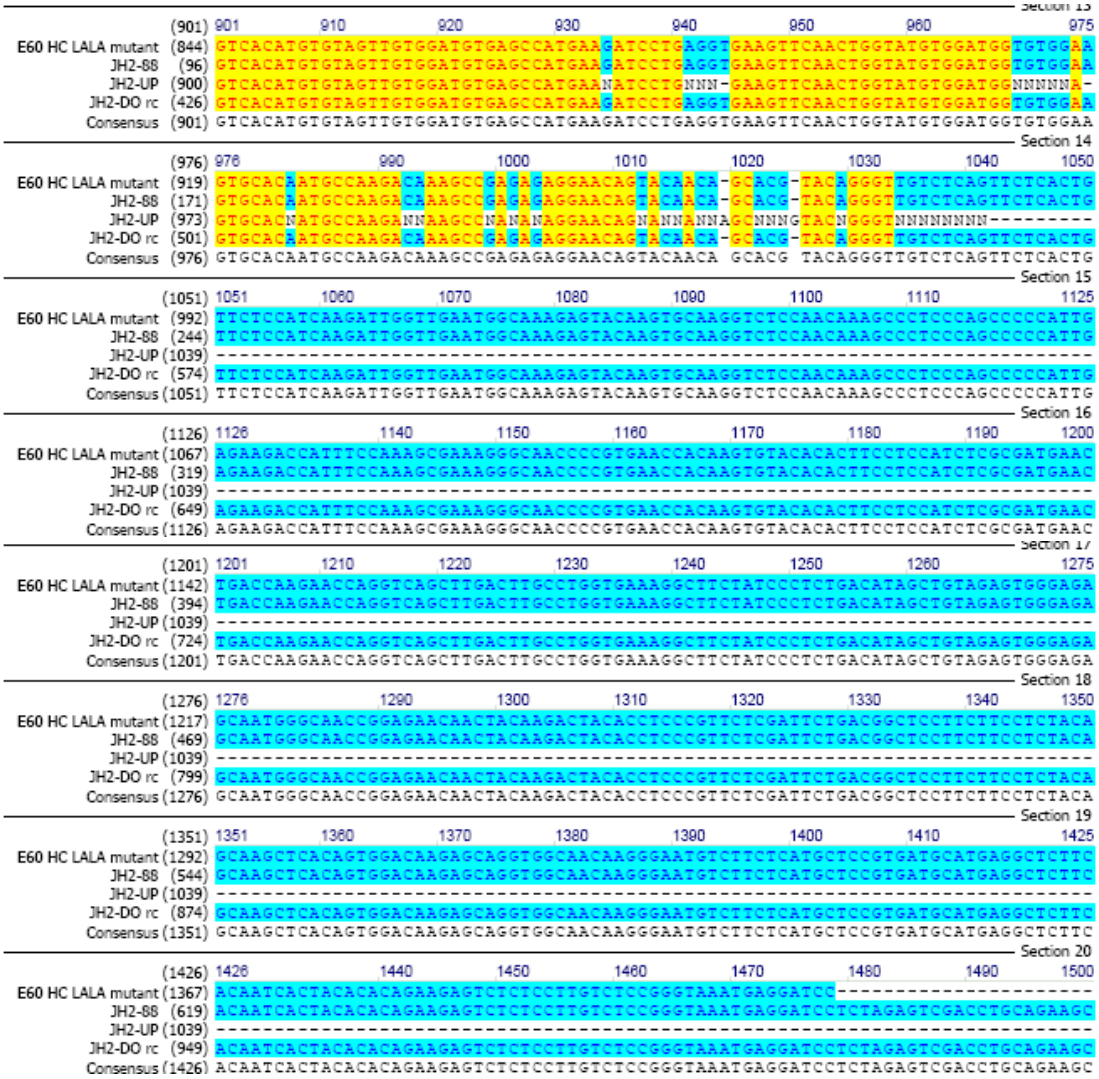
pE60 N297Q Hc Sequence Alignment



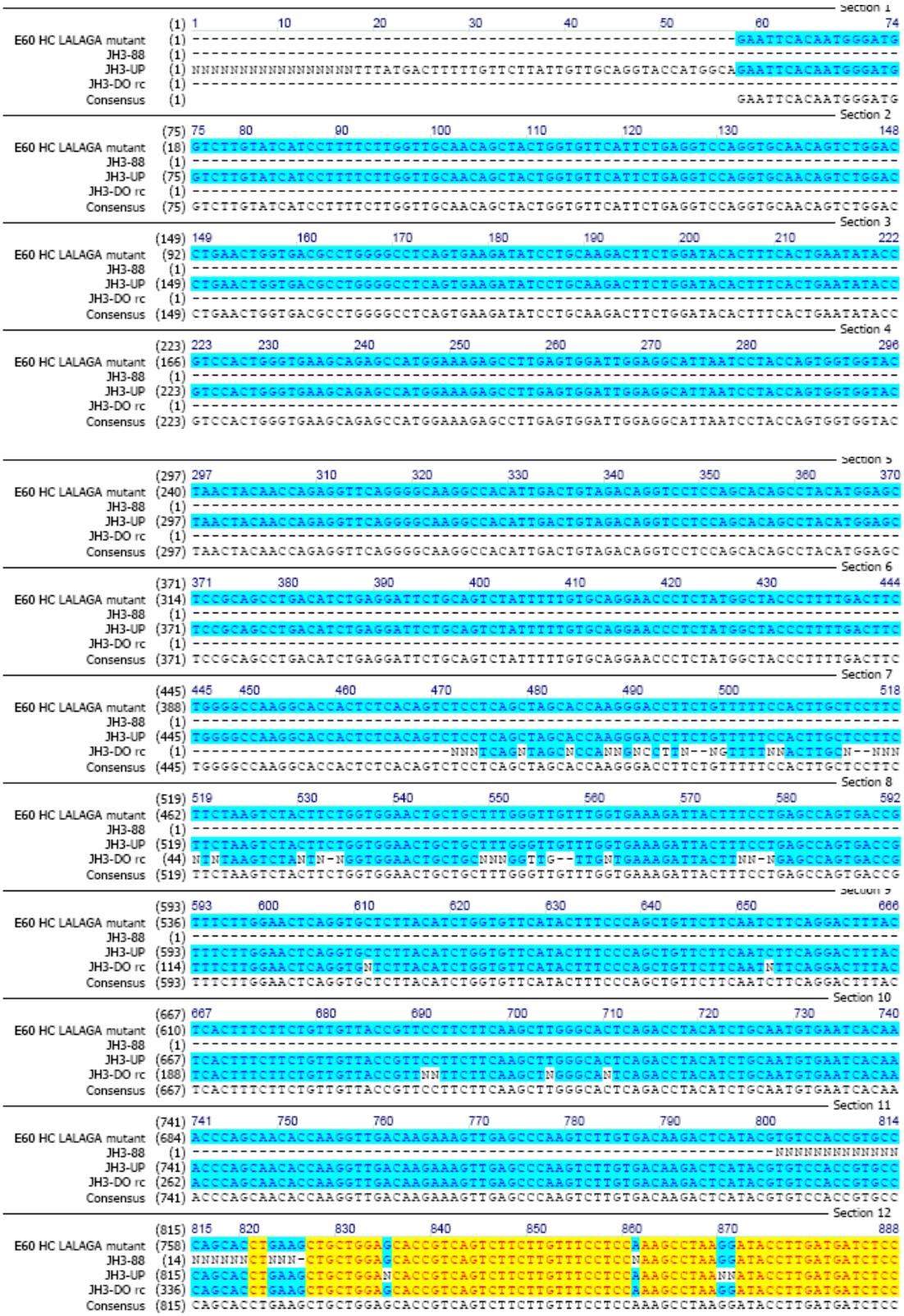

Section 13
(913) 913 920 930 940 950 960 970 988
E60 HC NQ mutant (857) TTGTGGATGTGAGCCATGAAGATCCTGAGGTGAAGTTCAACTGGTATGTGGATGGTGTGGAAGTGCACAATGCCAA
E60 HC NQ1-88 (111) TTGTGGATGTGAGCCATGAAGATCCTGAGGTGAAGTTCAACTGGTATGTGGATGGTGTGGAAGTGCACAATGCCAA
E60 HC NQ1-U (913) TTGTGGATGTGAGCCATGAANATCCTGNNN-GAAGTTCAACTGGTATGNNGNNGGNGNNNNNGTGCANNATGCCAA
E60 HC NQ1-D rc (444) TTGTGGATGTGAGCCATGAAGATCCTGAGGTGAAGTTCAACTGGTATGTGGATGGTGTGGAAGTGCACAATGCCAA
Consensus (913) TTGTGGATGTGAGCCATGAAGATCCTGAGGTGAAGTTCAACTGGTATGTGGATGGTGTGGAAGTGCACAATGCCAA
Section 14
(989) 989 1000 1010 1020 1030 1040 1050 1064
E60 HC NQ mutant (933) GACAAAGCCGAGAGAGGAACAGTACCAAAGCACGTACAGGGTTGTCTCAGTTCTCACTGTTCTCCATCAAGATTGG
E60 HC NQ1-88 (187) GACAAAGCCGAGAGAGGAACAGTACCAAAGCACGTACAGGGTTGTCTCAGTTCTCACTGTTCTCCATCAAGATTGG
E60 HC NQ1-U (988) NANAAAGCCNANANANGAACAGTACNNANNNNNNN----------
E60 HC NQ1-D rc (520) GACAAAGCCGAGAGAGGAACAGTACCAAAGCACGTACAGGGTTGTCTCAGTTCTCACTGTTCTCCATCAAGATTGG
Consensus (989) GACAAAGCCGAGAGAGGAACAGTACCAAAGCACGTACAGGGTTGTCTCAGTTCTCACTGTTCTCCATCAAGATTGG
Section 15
(1065) 1065 1070 1080 1090 1100 1110 1120 1130 1140
E60 HC NQ mutant (1009) TTGAATGGCAAAGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATTGAGAAGACCATTTCCAAAG
E60 HC NQ1-88 (263) TTGAATGGCAAAGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATTGAGAAGACCATTTCCAAAG
E60 HC NQ1-U (1023) ----------
E60 HC NQ1-D rc (596) TTGAATGGCAAAGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATTGAGAAGACCATTTCCAAAG
Consensus (1065) TTGAATGGCAAAGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATTGAGAAGACCATTTCCAAAG
Section 16
(1141) 1141 1150 1160 1170 1180 1190 1200 1216
E60 HC NQ mutant (1085) CGAAAGGGCAACCCCGTGAACCACAAGTGTACACACTTCCTCCATCTCGCGATGAACTGACCAAGAACCAGGTCAG
E60 HC NQ1-88 (339) CGAAAGGGCAACCCCGTGAACCACAAGTGTACACACTTCCTCCATCTCGCGATGAACTGACCAAGAACCAGGTCAG
E60 HC NQ1-U (1023) ----------
E60 HC NQ1-D rc (672) CGAAAGGGCAACCCCGTGAACCACAAGTGTACACACTTCCTCCATCTCGCGATGAACTGACCAAGAACCAGGTCAG
Consensus (1141) CGAAAGGGCAACCCCGTGAACCACAAGTGTACACACTTCCTCCATCTCGCGATGAACTGACCAAGAACCAGGTCAG
Section 17
(1217) 1217 1230 1240 1250 1260 1270 1280 1292
E60 HC NQ mutant (1161) CTTGACTTGCCTGGTGAAAGGCTTCTATCCCTCTGACATAGCTGTAGAGTGGGAGAGCAATGGGCAACCGGAGAAC
E60 HC NQ1-88 (415) CTTGACTTGCCTGGTGAAAGGCTTCTATCCCTCTGACATAGCTGTAGAGTGGGAGAGCAATGGGCAACCGGAGAAC
E60 HC NQ1-U (1023) ----------
E60 HC NQ1-D rc (748) CTTGACTTGCCTGGTGAAAGGCTTCTATCCCTCTGACATAGCTGTAGAGTGGGAGAGCAATGGGCAACCGGAGAAC
Consensus (1217) CTTGACTTGCCTGGTGAAAGGCTTCTATCCCTCTGACATAGCTGTAGAGTGGGAGAGCAATGGGCAACCGGAGAAC
Section 18
(1293) 1293 1300 1310 1320 1330 1340 1350 1368
E60 HC NQ mutant (1237) AACTACAAGACTACACCTCCCGTTCTCGATTCTGACGGCTCCTTCTTCCTCTACAGCAAGCTCACAGTGGACAAGA
E60 HC NQ1-88 (491) AACTACAAGACTACACCTCCCGTTCTCGATTCTGACGGCTCCTTCTTCCTCTACAGCAAGCTCACAGTGGACAAGA
E60 HC NQ1-U (1023) ----------
E60 HC NQ1-D rc (824) AACTACAAGACTACACCTCCCGTTCTCGATTCTGACGGCTCCTTCTTCCTCTACAGCAAGCTCACAGTGGACAAGA
Consensus (1293) AACTACAAGACTACACCTCCCGTTCTCGATTCTGACGGCTCCTTCTTCCTCTACAGCAAGCTCACAGTGGACAAGA
Section 19
(1369) 1369 1380 1390 1400 1410 1420 1430 1444
E60 HC NQ mutant (1313) GCAGGTGGCAACAAGGGAATGTCTTCTCATGCTCCGTGATGCATGAGGCTCTTCACAATCACTACACACAGAAGAG
E60 HC NQ1-88 (567) GCAGGTGGCAACAAGGGAATGTCTTCTCATGCTCCGTGATGCATGAGGCTCTTCACAATCACTACACACAGAAGAG
E60 HC NQ1-U (1023) ----------
E60 HC NQ1-D rc (900) GCAGGTGGCAACAAGGGAATGTCTTCTCATGCTCCGTGATGCATGAGGCTCTTCACAATCACTACACACAGAAGAG
Consensus (1369) GCAGGTGGCAACAAGGGAATGTCTTCTCATGCTCCGTGATGCATGAGGCTCTTCACAATCACTACACACAGAAGAG
Section 20
(1445) 1445 1450 1460 1470 1480 1490 1500 1510 1520
E60 HC NQ mutant (1389) TCTCTCCTTGTCTCCGGGTAAATGAGGATCC----------
E60 HC NQ1-88 (643) TCTCTCCTTGTCTCCGGGTAAATGAGGATCCTCTAGAGTCGACCTGCAGAAGCTTACTAGAGCGTGGTGCGCACGA
E60 HC NQ1-U (1023) ----------
E60 HC NQ1-D rc (976) TCTCTCCTTGTCTCCGGGTAAATGAGGANCCTCTAGAGTCGACCTGCAGAAGCTAN-TNGNGNNNNNNNNNNNNN-
Consensus (1445) TCTCTCCTTGTCTCCGGGTAAATGAGGATCCTCTAGAGTCGACCTGCAGAAGCT T G G

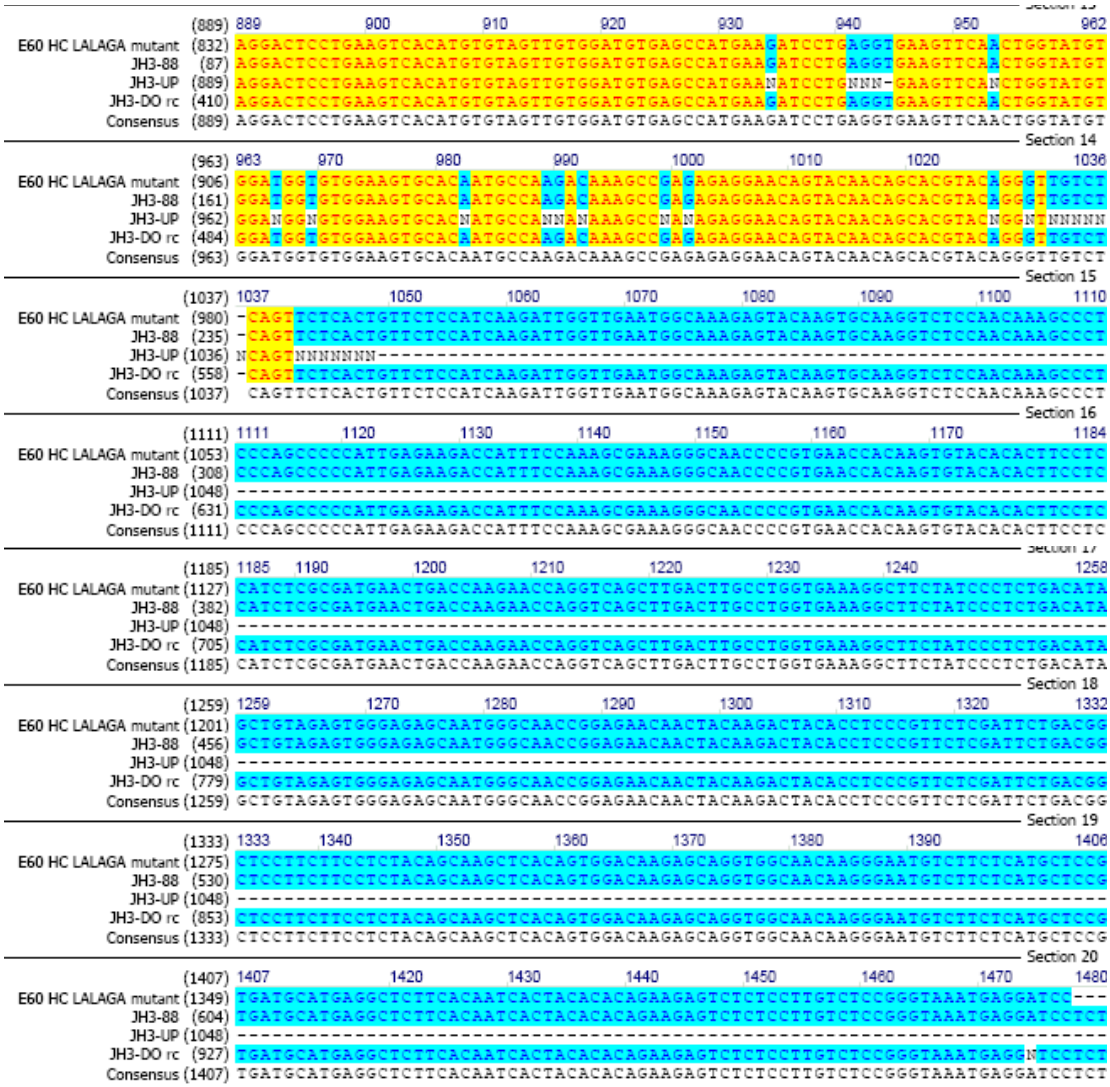
pE60 LALA Hc Sequence Alignment




Section 13
(901) 901 910 920 930 940 950 960 975
E60 HC LALA mutant (844) GTCACATGTGTAGTTGTGGATGTGAGCCATGAAGATCCTGAGGTGAAGTTCAACTGGTATGTGGATGGTGTGGAA
JH2-88 (96) GTCACATGTGTAGTTGTGGATGTGAGCCATGAAGATCCTGAGGTGAAGTTCAACTGGTATGTGGATGGTGTGGAA
JH2-UP (900) GTCACATGTGTAGTTGTGGATGTGAGCCATGAANATCCTGNNN-GAAGTTCAACTGGTATGTGGATGGNNNNNA-
JH2-DO rc (426) GTCACATGTGTAGTTGTGGATGTGAGCCATGAAGATCCTGAGGTGAAGTTCAACTGGTATGTGGATGGTGTGGAA
Consensus (901) GTCACATGTGTAGTTGTGGATGTGAGCCATGAAGATCCTGAGGTGAAGTTCAACTGGTATGTGGATGGTGTGGAA
Section 14
(976) 976 990 1000 1010 1020 1030 1040 1050
E60 HC LALA mutant (919) GTGCACAATGCCAAGACAAAGCCGAGAGAGGAACAGTACAACA-GCACG-TACAGGGTTGTCTCAGTTCTCACTG
JH2-88 (171) GTGCACAATGCCAAGACAAAGCCGAGAGAGGAACAGTACAACA-GCACG-TACAGGGTTGTCTCAGTTCTCACTG
JH2-UP (973) GTGCACNATGCCAAGANNAAGCCNANANAGGAACAGNANNANNAGCNNNGTACNGGGTNNNNNNNN---------
JH2-DO rc (501) GTGCACAATGCCAAGACAAAGCCGAGAGAGGAACAGTACAACA-GCACG-TACAGGGTTGTCTCAGTTCTCACTG
Consensus (976) GTGCACAATGCCAAGACAAAGCCGAGAGAGGAACAGTACAACA GCACG TACAGGGTTGTCTCAGTTCTCACTG
Section 15
(1051) 1051 1060 1070 1080 1090 1100 1110 1125
E60 HC LALA mutant (992) TTCTCCATCAAGATTGGTTGAATGGCAAAGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATTG
JH2-88 (244) TTCTCCATCAAGATTGGTTGAATGGCAAAGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATTG
JH2-UP (1039) ----------
JH2-DO rc (574) TTCTCCATCAAGATTGGTTGAATGGCAAAGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATTG
Consensus (1051) TTCTCCATCAAGATTGGTTGAATGGCAAAGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATTG
Section 16
(1126) 1126 1140 1150 1160 1170 1180 1190 1200
E60 HC LALA mutant (1067) AGAAGACCATTTCCAAAGCGAAAGGGCAACCCCGTGAACCACAAGTGTACACACTTCCTCCATCTCGCGATGAAC
JH2-88 (319) AGAAGACCATTTCCAAAGCGAAAGGGCAACCCCGTGAACCACAAGTGTACACACTTCCTCCATCTCGCGATGAAC
JH2-UP (1039) ----------
JH2-DO rc (649) AGAAGACCATTTCCAAAGCGAAAGGGCAACCCCGTGAACCACAAGTGTACACACTTCCTCCATCTCGCGATGAAC
Consensus (1126) AGAAGACCATTTCCAAAGCGAAAGGGCAACCCCGTGAACCACAAGTGTACACACTTCCTCCATCTCGCGATGAAC
Section 17
(1201) 1201 1210 1220 1230 1240 1250 1260 1275
E60 HC LALA mutant (1142) TGACCAAGAACCAGGTCAGCTTGACTTGCCTGGTGAAAGGCTTCTATCCCTCTGACATAGCTGTAGAGTGGGAGA
JH2-88 (394) TGACCAAGAACCAGGTCAGCTTGACTTGCCTGGTGAAAGGCTTCTATCCCTCTGACATAGCTGTAGAGTGGGAGA
JH2-UP (1039) ----------
JH2-DO rc (724) TGACCAAGAACCAGGTCAGCTTGACTTGCCTGGTGAAAGGCTTCTATCCCTCTGACATAGCTGTAGAGTGGGAGA
Consensus (1201) TGACCAAGAACCAGGTCAGCTTGACTTGCCTGGTGAAAGGCTTCTATCCCTCTGACATAGCTGTAGAGTGGGAGA
Section 18
(1276) 1276 1290 1300 1310 1320 1330 1340 1350
E60 HC LALA mutant (1217) GCAATGGGCAACCGGAGAACAACTACAAGACTACACCTCCCGTTCTCGATTCTGACGGCTCCTTCTTCCTCTACA
JH2-88 (469) GCAATGGGCAACCGGAGAACAACTACAAGACTACACCTCCCGTTCTCGATTCTGACGGCTCCTTCTTCCTCTACA
JH2-UP (1039) ----------
JH2-DO rc (799) GCAATGGGCAACCGGAGAACAACTACAAGACTACACCTCCCGTTCTCGATTCTGACGGCTCCTTCTTCCTCTACA
Consensus (1276) GCAATGGGCAACCGGAGAACAACTACAAGACTACACCTCCCGTTCTCGATTCTGACGGCTCCTTCTTCCTCTACA
Section 19
(1351) 1351 1360 1370 1380 1390 1400 1410 1425
E60 HC LALA mutant (1292) GCAAGCTCACAGTGGACAAGAGCAGGTGGCAACAAGGGAATGTCTTCTCATGCTCCGTGATGCATGAGGCTCTTC
JH2-88 (544) GCAAGCTCACAGTGGACAAGAGCAGGTGGCAACAAGGGAATGTCTTCTCATGCTCCGTGATGCATGAGGCTCTTC
JH2-UP (1039) ----------
JH2-DO rc (874) GCAAGCTCACAGTGGACAAGAGCAGGTGGCAACAAGGGAATGTCTTCTCATGCTCCGTGATGCATGAGGCTCTTC
Consensus (1351) GCAAGCTCACAGTGGACAAGAGCAGGTGGCAACAAGGGAATGTCTTCTCATGCTCCGTGATGCATGAGGCTCTTC
Section 20
(1426) 1426 1440 1450 1460 1470 1480 1490 1500
E60 HC LALA mutant (1367) ACAATCACTACACACAGAAGAGTCTCTCCTTGTCTCCGGGTAAATGAGGATCC----------
JH2-88 (619) ACAATCACTACACACAGAAGAGTCTCTCCTTGTCTCCGGGTAAATGAGGATCCTCTAGAGTCGACCTGCAGAAGC
JH2-UP (1039) ----------
JH2-DO rc (949) ACAATCACTACACACAGAAGAGTCTCTCCTTGTCTCCGGGTAAATGAGGATCCTCTAGAGTCGACCTGCAGAAGC
Consensus (1426) ACAATCACTACACACAGAAGAGTCTCTCCTTGTCTCCGGGTAAATGAGGATCCTCTAGAGTCGACCTGCAGAAGC

pE60 LALAGA Hc Sequence Alignment




(889) 889 900 910 920 930 940 950 962
E60 HC LALAGA mutant (832) AGGACTCCTGAAGTCACATGTGTAGTTGTGGATGTGAGCCATGAAGATCCTGAGGTGAAGTTCAACTGGTATGT
JH3-88 (87) AGGACTCCTGAAGTCACATGTGTAGTTGTGGATGTGAGCCATGAAGATCCTGAGGTGAAGTTCAACTGGTATGT
JH3-UP (889) AGGACTCCTGAAGTCACATGTGTAGTTGTGGATGTGAGCCATGAANATCCTGNNN-GAAGTTCANCTGGTATGT
JH3-DO rc (410) AGGACTCCTGAAGTCACATGTGTAGTTGTGGATGTGAGCCATGAAGATCCTGAGGTGAAGTTCAACTGGTATGT
Consensus (889) AGGACTCCTGAAGTCACATGTGTAGTTGTGGATGTGAGCCATGAAGATCCTGAGGTGAAGTTCAACTGGTATGT
Section 14
(963) 963 970 980 990 1000 1010 1020 1036
E60 HC LALAGA mutant (906) GGATGGTGTGGAAGTGCACAATGCCAAGACAAAGCCGAGAGAGGAACAGTACAACAGCACGTACAGGGTTGTCT
JH3-88 (161) GGATGGTGTGGAAGTGCACAATGCCAAGACAAAGCCGAGAGAGGAACAGTACAACAGCACGTACAGGGTTGTCT
JH3-UP (962) GGANGGNGTGGAAGTGCACNATGCCANNANAAAGCCNANAGAGGAACAGTACAACAGCACGTACNGGNTNNNNN
JH3-DO rc (484) GGATGGTGTGGAAGTGCACAATGCCAAGACAAAGCCGAGAGAGGAACAGTACAACAGCACGTACNGGGTTGTCT
Consensus (963) GGATGGTGTGGAAGTGCACAATGCCAAGACAAAGCCGAGAGAGGAACAGTACAACAGCACGTACAGGGTTGTCT
Section 15
(1037) 1037 1050 1060 1070 1080 1090 1100 1110
E60 HC LALAGA mutant (980) -CAGTTCTCACTGTTCTCCATCAAGATTGGTTGAATGGCAAAGAGTACAAGTGCAAGGTCTCCAACAAAGCCCT
JH3-88 (235) -CAGTTCTCACTGTTCTCCATCAAGATTGGTTGAATGGCAAAGAGTACAAGTGCAAGGTCTCCAACAAAGCCCT
JH3-UP (1036) NCAGTNNNNNNN----------
JH3-DO rc (558) -CAGTTCTCACTGTTCTCCATCAAGATTGGTTGAATGGCAAAGAGTACAAGTGCAAGGTCTCCAACAAAGCCCT
Consensus (1037) CAGTTCTCACTGTTCTCCATCAAGATTGGTTGAATGGCAAAGAGTACAAGTGCAAGGTCTCCAACAAAGCCCT
Section 16
(1111) 1111 1120 1130 1140 1150 1160 1170 1184
E60 HC LALAGA mutant (1053) CCCAGCCCCCATTGAGAAGACCATTTCCAAAGCGAAAGGGCAACCCCGTGAACCACAAGTGTACACACTTCCTC
JH3-88 (308) CCCAGCCCCCATTGAGAAGACCATTTCCAAAGCGAAAGGGCAACCCCGTGAACCACAAGTGTACACACTTCCTC
JH3-UP (1048) ----------
JH3-DO rc (631) CCCAGCCCCCATTGAGAAGACCATTTCCAAAGCGAAAGGGCAACCCCGTGAACCACAAGTGTACACACTTCCTC
Consensus (1111) CCCAGCCCCCATTGAGAAGACCATTTCCAAAGCGAAAGGGCAACCCCGTGAACCACAAGTGTACACACTTCCTC
Section 17
(1185) 1185 1190 1200 1210 1220 1230 1240 1258
E60 HC LALAGA mutant (1127) CATCTCGCGATGAACTGACCAAGAACCAGGTCAGCTTGACTTGCCTGGTGAAAGGCTTCTATCCCTCTGACATA
JH3-88 (382) CATCTCGCGATGAACTGACCAAGAACCAGGTCAGCTTGACTTGCCTGGTGAAAGGCTTCTATCCCTCTGACATA
JH3-UP (1048) ----------
JH3-DO rc (705) CATCTCGCGATGAACTGACCAAGAACCAGGTCAGCTTGACTTGCCTGGTGAAAGGCTTCTATCCCTCTGACATA
Consensus (1185) CATCTCGCGATGAACTGACCAAGAACCAGGTCAGCTTGACTTGCCTGGTGAAAGGCTTCTATCCCTCTGACATA
Section 18
(1259) 1259 1270 1280 1290 1300 1310 1320 1332
E60 HC LALAGA mutant (1201) GCTGTAGAGTGGGAGAGCAATGGGCAACCGGAGAACAACTACAAGACTACACCTCCCGTTCTCGATTCTGACGG
JH3-88 (456) GCTGTAGAGTGGGAGAGCAATGGGCAACCGGAGAACAACTACAAGACTACACCTCCCGTTCTCGATTCTGACGG
JH3-UP (1048) ----------
JH3-DO rc (779) GCTGTAGAGTGGGAGAGCAATGGGCAACCGGAGAACAACTACAAGACTACACCTCCCGTTCTCGATTCTGACGG
Consensus (1259) GCTGTAGAGTGGGAGAGCAATGGGCAACCGGAGAACAACTACAAGACTACACCTCCCGTTCTCGATTCTGACGG
Section 19
(1333) 1333 1340 1350 1360 1370 1380 1390 1406
E60 HC LALAGA mutant (1275) CTCCTTCTTCCTCTACAGCAAGCTCACAGTGGACAAGAGCAGGTGGCAACAAGGGAATGTCTTCTCATGCTCCG
JH3-88 (530) CTCCTTCTTCCTCTACAGCAAGCTCACAGTGGACAAGAGCAGGTGGCAACAAGGGAATGTCTTCTCATGCTCCG
JH3-UP (1048) ----------
JH3-DO rc (853) CTCCTTCTTCCTCTACAGCAAGCTCACAGTGGACAAGAGCAGGTGGCAACAAGGGAATGTCTTCTCATGCTCCG
Consensus (1333) CTCCTTCTTCCTCTACAGCAAGCTCACAGTGGACAAGAGCAGGTGGCAACAAGGGAATGTCTTCTCATGCTCCG
Section 20
(1407) 1407 1420 1430 1440 1450 1460 1470 1480
E60 HC LALAGA mutant (1349) TGATGCATGAGGCTCTTCACAATCACTACACACAGAAGAGTCTCTCCTTGTCTCCGGGTAAATGAGGATCC---
JH3-88 (604) TGATGCATGAGGCTCTTCACAATCACTACACACAGAAGAGTCTCTCCTTGTCTCCGGGTAAATGAGGATCCTCT
JH3-UP (1048) ----------
JH3-DO rc (927) TGATGCATGAGGCTCTTCACAATCACTACACACAGAAGAGTCTCTCCTTGTCTCCGGGTAAATGAGGNTCCTCT
Consensus (1407) TGATGCATGAGGCTCTTCACAATCACTACACACAGAAGAGTCTCTCCTTGTCTCCGGGTAAATGAGGATCCTCT

APPENDIX C
ANTI-CHIKV SYNTHESIZED GENE SEQUENCES

Synthesized CHK152VH Sequence (Highlighted in Red)

421 AAATTTTGTG TCGCCCTTTA CACGTACTTA GTCGCTGAAG AATTCACAAT GGGATGGTCT
481 TGTATCATCC TTTTCTTGGT TGCAACAGCT ACTGGTGTTC ATTCTCAAGT TCAATTGCAG
541 CAGCCTGGTG CAGCTCTCGT GAAGCCAGGT GCTTCAGCAA TGATGTCTTG TAAGGCTTCT
601 GGTTACACAT TCACAAGCTA TTGGATTACA TGGGTGAAAC AAAGACCTGG TCAGGGTCTT
661 GAGTGGATAG GAGATATTTA TCCCGGAACT GGAAGAACTA TCTACAAAGA GAAGTTTAAA
721 ACCAAGGCTA CTCTGACTGT AGATACCAGT TCTTCTACTG CTTTTATGCA ACTTAATTCT
781 TTGACATCAG AAGATAGTGC TGTGTACTAT TGTGCTAGGG GGTATGGCTC ACCATATTAC
841 GCTTTAGACT ATTGGGGACA AGGTACTTCC GTTACTGTGT CTTCTGCTAG CCGTCAATCG

Synthesized CHK152VL Sequence (Highlighted in Red)

1141 GCCTTTCGCC CGGGCTAATT AGGGGGTGTC GCCCTTTACA CGTACTTAGT CGCTGAAGAA
1201 TTCACAATGG GATGGTCTTG TATCATCCTT TTCTTGGTTG CAACAGCTAC TGGTGTTCAT
1261 TCTGATATCG TTTTAACACA ATCTCCAGCA TCTTTGGCTG TTTCTCAAGG ACAAAGGGCT
1321 ACTATCAGTT GCAAGGCTTC TCAATCAGTG GACTATGACG GTGATTCTTA TGTTAACTGG
1381 TACCAACAGA AACCTGGACA GTCCCCAAAA CTTCTTATCT ACGACGCATC TAATCTCGAA
1441 TCTGGAATAC CAGCCAGATT TTCTGGATCT GGATCTGGAA CAGATTTCAC TCTTAACATC
1501 CATCCTGTCG AGGAAGAGGA TGTTGCAACT TATTACTGTC AGGAATCAAA TGAAGATCCA
1561 CGTACTTTCG GTGGAGGAAC TAAGTTGGAG ATCAAAGCTA GCCGTCAATC GAGTTCGTAC

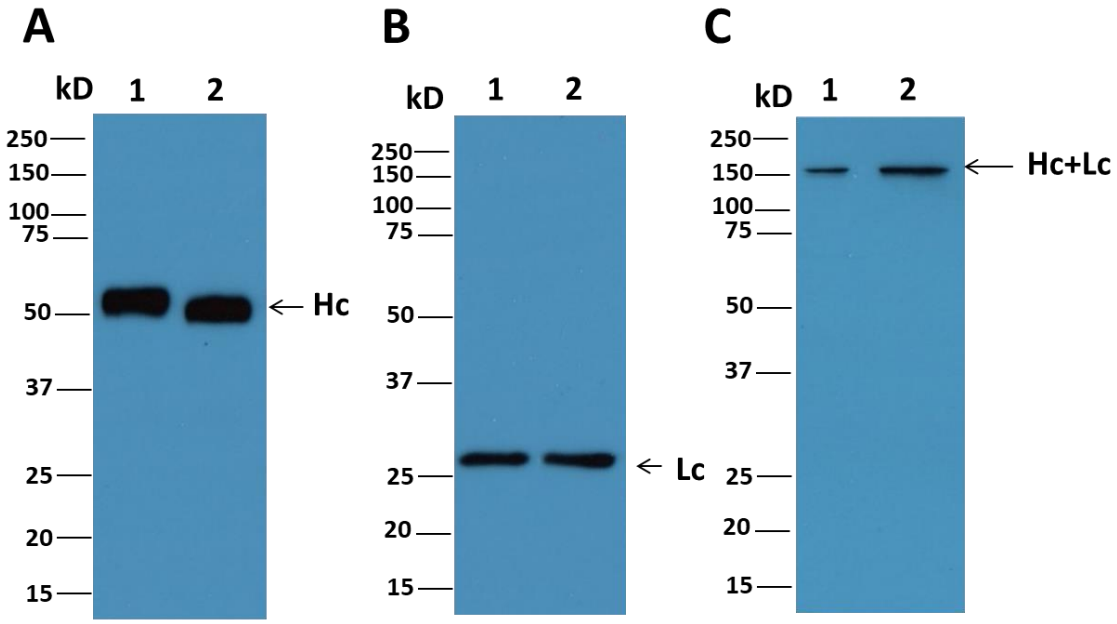
Synthesized CHK166VH Sequence (Highlighted in Red)

1141 GCCTTTCGCC CGGGCTAATT AGGGGGTGTC GCCCTTTACA CGTACTTAGT CGCTGAAGAA
1201 TTCACAATGG GATGGTCTTG TATCATCCTT TTCTTGGTTG CAACAGCTAC TGGTGTTCAT
1261 TCTGAAGTTA GGTTGGTGGA ATCAGGAGGC GGTCTTGAGC AGCCAGGGGG TTCACTGAAG
1321 CTATCTTGTG CAGCTTCTGG TTTTACATTC AGTGATTACT TTATGTATTG GGTGAGACAA
1381 ACCCCTGAGA AACGTCTTGA GTGGGTCGCC TACATTAGTA ATGGGGGAAT TTCCACTTTC
1441 TATAGCGATG CAGTAAAAGG AAGATTCACT ATATCCAGAG ACAACGCTAG AAATACTCTT
1501 TATCTCCAAA TGTCTAGATT GAAGTCAGAA GATACTGCTA TCTACTATTG TGTTAGGCAG
1561 GTATATGGCC AGGGATACTT TGACTATTGG GGACAAGGTA CTACTTTAGC AGTTTCTTCT
1621 GCTAGCCGTC AATCGAGTTC GTACCTAAGG GCGACACAAA ATTTATTCTA AATGCATAAT

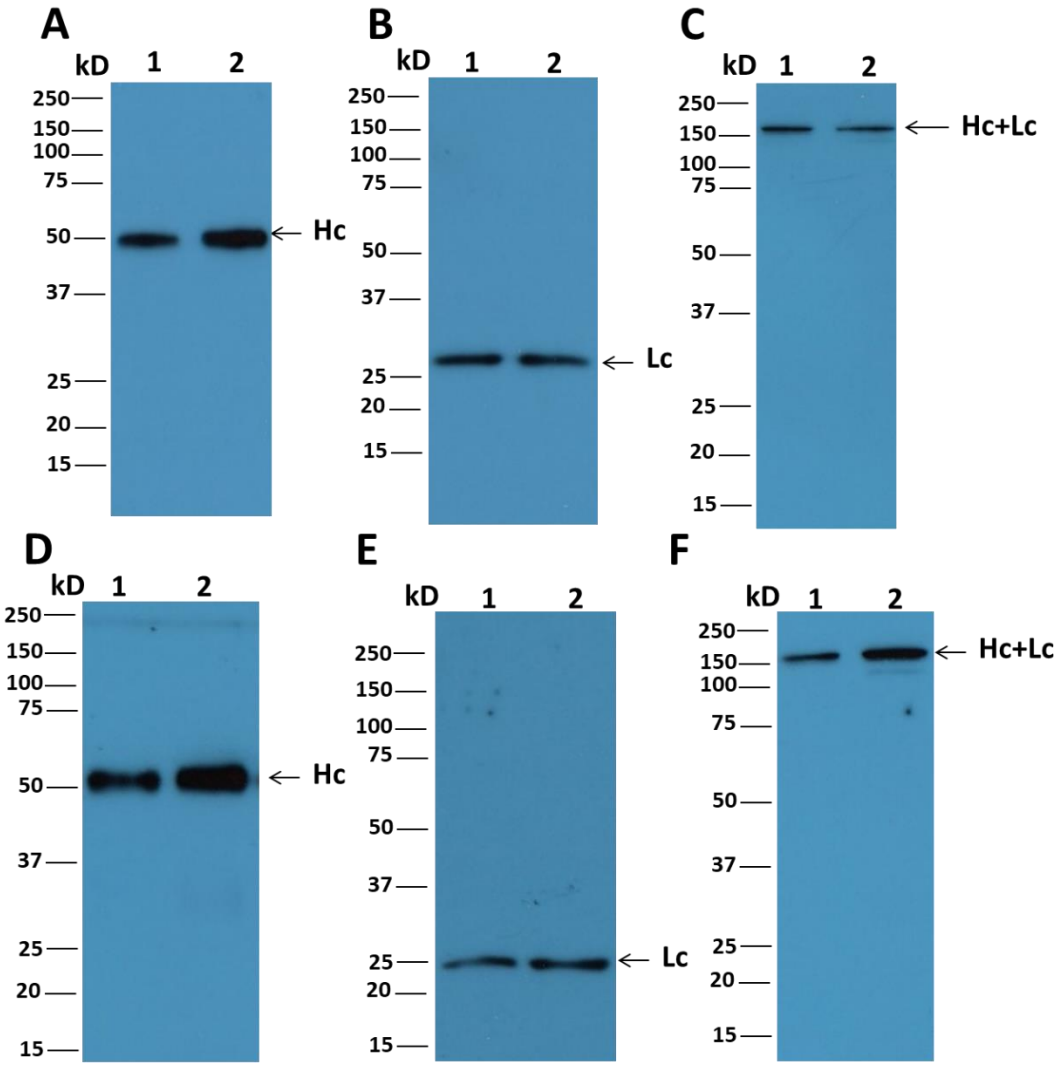
Synthesized CHK166VL Sequence (Highlighted in Red)

421 AAATTTTGTG TCGCCCTTTA CACGTACTTA GTCGCTGAAG AATTCACAAT GGGATGGTCT
481 TGTATCATCC TTTTCTTGGT TGCAACAGCT ACTGGTGTTC ATTCTCAAAT CGTTCTGATT
541 CAATCTCCAG CAATTATGTC TGCTTCTCTT GGAGAGAGGG TGACTATGAC TTGCACCGCT
601 AGTAGCTCTG TTTCAAGTTC TTATTTGCAC TGGTACCAAC AGAAACCTGG ATCATCACCA
661 AAACTTTGGA TCTACTCCTC ATTTTCTTTA GCATCTGGAG TTCCAGCCAG ATTTTCTGGA
721 TCTGGATCTG GAACATCCTA TAGCCTTACT ATCTCAACAA TGGAAGCTGA GGATGCTGCA
781 ACATATTACT GTCATCAGTA TCTCCGTTCC CCATGGACTT TCGGTGGAGG AAGTAAGTTG
841 GAGATCAAAG CTAGCCGTCA ATCGAGTTCG TACCTAAGGG CGACACCCCC TAATTAGCCC

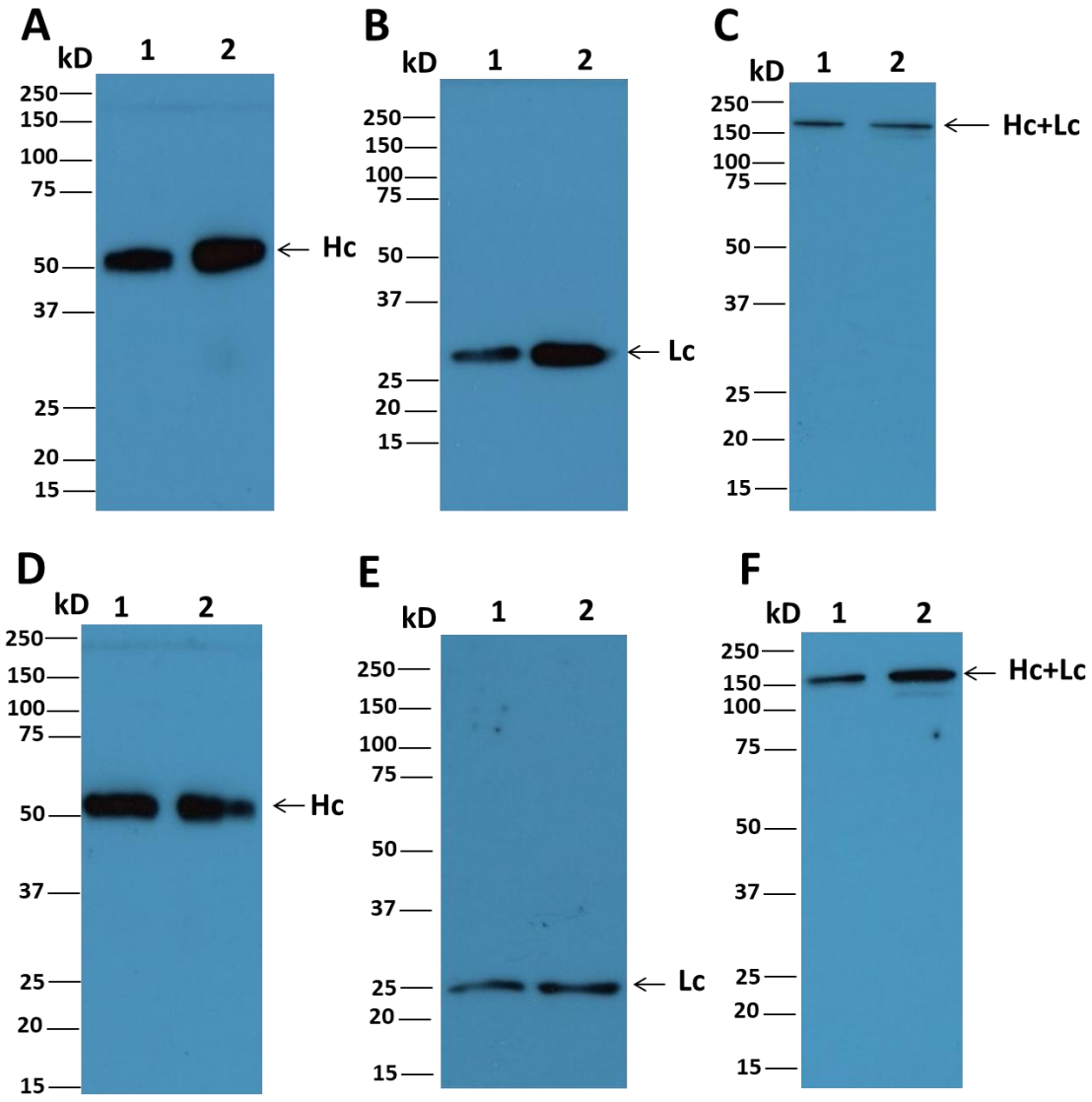
APPENDIX D
WESTERN BLOT ANALYSIS OF PE60 BACKBONE MUTANTS



Western blot analysis of pE60 N297Q. Purified WTpE60 (lane 1) and pE60 N297Q (lane 2) were initially ran on an SDS PAGE and later transferred onto a PDVF membrane for analysis by HRP conjugated goat anti-human gamma (A) and kappa (B and C) antibodies under reducing (A and B) and non-reducing (C) conditions. Hc, heavy chain; Lc, light chain.



Western blot analysis of pE60 LALA. Purified WTpE60 (lane 1) and pE60 LALA (lane 2) from WT (A, B, and C) and GnGn (D, E, and F) plants were initially ran on an SDS PAGE and later transferred onto a PDVF membrane for analysis by HRP conjugated goat anti-human gamma (A) and kappa (B and C) antibodies under reducing (A and B) and non-reducing (C) conditions. Hc, heavy chain; Lc, light chain.



Western blot analysis of pE60 LALAGA. Purified WTpE60 (lane 1) and pE60 LALAGA (lane 2) from WT (A, B, and C) and GnGn (D, E, and F) plants were initially ran on an SDS PAGE and later transferred onto a PDVF membrane for analysis by HRP conjugated goat anti-human gamma (A) and kappa (B and C) antibodies under reducing (A and B) and non-reducing (C) conditions. Hc, heavy chain; Lc, light chain.

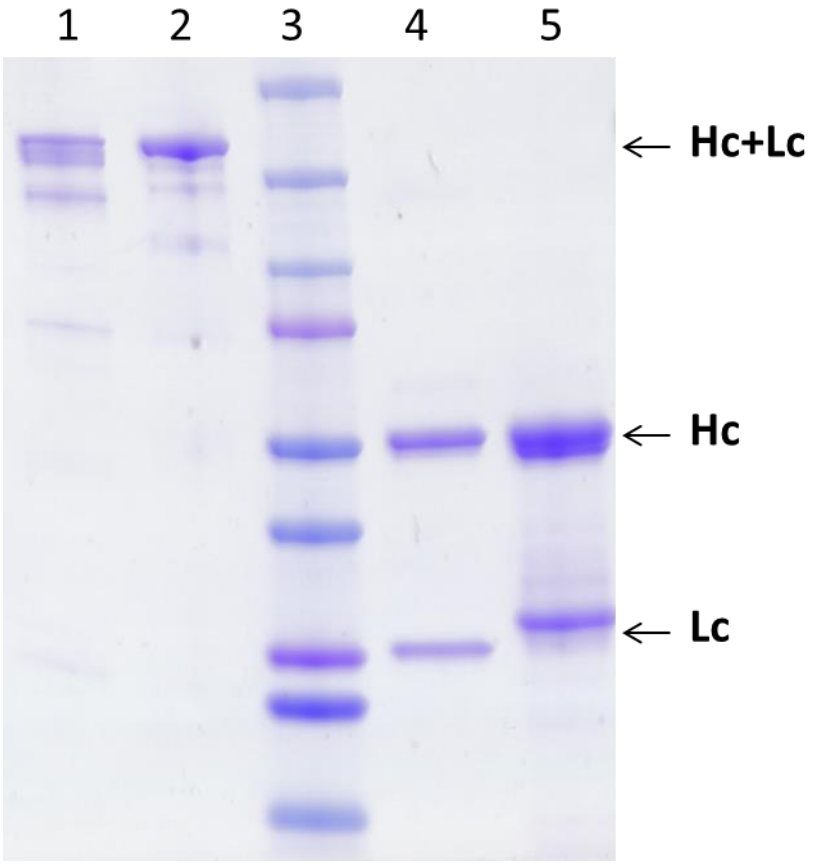
APPENDIX E
CONDITIONS FOR SURFACE PLASMON RESONANCE EXPERIMENT

Table 1: Experimental conditions of Surface Plasmon Resonance (SPR) experiments

Assay	Chip	Immobilized on chip (Standard amine coupling)	Running buffer	Temperature (°C)	Ligand	Ligand flow rate (uL/min)	Rmax
hCD64 (FcγRI)	CM5	Protein A	HBS-EP+	25	Individual SMAb	20	50
hCD32a (FcγRIIa)	CM5	Protein A	HBS-EP+	37	Individual SMAb	25	100
hCD32bc (FcγRIIb/c)	CM5	Protein A	HBS-EP+	37	Individual SMAb	25	100
hCD16a (FcγRIIIa)	CM5	Protein A	HBS-EP+	25	Individual SMAb	20	50
hc1q	CM5	Individual SMAb (~13000RU)	HBS-EP+	25	N/A	N/A	N/A
hFcRn pH6.0	CM5	Anti-Fab	PBS-T pH6.0	25	Individual SMAb	20	100
hFcRn pH7.4	CM5	Anti-Fab	PBS-T pH7.4	25	Individual SMAb	20	100

Assay	Analyte	Analyte concentration	Contact time (s)	Dissociation time (s)	Flow rate (uL/min)	Regeneration conditions
hCD64 (FcγRI)	hCD64	0, 3.75, 7.5, 15, 30, 60nM	135	600	40	Glycine pH1.5 x 2
hCD32a (FcγRIIa)	hCD32a	0, 0.125, 0.25, 0.5, 1, 2 uM	30	60	45	Glycine pH1.5 x 2
hCD32bc (FcγRIIb/c)	hCD32bc	0, 0.125, 0.25, 0.5, 1, 2 uM	30	60	45	Glycine pH1.5 x 2
hCD16a (FcγRIIIa)	hCD16a	0, 0.0625, 0.125, 0.25, 0.5, 1uM	135	600	40	Glycine pH1.5 x 2
hc1q	hc1q	0, 6.25, 12.5, 25 ,50,100ug/mL	120	600	30	Glycine pH2.1
hFcRn pH6.0	hFcRn	0, 75, 100, 150, 200, 250nM	60	90	30	Glycine pH2.1
hFcRn pH7.4	hFcRn	0, 250nM	60	90	30	Glycine pH2.1

APPENDIX F
SDS PAGE ANALYSIS OF pCHK152 IN GEMINIVECTOR



SDS PAGE analysis of pCHK152 inpBYR11eK2Md. Purified pCHK152 (lanes 2 and 5) from WT plants and mammalian produced anti-WNV (lanes 1 and 4) were initially ran on an SDS PAGE under reducing (lanes 4 and 5) and non-reducing (lanes 1 and 2) conditions and stained with Coommassie for visualization. Hc, heavy chain; Lc, light chain.

BIOGRAPHICAL SKETCH

Jonathan Hurtado
School of Life Sciences
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EDUCATION

August 2014-Present **(April 2019)**	**PhD in Molecular and Cellular Biology** School of Life Sciences Arizona State University Current GPA- 3.88
May 2014	**Master of Science in Applied Biological Sciences** College of Letters and Sciences Arizona State University GPA- 4.0 • Dean's List Spring 2013
May 2013	**Bachelors of Science in Applied Biological Sciences** College of Letters and Sciences Arizona State University GPA- 4.00 • Presidents Honor List Fall 2011, Spring 2012, Fall 2012
July 2011	**Associate in Science with Highest Distinction** Chandler-Gilbert Community College GPA- 3.912 • Presidents Honor List Spring 2010, Fall 2010, Spring 2011

RESEARCH EXPERIENCE

PhD Graduate Research, 08/2014- present
Center for Immunotherapies, Vaccines, and Virotherapies, Biodesign Institute, Arizona State University
Mentor: Dr. Qiang Chen
Topic: The impact of antibody structure variation and glycosylation on its effector function.

- Designed and cloned anti-Dengue Virus MAbs heavy chain mutants and anti-Chikungunya Virus MAbs
- Assayed their expression and accumulation levels in both wild-type and a glycoengineered *N. Benthamiana* lines
- Investigated the binding affinity of anti-DENV MAb variants to their antigens

- Investigated the neutralization profile of anti-DENV Mab variants to DENV2
- Expression and purification of ZIKV and Hepatitis B virus core virus like particle from plants
- Published two peer-reviewed articles from this research and book chapter

MS Graduate Research, 08/2013 - 07/2014
Center for Infectious Diseases and Vaccinology, Biodesign Institute, Arizona State University
Mentor: Dr. Qiang Chen
Topic: Molecular Design and Functional Characterization Portfolio of Flavivirus Therapeutics

- Designed and cloned several diabodies, Fabs, and scFv for a MAb against West Nile virus (WNV) and two full monoclonal antibodies (MAbs) against Dengue virus
- Assayed their expression and accumulation levels in both wild-type and a glycoengineered *N. Benthamiana* lines
- Investigated the binding affinity of anti-WNV MAb variants to their antigens
- Published one peer reviewed journal article from this research

Undergraduate Research, 08/2012-07/2013
Center for Infectious Diseases and Vaccinology, Biodesign Institute, Arizona State University
Mentor: Dr. Qiang Chen
Topic: Enhance the expression of recombinant proteins in plants

- Cloned marker gene of DsRed into MagnICON Vectors
- Infiltrated this gene into *Nicotiana Benthamiana* plants
- Investigated the expression kinetics of this gene in *N. Benthamiana*
- Published two peer reviewed journal articles from this research

PRESENTATIONS

- Oral presentation at SACNAS 2018 in San Antonio, Texas.
- Presented poster at SACNAS 2017 in Salt Lake City, Utah.
- Presented poster at NEST Training Retreat in Woods Hole, Massachusetts (2016)
- Presented poster at the American Society of Plant Biologist Conference Minneapolis, Minnesota (2015)
- Presented research at the "Show Case" of College of Technology and Innovation, 2013

TEACHING EXPERIENCE

Mentoring Undergraduate Students – Supervision of projects including teaching of basic molecular techniques and analysis of results.

Teaching Assistant, School of Life Sciences, University of Arizona - Human Anatomy and Physiology Lab in the Fall 2016, Spring 2017, and Fall 2017.

PUBLICATIONS

1. **Hurtado, J.;** Chen, Q. 2018. Plant-produced Anti-Dengue Virus Monoclonal Antibodies Exhibit Potent Protection in Antibody-Dependent Enhancement Mouse Model. ***In preparation.***
2. **Hurtado, J.;** Chen, Q. 2018. Neutralization and Reduction of Mouse Ankle Inflammatory Pathology by Plant Produced Anti-Chikungunya Virus Monoclonal Antibody with Specific N-glycosylation. ***In preparation.***
3. Yang, M.; Sun, H.; Lai, H.; **Hurtado, J.**; Chen, Q. 2017. Plant-produced Zika virus envelope protein elicits neutralizing immune responses that correlate with protective immunity against Zika virus in mice. Plant Biotech Journal.
4. Dent, M.; **Hurtado, J.;** Paul, A.; Sun, H.; Lai, H.; Yang, M.; Esqueda, A.; Bai, F.; Steinkellner, H.; Chen, Q. 2016. Plant-produced anti-dengue virus monoclonal antibodies exhibit reduced antibody-dependent enhancement of infection activity. Journal of General Virology, 97(12), 3280-3290. doi:10.1099/jgv.0.000635
5. Chen Q, Dent M, **Hurtado J**, Stahnke J, McNulty A, Leuzinger K, Lai H. 2014. "Large Scale Transient Expression by Agroinfiltration in Lettuce". In: Methods in Molecular Biology - Recombinant Proteins from Plants, ed. by Menassa R. S, Springer New York, 2014.
6. He J, Peng L, Lai H, **Hurtado J**, Stahnke J, and Chen Q. A plant-produced antigen elicits potent immune response against West Nile virus in mice. 2014. BioMed Research International (IF 2.88) 2014;2014:952865.
7. Junyun He, Huafang Lai, **Jonathan Hurtado**, Jake Stahnke, and Qiang Chen. Structural and functional characterization of an anti-West Nile virus monoclonal antibody and its single-chain variant produced in glycoengineered plants. 2014. Plant Biotech J 2014 12: 1098–1107. doi: 10.1111/pbi.12217 (IF 5.6)
8. Q Chen, H Lai, **J Hurtado**, J Stahnke, K Leuzinger, M Dent. Agroinfiltration as an Effective and Scalable Strategy of Gene Delivery for Production of Pharmaceutical Proteins. 2013. ATBM 1: 103. doi:10.4172/atbm.1000103
9. K Leuzinger, M Dent, **J Hurtado**, J Stahnke, H Lai, X Zhou, Q Chen. Efficient Agroinfiltration of Plants for High-level Transient Expression of Recombinant Proteins. 2013. Journal of visualized experiments: J. Vis. Exp. (77), e50521, doi:10.3791/50521

PATENT

"Plant-Derived Antibodies and Derivatives that Reduce Risk of Antibody-Dependent Enhancement (ADE) of Infection" U.S. Patent Application filed 01/27/14. No. 61/932,033. Patent Approved by USPTO on January 27, 2015. Inventors: Q. Chen, H. Lai, J. Hurtado

GRANTS

- SACNAS 2017 Travel Grant Salt Lake City, Utah (2017)
- American Society of Plant Biologist Conference Travel Grant Minneapolis, Minnesota (2015)
- Initiative for Maximizing Student Development (IMSD) NIH Training Fellowship (R25) August 2014 to July 2016
- Undergraduate Research Grant 2012 "Development of novel detection and diagnostic reagents of West Nile virus, Phase II" $1,000, Department of Applied Sciences and Mathematics, ASU

HONORS AND AWARDS

- SACNAS 2018 Best Oral Presentation Award San Antonio, Texas (2018)
- Undergraduate Student Research Award for Graduation Class of 2013, Department of Applied Sciences and Mathematics, College of Letters and Sciences, Arizona State University (2013)