Immunogenic Subviral Particles Displaying Domain III of Dengue 2 Envelope Protein Vectored by Measles Virus

by

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ABSTRACT

Vaccines against the arthropod-borne dengue virus (DENV) are still commercially nonexistent. A subunit immunization strategy may be of value, especially if a safe viral vector acts as a biologically active adjuvant. The DENV envelope protein (E), the main target for neutralizing immune responses, has three conformational domains. The immunoglobulin-like and independently folding domain III (DIII) contains epitopes that elicit highly specific neutralizing antibodies. The hepatitis B small surface antigen (HBsAg, S) was used as a scaffold to display DENV 2 DIII on a virus-like particle (VLP). A measles virus (MV) was engineered to vector HBsAg and the hybrid glycoprotein DIII-HBsAg in two different loci (DIII-S). Despite the relatively deleterious effect on replication caused by the insertion of two transcription cassettes, the recombinant virus MVvac2(DIII-S,S)P induced the secretion of DIII-S hybrid VLP with a similar sucrose density as HBsAg particles (1.10-1.12g/ml) and peaked at 48 h post-infection producing $1.3x10^6$ $TCID_{50}$/ml infectious MV units *in vitro*. A second recombinant virus, MVvac2(DIII-S)N, was engineered to vector only the hybrid DIII-S. However, it did not induce the secretion of hybrid HBsAg particles in the supernatant of infected cells. The immunogenicity of the recombinant viruses was tested in a MV-susceptible small animal model, the experimental group which received two 10^5 $TCID_{50}$ I.P. doses of MVvac2(DIII-S,S)P in a 28 day interval developed a robust immune response against MV (1:1280), HBsAg (787 mIU/ml) and DENV2 (Log_{10} neutralization index of 1.2) on average. In summary, it is possible to display DENV E DIII on hybrid HBsAg particles vectored by MV that elicit an immune response. This forms the basis for a potential vaccine platform against DENV.

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ABBREVIATION

Term	Definition
ATU	Additional Transcription Unit
ADE	Antibody-dependent Enhancement
CDC	Center for Disease Control and Prevention
DC	Dendritic Cell
DENV	Dengue Virus
DF	Dengue Fever
DHF	Dengue Hemorrhagic Fever
dsRNA	Double-stranded RNA
DSS	Dengue Shock Syndrome
DI	Domain I of the DENV E Protein
DII	Domain II of the DENV E protein
DIII	Domain III of the DENV E protein
DIII-S	Hybrid DIII-HBsAg Antigen
EC	Epithelial Cell
E Protein	Envelope Protein
ER	Endoplasmic Reticulum
GTase	Guanylytransferase
HBsAg	Hepatitis B small surface antigen
HBV	Hepatitis B Virus
HPV	Human Papilloma Virus
IFN	Interferon

I.P.	Intraperitoneal
IRES	Internal Ribosome Entry Site
LAV	Live Attenuated Vaccine
LN	Lymph Node
MOI	Multiplicity of Infection
MTase	Methyltransferase
MV	Measles Virus
NIH	National Institute of Health
NS	Non-structural Protein
NTPase	Nucleoside Triphosphatase
PDK	Primary Dog Kidney Cells
PIV	Purified Inactivated Vaccine
Pre-M/M Protein	Pre-Membrane/Membrane Protein
RdRp	RNA-dependent RNA Polymerase
RTPase	RNA Triphosphatase
TGN	Trans-Golgi Network
TMB	Tetramethylbenzemidine
UTR	Untranslated region
VLP	Virus-like Particles
YFV	Yellow Fever Virus

Chapter 1. Introduction

1.1 Dengue Epidemiology. Dengue is the most important arthropod borne viral disease in the world. It is caused through infection by dengue virus (DENV). In 2013, it was estimated that 390 million people has been infected with DENV, causing 96 million clinical cases annually (1). Dengue has become endemic in various countries in the Americas, the Middle East, Africa, the Western Pacific and Southeast Asia (2). About 40% of the world's populations live in locations that are highly at risk for the transmission of DENV (Fig. 1). Areas at risk are mainly tropical or sub-tropical bearing the optimal breeding ground for the arthropod vectors that transmits DENV, which are the mosquitoes: *Aedes aegypti* and *Aedes albopictus* (3).

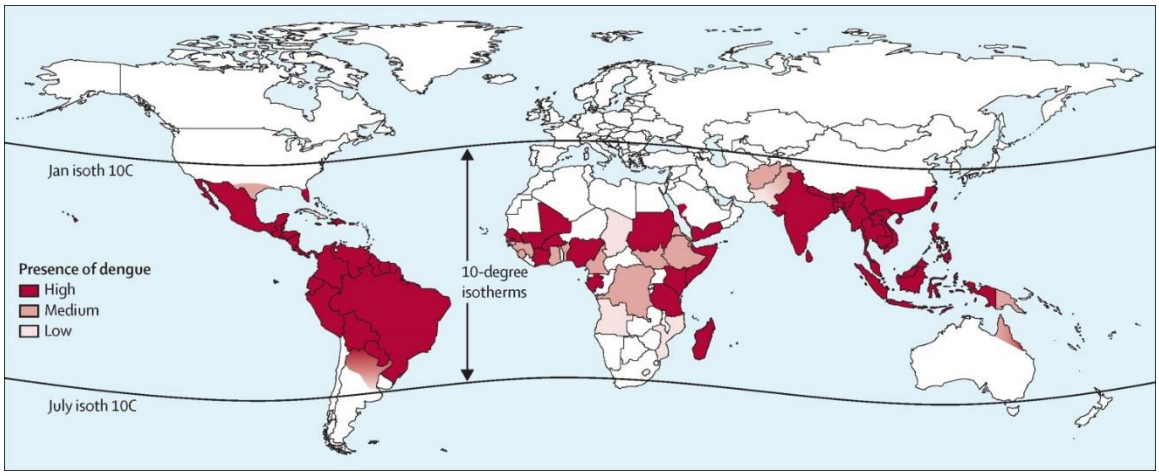

Figure 1. Diagram Showing a World Map Highlighting Countries where Dengue Virus is Prevalent. Dengue is endemic in over 100 countries. The three different shades of red indicate the level of prevalence of the virus in each country. Dengue and dengue hemorrhagic fever are prevalent in urban and suburban areas in the Americas, South-East Asia, Eastern Mediterranean, and Western Pacific; it is mainly prevalent in rural areas in Africa (3).

Modernization and societal changes have aided in the geographic expansion of the mosquito vectors. These changes include globalization, increased international travel, as well as unplanned urbanization (2). Unplanned urbanization has pushed human dwellings to invade the rainfall forest, placing humans in mosquitoes' home environment, which increases exposure rate (4). In addition, inadequate waste and sewer management, along with lack of a vector control program that is usually present in unplanned urbanizations exacerbate epidemic activity of DENV (2). These types of scenarios can be seen in developing countries where DENV infections run rampant, like the Philippines, Thailand, and Vietnam, which are the Asian countries that has the highest dengue prevalence (2). In these countries, DENV infection is one of the top 10 causes of death in hospitalized children (5). In addition to the burden that DENV infection place on the health care system of each country, it also imposes a high economic burden on the country's government and on each individual (3). In Southeast Asia, clinical DENV infection cause an annual economic burden of ~$950 million. Therefore a reduced transmission of DENV would greatly lessen the burden placed on both the healthcare system and the economic stability of developing countries where DENV infection is endemic.

Many other factors have been associated to the 30-fold increases in DENV prevalence in the past 50 years. The main factor being the expansion of the habitat for *A. aegypti* and *A. albopictus*; due to globalization, international travel, climate changes, arthropod vector's adaptation, etc. In the U.S. recent outbreaks have happened in Hawaii in 2001, Texas in 2005, and Florida in 2009 to 2011 (6). In 2010, the 1st cases of DENV infections due to indigenous transmission in Europe was reported (2). This was acknowledged to be caused by *A. albopictus* whose eggs have adapted to the subfreezing

temperature in Europe. All of these factors only magnify the dire need of a viable vaccine against DENV as the habitats for the vector transmitting DENV broaden (6). Most severe cases of DENV infection reported annually originate from the Asia-Pacific region, the Americas, and Africa; where there is a high concentration of all four heterologous

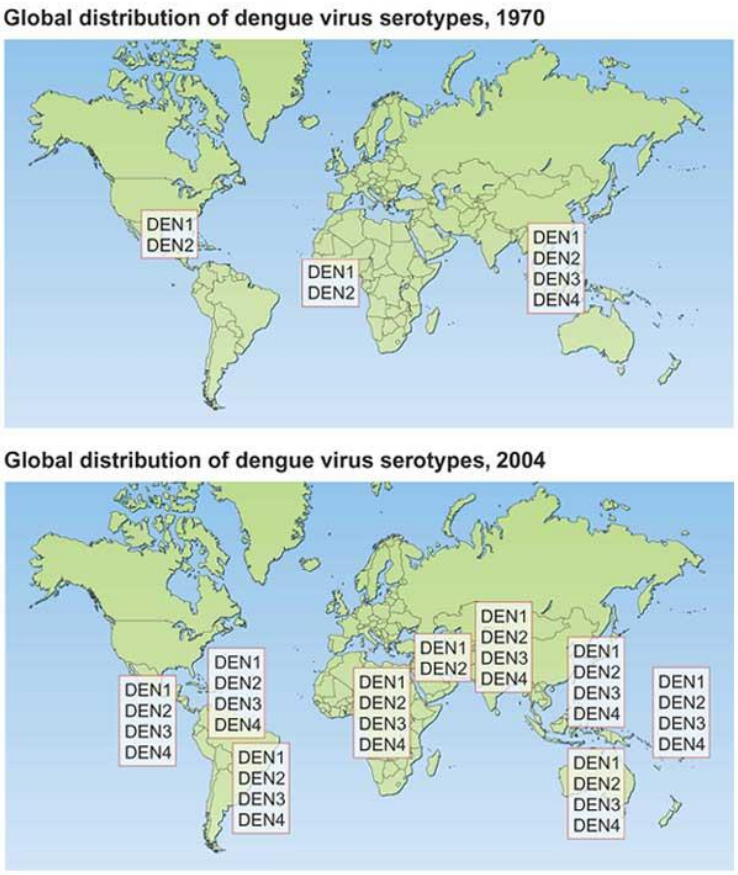

Figure 2. Two World Maps Presenting the Increased International Distribution of the Four Different Serotypes of DENV, Over 34 Years. The top world map shows that only one area of the world, the Asia-Pacific region, have all four DENV serotypes circulating. 34 years later, the bottom world map shows that now the majority of the world harbors all four DENV serotypes (8).

serotypes circulating (7). In addition, the spread of the different DENV serotypes have steadily increased all over the world in recent years (Fig. 2). The increase in international travel have aided in the spreading of the mosquito vectors that transmits DENV; this includes the movement of the virus in infected individuals who travels internationally as well as cases where the mosquito vector's eggs were unknowingly transported in tires

being shipped overseas (8). The broadening of the habitat for *A. aegypti* and *A. albopictus* have also been encouraged by international travel, where they become adapted to their new environment. The expansion and development of human housings into the natural habitat for these vectors have also increase the rate of exposure between humans and mosquitoes (8). Overall, many factors have encouraged the increased distribution of DENV, which have promoted more frequent and larger dengue epidemics that are associated with more severe symptoms of the illness.

1.2 Transmission of Dengue Virus. DENV is an arbovirus, transmitted by the bite of female mosquitoes, specifically: *A. aegypti* and *A. albopictus* (2). *A. aegypti* originated from Africa and has now spread throughout most of the world, whereas *A. albopictus* originated from Southeast Asia, with a cold-resistant strain in Europe (5). Transmission of DENV happens during the feeding activity for these female mosquitoes, which peaks in the morning time and late afternoon. The virus is transported from person to person while these mosquitoes have their blood meal, at which point the virus is subcutaneously injected into the person (2). The transmission is carried exclusively by female mosquitoes due to their dependence on blood for oviposition. It has been demonstrated that there is vertical transmission from infected females to their ova, this is the main mechanism that explain the persistence of infected mosquitoes during winter in some non-tropical regions. Male mosquitoes feed from fruit.

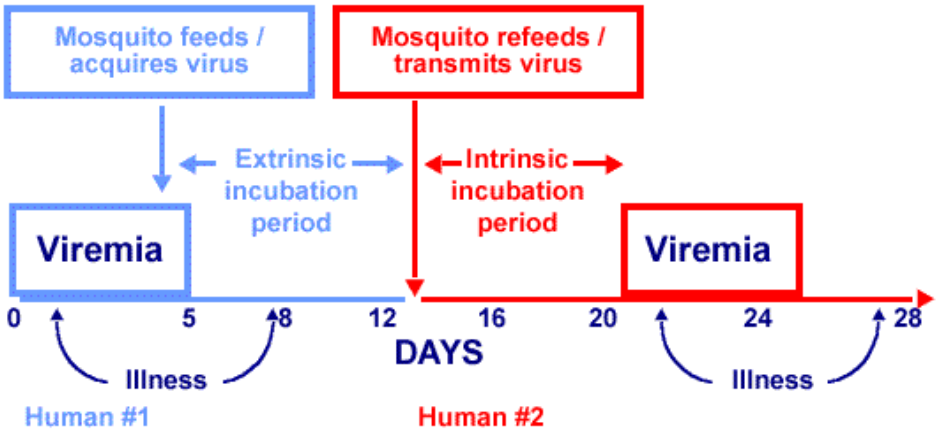

Figure 3. Timeline Representing the Transmission of DENV, Following the Feeding Schedule and Life Cycle of the Mosquito Vector. A mosquito may obtain DENV when it feeds from a viremic host, who has a high titer of DENV in their bloodstream, enough to be taken up by the mosquito. Mosquito may infect a second host if it directly takes another blood meal after it obtained DENV. As well as after 8-10 days post-blood meal from a viremic first host, because the DENV would have had time to replicate and reach the salivary gland of the mosquito [CDC].

Viremia, the presence of a high titered virus in the bloodstream, can usually be observed 24-48 hours before the appearance of clinical symptoms. Viremia may last up to 10 days in patients infected with DENV (2). If the mosquito feed on an individual with viremia, the mosquitoes can become infected by DENV which will target the epithelial cells of the mosquito's mid-gut (2). These mosquitoes will then become infectious, after an incubation period of 8-10 days post-feeding. After spreading systemically through the haemocele, DENV reaches the salivary glands where it can be easily transmitted to humans (2). In summary, the mosquito can transmit DENV by directly changing host during a blood meal or after 8-10 days when DENV has multiplied in the salivary glands (Fig. 3). The mosquito will then be able to transmit DENV for the rest of its life in addition to the possibility of transmitting DENV to its eggs, as mentioned (5).

Initially in humans, DENV targets macrophages and dendritic cells (DC) because they are present in the epidermis and subcutaneous tissue where the virus would be injected by the mosquito (2). The infected macrophages and DCs will then migrate to the lymph node (LN) where the virus will spread to other macrophages and ultimately peripheral monocytes. This will result in the first viremia, as viruses are moved in draining and remote LN. The major sites of viral replication for DENV in humans are posited to be DCs, macrophages, and monocytes. In addition, DENV may also be found in other tissues throughout the body, such as the lungs, the liver, the spleen and the kidneys (2). Cells that are infected with DENV usually die through apoptosis or necrosis. Necrosis causes the increased production of toxins which in turn will activates the fibrinolytic and coagulation systems. Viremia, platelet dysfunction, and severe thrombocytopenia, will result in the fragility of the capillaries, presented as bruising, petechiae, and gastrointestinal mucosal bleeding; these are the characteristics of DHF (9). In addition, increased vascular permeability and coagulopathy is amplified when IgM antibodies reacting with the epithelial cells (EC), platelets, and plasmin are produced. During secondary infection, enhancement of infection by IgG (discussed below) will contribute to the high viral load that will lead to the secondary viremia (9).

1.3 Dengue Virus Structure and Replication Cycle. DENV is a member of the family of *Flaviviridae*; It is an enveloped virus that has an icosahedral nucleocapsid containing ~10.7kb of positive-sense, single-stranded RNA as genome (1). This encodes a polyprotein that will be cleaved into seven nonstructural proteins and three structural proteins that will be processed co- and post-translation by cellular and viral encoded proteases. The three structural proteins are C, pre-M/M, and E proteins. The seven

nonstructural proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (2). The capsid protein (C protein) serve as RNA chaperone and binds at a high affinity with the viral RNA; this protein is confined to the nucleus and nucleoli when independently expressed on its own in infected cells. The pre-membrane or membrane protein (pre-M/M protein) plays a vital role in viral fusion and entry; it has been shown that mutations in the M protein resulted in lower rates of virus assembly and entry (2). The envelope protein (E protein) in DENV lies parallel to the surface of the virion. Since it has been shown that anti-E antibodies will neutralize viral infectivity through the inhibition of viral binding to cells, the E protein has been concluded to play a vital part in virus attachment and virus-specific membrane fusion (10). In addition, it has been observed that the E protein's stem region plays a vital role in heterodimerization of pre-M, hence affecting membrane binding and virus assembly (2). The non-structural protein 1 (NS1) is 46 kDa, it is a glycoprotein that co-localizes with double-stranded RNA (dsRNA) in mammalian and insect cells, which makes it vital for viral replication. The non-structural protein 2-A (NS2A) is 22 kDa, it is important in the replication of the genome, RNA. The non-structural protein 2-B (NS2B) is 14 kDa, it is a membrane associated protein that is a required cofactor for the NS3 protein, as it recruits NS3 to the endoplasmic reticulum (ER) membrane. The non-structural protein 3 (NS3) is 69 kDa, it serves as a multifunctional viral protease; it has the protease domain at the N-terminal and a domain that includes nucleoside triphosphatase (NTPase), RNA triphosphate (RTPase), and helicase activities at the C-terminal. The non-structural protein 4-A (NS4A) is 16 kDa, it co-localizes with NS3 and the viral dsRNA in cytoplasmic foci, which contains viral replication complexes. Constitutes a cofactor for the viral protease. It may be important

in formation of replication vesicles, making it a viral membrane protein. The non-structural 4B (NS4B) protein weighs 28 kDa, it also co-localizes with NS3 and the viral dsRNA at the site of RNA replication. The non-structural protein 5 (NS5) is the largest protein (105 kDa). NS5 is also the most conserved protein because it encodes the RNA-dependent RNA polymerase (RdRp) on its C-terminal domain, which makes it vital for viral RNA synthesis; methyltransferase (MTase) and guanylytransferase (GTPase) are also encoded on its N-terminal domain. In addition, this protein is also involved in counteracting the interferon (IFN) system of the innate immune response (2, 5, 6).

During host cell infection, DENV enters the cell via clathrin-dependent receptor-mediated endocytosis (3). After endocytosis, the environment inside the endosomes becomes acidified, which causes a pH-dependent conformational change that rearranges the E and M proteins prompting a class II membrane fusion machinery; exposing the fusion loop into the endosomal membrane (Fig. 4). This allows the fusion of the viral envelope with the endosomal membrane; releasing the viral nucleocapsid into the cytoplasm (2). The nucleocapsid will then dissociate in the cytoplasm, releasing the viral RNA to be translated by the ribosomes on the rough ER. The polyprotein is then translated and co-translationally processed and cleaved by host and viral proteases into the respective structural and non-structural viral proteins. The RdRp generates the negative complementary strand to the viral genome, which will be used as a template to

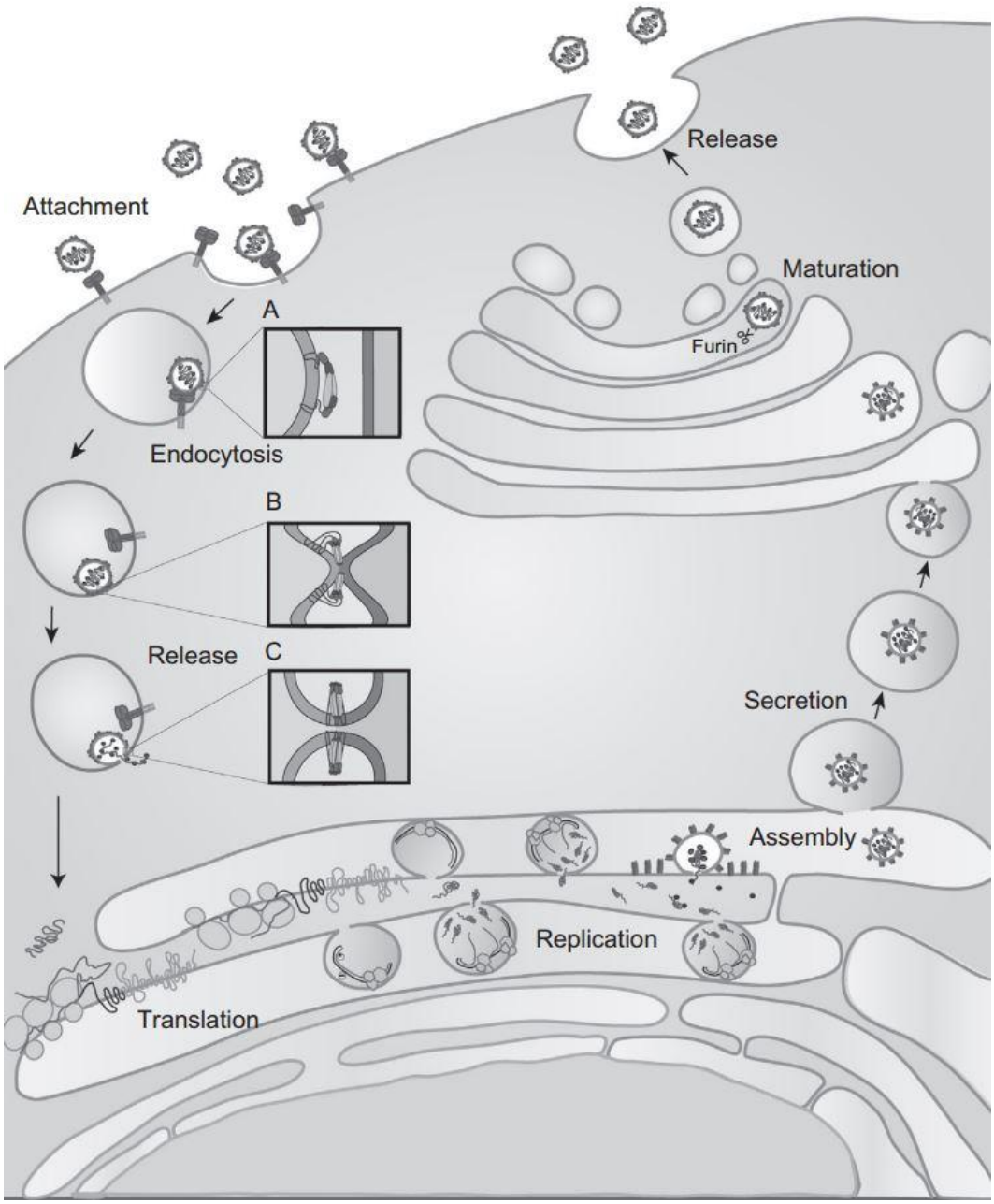

Figure 4. Diagram Representing the Replication Cycle of DENV in Infected Host Cell. DENV binds to the host cell's surface receptor and enter the cell via receptor-mediated endocytosis. The acidification of the internal environment of the endosome allows for the rearrangement of the DENV E and M protein, resulting in the fusion of the viral envelope with the endosomal membrane. Hence, the viral RNA is released into the cell cytoplasm. The viral genome is then translated creating a polyprotein, which is cleaved into the respective 3 structural and 7 non-structural viral proteins. The RdRp encoded by NS5 generates the complementary strand to the viral genome for viral replication. The viral RNA will then associate with capsid proteins to form the nucleocapsid. The nucleocapsid will associate with the E and pre-M rich ER membranes and buds into the ER lumen to create immature virion. This will exit the cell via exocytosis through a secretory pathway (2).

replicate more copies of the viral RNA. The nucleocapsid is then formed by the association of the viral RNA with the capsid protein. The nucleocapsid buds into the lumen of the ER and obtain the lipid membrane proteins pre-M/M and E (2, 3). After the assembly of the immature virions, it is transported out of the host cell through the classical secretory pathway. The viral particles will be contained in a vesicle, transported through the Golgi stacks and secreted by exocytosis. During its transport through the trans-Golgi network (TGN), the protease furin cleaves the pre-M protein to its mature form, M protein that remains on the mature and infectious virion.

1.4 Manifestation of Symptoms in Dengue Virus Infected Patients. There are 4 different serotypes of DENV: DENV-1, DENV-2, DENV-3, and DENV-4 (1). The presence of multiple serotype represents a challenge in controlling illnesses and symptoms in patients infected with DENV as well as in creating a viable commercial vaccine with complete protection against DENV. Clinically, DENV infection has been divided in two main syndromes: Classic Dengue and severe Dengue infection. The patient can evolve clinically from non-significant, dengue fever (DF), to the extremes of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (4). After infection with a mosquito bite, an individual may be asymptomatic, or may develop initially a flu-like syndrome. In most cases, the infection will be resolved without any complications (1). The majority of severe DENV infection cases will result in DF, where the symptoms include: sudden onset of fever reaching 102.2ºF, headache, myalgia, macular or maculopapular rashes, joint pains, body aches, and retro-orbital pain (5, 6). Usually, this acute phase is limited to last only 1 week, followed by recovery. However, 1% to 7% of DENV patients may develop DF with hemorrhage, where symptoms include: petechial

purpura, ecchymosis, and epistaxis. Furthermore, up to 2% of DENV patients may develop the more severe and life-threatening DHF, where complications include: increased vascular permeability, liver damage, thrombocytopenia, and hemorrhaging from the skin, gum, nose, and gastrointestinal tract. These cases may also worsen and progress to DSS, where a redistribution of the effective central vascular volume towards the periphery is caused by vasodilatation and diapedesis, resulting in a sudden drop in effective perfusion pressure (2). The main problem in DHF or DSS is not blood loss but intravascular fluid loss. Therapy at this stage of symptoms is the management of blood volume and blood pressure. The current therapy for patients infected with DENV is focused on support, mainly with bed rest, fluid intake, and the control of fever or pain with antipyretics and analgesics. In mild cases, patients with DENV infection will respond in one or two days after plasma expansion with isotonic saline solution (5).

1.5 Enhancement of Pathogenesis. Pathogenesis in an individual infected with DENV may be enhanced due to the existence of the four different serotypes. Two different pathways of pathogenesis enhancement non-mutually exclusive have been considered: antibody dependent enhancement (ADE) and the original antigenic sin. Upon DENV infection, serotype-specific life-long immunity is acquired; however it will only confer a transient immunity against another serotype (11). Studies have shown that antibodies elicited from a primary infection are a major factor in the rapid development of the more severe DHF and DSS after a secondary infection with a heterologous serotype (12, 13).

The explanation for ADE is that the non-neutralizing, cross-reactive antibodies gained from the primary infection will bind to the heterotypic virus (1, 9, 13). The antibody-virus complex will increase the uptake of the viral particle by Fc-receptor expressing cells such as monocytes, macrophages and DCs (Fig. 5). This will in turn increase the viral replication and increase the severity of infection (14). In addition, ADE may also occur in situations when there is a low antibody concentration or when there is low antibody avidity for the current DENV infecting serotype. Specifically, when the amount of antibodies bound the virion is below the amount needed for neutralization,

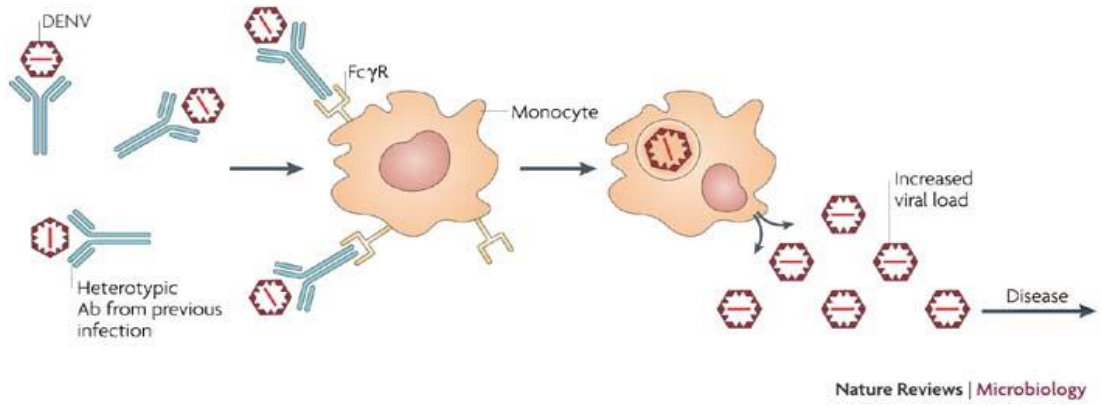

Figure 5. Diagram Representing the Stages Leading to Antibody-dependent Enhancement. Cross-reactive heterotypic antibodies from the primary DENV infection bind to the viral particles from the current DENV infection. These antibodies are non-neutralizing that creates an Ab-virus complex which enhances its uptake by Fc-receptor expressing cells such as monocytes, macrophages, and DCs. Resulting in an increased viral load (14).

which is a minimum of ~30 for each virion (1, 2). The occurrence of ADE has been supported through its observation *in vitro*; where non-neutralizing antibodies that were specific to DENV were able to increase viral replication in peripheral blood leukocytes (6). In addition, ADE incidences *in vivo* have also been observed in monkey. Scientists observed a higher viral load in monkeys that received a passive transfer of humanized anti-DENV monoclonal antibody or human sera that are immune against DENV (6).

Original antigenic sin occurs when pathogenesis enhancement is mediated by the cellular immune response (2). Scientists have found that the memory T cells that are specific for DENV serotype responsible of the primary infection may be reactivated due to cross-reactive characteristics. These T cells show suboptimal degranulation, but it will induce an increased secretion of cytokines, resulting in apoptosis of infected and non-infected bystander cells (6). This excessive activation of the cellular immune system causes a cytokine storm, which increases vascular permeability. This reaction has been concluded to be the main reason behind life-threatening DSS cases (1, 2, 6).

Since all of the DENV vaccines that are most developed target the humoral immune system, ADE is the major cause of difficulties in the generation of a vaccine against DENV. This created a fear in the production of DENV vaccines because a vaccine that could not generate an equilibrated neutralizing immunity against all four serotypes could increase the risk of vaccinees developing the more severe and life-threatening DHF and DSS. Therefore it has been concluded that a tetravalent vaccine is mandatory (15). A successful vaccine against DENV must induce a balanced and long-lasting immunity against all four of the heterologous DENV serotypes. In addition, it must target an antigenic epitope on DENV, which is conserved in all four DENV serotypes, yet also be highly specific to each serotype to avoid cross-reactivity. This challenge in creating a safe vaccine for DENV has resulted in a lack of commercially available vaccines against DENV, which has been approved.

1.6 Various Approaches to Creating a Vaccine and its Challenges. An ideal dengue vaccine must have a couple of vital characteristics. First, it must be a tetravalent vaccine formulation and induce a long-lasting balanced immune response to each serotype. The vaccine should also be safe in 9-12 months old children, so it can be used as a pediatric vaccine, since this is the population most at risk of complicated Dengue infections. A vaccine that can be produced at a low cost with high efficiency for the ease of scaling up production would also be desirable (10). The main targets that have been used in vaccines against DENV infection in humans are the pre-M-E, and NS1 proteins

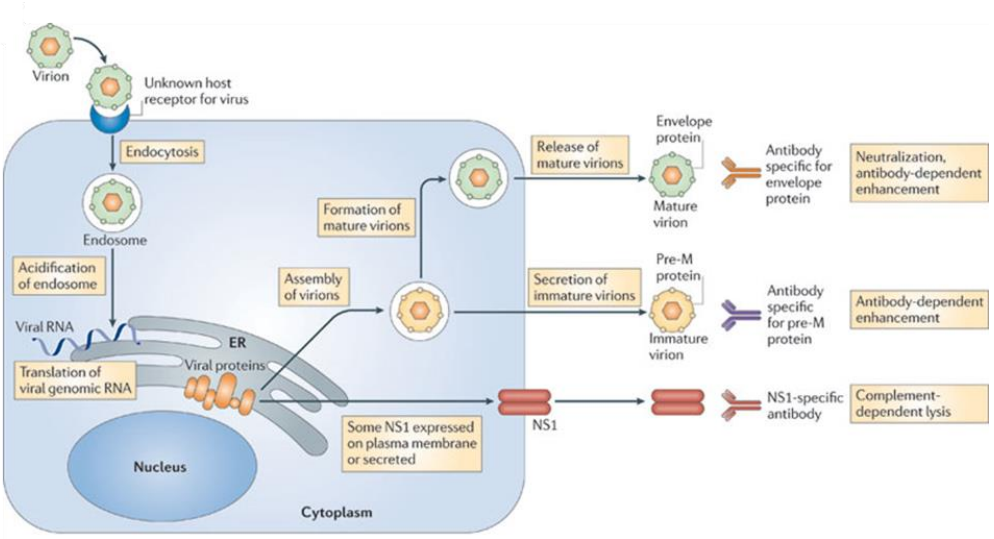

Figure 6. Illustration of the Three Main DENV Proteins Targeted for an Antibody Response and the Function of those Specific Antibodies. Antibodies against the envelope protein of the mature virion is found to be neutralizing, but may induce antibody-dependent enhancement. Antibodies against the pre-M protein on the immature virion are found to be serotype cross-reactive and may induce antibody-dependent enhancement. Antibodies against NS1 will cause complement-dependent lysis of the infected host cell (1).

(Fig. 6). The pre-M protein creates a heterodimer with the E proteins and is cleaved when the virion matured. The M protein is completely inaccessible for antibody binding because it is hidden beneath the E protein dimer. However, in some cases where incomplete cleavage of pre-M by furin results in partially mature/immature virion, this

can be recognized by M protein-specific antibodies. Yet, antibodies against the pre-M protein are very cross-reactive across the four different serotypes (1). The NS1 protein has a multimeric structure and it can be found displayed on the surface of the infected host cells or released as a soluble particulates. Although antibodies for NS1 are very serotype specific, this glycoprotein is not incorporated into the virion; leading scientists to conclude that ADE can be avoided by utilizing NS1 as the target epitope. In addition, antibodies against NS1 also cause complement-dependent lysis of the DENV infected host cell (1). The E glycoprotein is the main membrane surface component of DENV. In its tightly packed mature form the cross reactivity of the E protein is controlled by the dimeric conformation on the virion surface. Each virion is composed of ninety dimers tightly packed with an icosahedral symmetry, which limits the accessibility of epitopes to bind antibodies. It has been found that antibodies against the E protein after the first DENV infection are very serotype cross-reactive. These antibodies are also short-lived, since most of them are not represented in the memory immune response that protects the individual against the serotype that caused the prime infection. Structurally, it has been described three domains in each monomer of E. The fist two domains are not sequential and represent antibody epitopes with a high cross-reactive potential. However, domain III (DIII) of the E protein has the highest variable in amino acid sequence between the four different serotypes. Therefore, it has been observed that antibodies specifically against DIII of the E protein are highly serotype-specific.

In addition to ADE, there are multiple challenges that are faced in the production of a DENV vaccine. The mechanism of protective immune response against DENV is not completely understood (16). In addition, vaccine development is further halted due to the

lack of animal model for DENV. Normal mice infected with human DENV do not show measurable viremia or disease (17, 18). Therefore researchers have to use a mouse adapted strain of the virus or infect an immunocompromised mouse model (17). Some have argued the relevance of the results from these studies lacking a reliable animal model when applied to human infection.

Despite these challenges, there are multiple DENV vaccine platforms that have been pursued and some of which have been approved for clinical testing. These include live attenuated virus (LAV), purified inactivated virus (PIV), recombinant subunits, virus-like particles (VLPs), and plasmid/viral vectors. LAV vaccines make up the majority of vaccine candidates in clinical trials (Table 1). It has been shown to produce a large, long-lasting, and broad humoral and cellular immune response. DENV strain for LAV were generated by some research groups through serial passaging in tissue culture, and these were found to induce T cell and antibody responses that are similar to those caused by a natural infection (1). However, it has been difficult for LAV vaccines to achieve a balance between an optimal level of attenuation and a low level of reactogenicity (16, 17). The top 3 most developed DENV vaccines are the recombinant LAV from Sanofi Pasteur, the US National Institute of Health (NIH), and the US Centers for Disease Control and Prevention (CDC) – partnered with Inviragen (Fig. 7). The vaccine developed by Sanofi Pasteur utilized the attenuated vaccine strain 17D of the yellow fever virus (YFV) as the backbone of their recombinant virus. They replace the gene encoding pre-M and E proteins with those from each DENV serotypes (1). The vaccine developed by the NIH utilizes the attenuated version of DENV-1, 3, and 4 genome, the attenuating mutation can be found in the 3’ untranslated region (UTR).

However, they developed a recombinant LAV against DENV-2, they utilized the genome of DENV-4 as the backbone and replaced the pre-M and E gene with those of DENV-2. The vaccine developed by CDC-Inviragen utilized the DENV-2 genome as the backbone of their recombinant virus; replacing the pre-M and E gene with those of DENV-1, 3, and 4 (1).

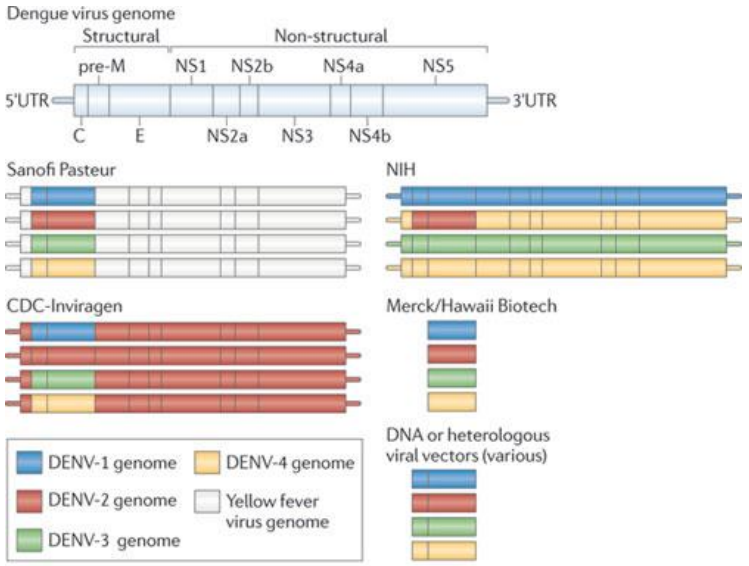

Figure 7. Diagram Representing the Different Approaches that has been done to Create a Viable Tetravalent Vaccine against DENV. At the top is the DENV RNA genome. The Sanofi Pasteur vaccine used the vaccine strain of yellow fever virus (YFV17D) as the backbone, replacing the pre-M and E gene with the respective pre-M and E gene from each DENV serotype. The NIH vaccine used attenuated DENV genome, as well as a replacing the pre-M and E gene of the DENV-4 genome with those of DENV-2. The CDC-Inviragen vaccine used attenuated DENV-2 genome as the backbone, replacing the pre-M and E gene with those of DENV-1, DENV-3, and DENV-4. The newer approach to creating a DENV vaccine is in using plasmids or viral vectors to only express the E and pre-M gene (1).

The results from the clinical trial for some of these vaccines found a problem seen in tetravalent vaccines, where the simultaneous vaccination with all 4 different serotypes caused an inter-serotype interference. The consequence was observed in viremic patients caused by one or two dominant serotype with higher viral replication fitness compared to the other serotypes. The newer approaches followed by some research groups in creating a vaccine against DENV are utilizing protein subunit vaccines, heterologous viral vectors, inactivated virion containing only structural proteins, or nucleic acid vaccines encoding pre-M and E proteins (1).

A different approach, the PIV vaccine, contains all of the viral structural protein and RNA, since it is an inactivated wild-type virus. The inclusion of structural proteins allows the induction of an immune response against them. Recombinant subunit vaccines allow for the targeting of a specific viral antigen to induce an immune response. Most subunit vaccines express truncated parts of the envelope protein. Various protein expression systems has been used such as bacterial, yeast, insect, mammalian cells, etc. (17). VLP vaccines are advantageous because it does not contain any replicative gene material, but it is able to express a high and repetitive amount of antigens on its membrane. Studies have shown that this increases the immunogenicity of the vaccine. DNA and virus-vectored vaccines is a great design for *in vivo* expression of antigens; most are in the form of VLPs. DNA vaccines are also advantageous in their thermo-stability, which causes it to be cost-efficient for vaccine storage and transport. Virus-vectored vaccine may induce immune response against both the expressed DENV antigen as well as the viral vector itself. The utilization of a well-known and well-characterized licensed vaccine has been established as a vaccine vector. Some concerns have arisen,

however, that pre-existing immunity against the viral vector itself may halt the immunogenicity of the virus-vectored DENV vaccines (17, 19). All of the vaccine designs against DENV have its advantages and disadvantages.

Type	Vaccine approach	Developer	Stage of Development
LAV, Tetravalent, Chimeric	Chimeric viruses YFV17D-DENV	Sanofi Pasteur	Phase III
LAV. Tetravalent, Chimeric	Chimeric DENV with 3' UTR deletion mutations (Δ30)	NIH/ NIAID	Phase I
LAV, Tetravalent, Chimeric	Empirically attenuated (passaged in tissue culture) Recombination using DENV-2 backbone and pre-M/E from DENV-1 to DENV-4	CDC-Inviragen	Phase II
LAV, Tetravalent	Empirically attenuated by passage in primary dog kidney cells (PDK)	WRAIR/GSK	Phase II
PIV, Tetravalent	Purified and inactivated DENV-1 to DENV-4	WRAIR	Phase I
PIV, Monovalent	Purified and inactivated DENV-1	WRAIR	Phase I
Recombinant Subunit	E protein of DENV, affinity purified, produced in insect cells	Hawaii Biotec/Merck	Phase I
Recombinant Subunit	DIII of the E protein fused to a carrier protein		Preclinical
DNA	Targeting NS1	Multiple	Preclinical
DNA Tetravalent	DENV-1 to DENV-4, pre-M and E proteins	NMRC	Phase I
DNA Tetravalent	DIII of the E protein from DENV-1 to DENV-4, synthetic consensus (SynCon^{TM}) Human codon optimized	Inovio	Precilinical
DNA Monovalent	DENV-1 pre-M and E proteins	NMRC	Phase I
DNA Shuffle	E protein from DENV-1 to DENV-4, codon optimized DNA shuffling, generating single chimeric antigen	NMRC/ Maxygen	Preclinical
Adenoviral Vector	Expression of the pre-M and E proteins from DENV-1 to DENV-4 in recombinant adenoviral vector	NMRC/GenPhar	Preclinical
Alphavirus replicon particles	Expression of the pre-M and E proteins or soluble E dimers from DENV-1 to DENV-4, in VRP	Global Vaccines	Preclinical

Table 1. Dengue Vaccine Candidates in Various Stages of Development (6).

1.7 Domain III as a Vaccine Target. The E protein of DENV forms a dimeric assembly of ninety subunits displayed in icosahedral symmetry. Each monomer contains three structural domains (Fig. 8): Domain I (DI), Domain II (DII) and Domain (DIII). DI of the E protein has an 8-stranded central β-barrel structure and two large loops that form the elongated DII, which has the highly conserved hydrophobic fusion peptide (20, 21). The C-terminal of DIII can fold independently from the other two domains in an immunoglobulin-like manner as a core protein, stabilized by a single conserved disulfide bridge (10). During the fusion transition, DIII undergoes the most significant displacement (20).

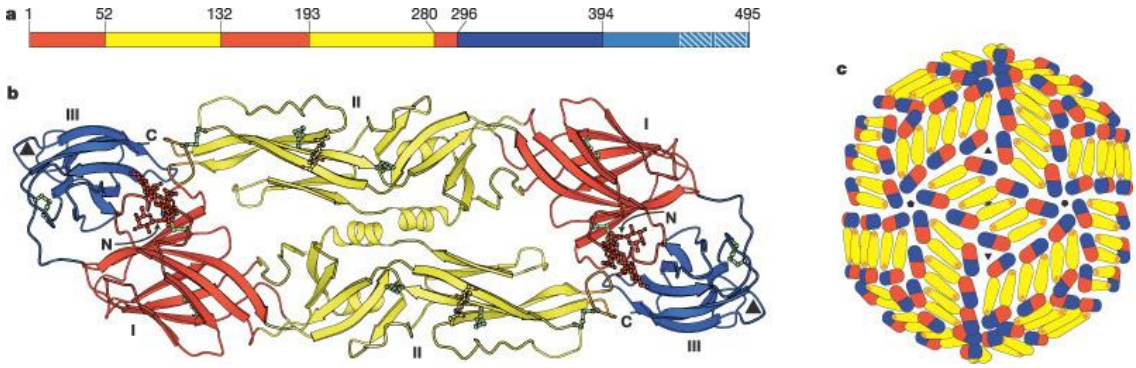

Figure 8. Structure of the Dengue E Protein with its Three Domains. A, The three domains of dengue E protein: DI (red), DII (yellow), and DIII (blue). **B,** Conformation of the E protein in the mature virus particle. **C,** Arrangement of each domain of the E protein on the surface of the mature virus particles; 90 dimers in an icosahedral lattice (21).

DIII of the envelope protein has been known to contain a cell surface receptor recognition site, and in turn it is important in host cell binding. Previous studies have observed that recombinant DIII from the E protein of DENV have inhibited viral infectivity through binding competition with the host receptor (20). This suggests that DIII contains a neutralizing type-specific and sub complex-specific epitopes. Anti-DIII antibodies that have been found to be potently neutralizing and type-specific, hence less cross-reactive with a lower risk of causing ADE (17, 19). Although DIII displays an

identical fold across all four different serotypes of DENV, it also has the most variable in amino acid sequences between the four serotypes (1, 20). Moreover, studies have shown that neutralizing serotype-specific antibodies against DENV focuses on DIII (22). Mutations to DIII residues have also lead to the decrease in the binding of neutralizing monoclonal antibodies (23). ***Therefore, for our project, we have decided to focus on DENV 2 E DIII as the antigenic epitope to target in creating our recombinant vaccine.***

1.8 Hepatitis B Virus-like Particles. Virus-like particles (VLPs) are multi-protein complexes that are similar in structure to complete viruses, but it does not contain a viral genome, making them non-infectious, thus safer (1). VLPs formation is completed through budding from the usual cellular mechanism, which allows it to be similar to the infective virus structure (24). VLPs have been used in production of vaccines in the pharmaceutical industry due to its ability to cause a good immune system response; VLPs has been shown to elicit both humoral and cellular immune response (25). This is partly due to their highly repetitive surface proteins that form its structure, which will induce the activation of the immune system. Two of the well-known vaccines that have been developed through the use of VLPs are human papilloma virus (HPV) and hepatitis B virus (HBV) (24). This was done using the hepatitis B virus small surface antigen (HBsAg) and the VLP produced by the recombinant L1 protein of HPV. These VLPs induce both neutralizing and protective immune responses and has been shown to be stable and safe. Hepatitis B virus creates surface antigens that make up the viral envelope protein. There are three different types of surface antigens that are produced: the large surface antigen (L), made up of preS1, preS2 and the S domain, the middle surface antigen (M), made up of the preS2 and the S domain, and the small surface antigen

(HBsAg), made up of only the S domain (26). The small surface antigen of hepatitis B virus (HBsAg) has been shown to be produced in the highest quantity. In addition, HBsAg have been observed to spontaneously assemble to create empty VLPs in the absence of any other viral. The VLPs created by HBsAg is made up of 100 to 150 subunits of the 226 amino acid of the HBsAg protein (26). Due to the small size of DIII of the E protein (~100 amino acids), expressing it by itself will not elicit a strong immune response; hence we will use chimeric VLPs as our vaccine platform (Fig. 9). Chimeric VLPs are an adaptation to the VLP design and structure, where the immunogenic antigens are incorporated onto their surface (25, 27). This will be done through the fusion of the target epitope to the envelope protein of a virus used to form the VLP. This process allows the expression of any specific epitope that is targeted by neutralizing antibodies to be expressed on a highly immunogenic VLP, which may act as an adjuvant and boosts the immune response (27, 28). As previously mentioned, purified HBsAg VLPs are used in the currently licensed vaccine against HBV. Antibody protection induced by this vaccine is mainly specific for the "a"-determinant of the viral envelope. Previous studies have shown the successful insertion of foreign sequences into the N-terminus of HBsAg without affecting the conformational epitopes of the "a"-determinant (26, 29, 30). Therefore, it is possible to create a chimeric HBsAg VLP to function as a bivalent immunogens that will induce immune response against HBV and the foreign epitope.

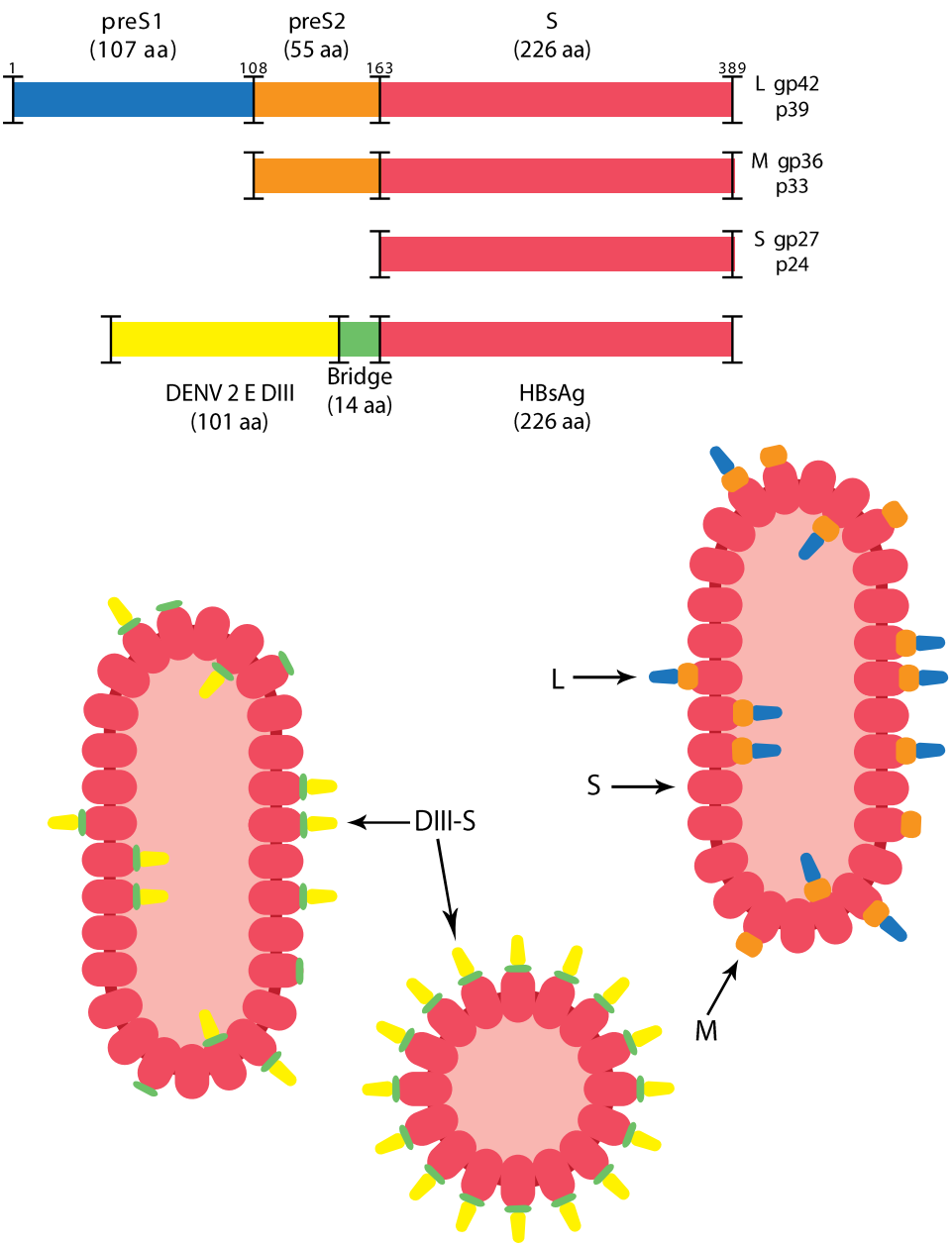

Figure 9. Illustration of the Chimeric VLPs Engineered to Express DIII and HBsAg. At the top is the representation of the Hepatitis B surface antigen coding sequence, breaking down the domains that make up the large, middle, or small surface antigens; with its respective preS1, preS2, and S domains. The last coding sequence represent the chimeric VLP, which includes the 101 amino acid of DIII fused to the N-terminus of the 226 amino acid of S domain, by a 14 amino acid long bridge. The bottom part of the diagram shows the native VLP produced by HBC which includes the L (blue), M (orange) and S (pink) antigens. To its left is the representation of the hybrid DIII-HBsAg VLPs, which includes the S antigen, the DIII (yellow) and the bridge (green). The chimeric VLP on the far left side is expected to be produced from the recombinant virus, MVvac2(DIII-S,S)P, and the chimeric VLP on the middle is expected to be produced by the recombinant virus MVvac2(DIII-S)N.

1.9 Measles Virus as Viral Vector. Measles virus (MV), a member of the *Paramyxoviridae* family, is an enveloped, negative-sense single-stranded RNA virus. The negative sense genome functions as template to generate sub-genomic mRNAs though a

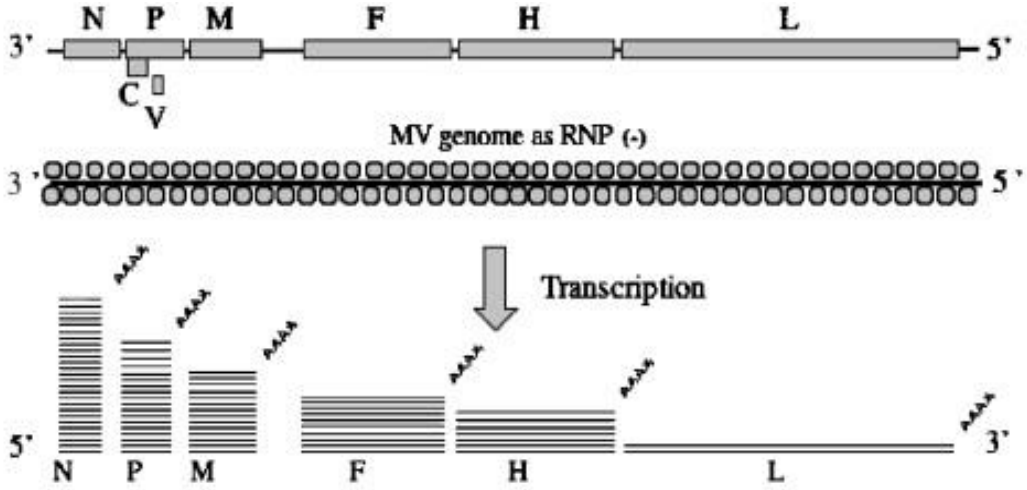

Figure 10. Schematic of the Transcription and Replication Mechanism of the Measles Virus Genome. The top image of the diagram depicts the genomic map of the measles virus genome, a negative sense, single-stranded RNA. The bottom image of the diagram shows the viral transcripts, 5'-3' mRNAs, which represent the amount of each mRNA produced due to the viral transcription gradient (32). This in turn will cause a gradient in the amount of proteins translated based on the location of the gene encoding that specific protein.

conserved mechanism (31). There are six cistrons coding for eight proteins in MV, which includes coding for: N, P/C/V, M, F, H, and L proteins (32, 33). The transcription mechanism of *mononegavirale*s posits that boundary signals between cistrons determine a gradient transcription from 3' to 5' extremes (Fig. 10). The gene closest to the 3' end (coding for N protein) is to be transcribed in higher amount and the gene towards the 5' end (coding for L protein) is to be transcribed in the least amount (31, 32).

The recognition of these sequences allows us to insert additional transcription units (ATUs) between native cistrons. Thus recombinant strain of MV can be created by various ATUs that may be placed strategically within the viral genome, generating a viral

vector that expresses foreign proteins (34). The locations of these ATUs determine the amount of protein that will be produced from that inserted gene (32). Theoretically, ATU sites cloned after the coding region of each structural protein can be generated. Practically, we have available in the laboratory ATUs cloned in N-P, P-M, H-L and L-trailer gene boundaries and some combinations; the presence of convenient restriction sites allows the straightforward cloning strategy.

There are several features that make MV an attractive option as viral vector. Its genome only replicates in the cytoplasm, which confirms that it will not integrate into the host's cell DNA, and the attenuated strain of MV has never reversed back to pathogenicity, pointing at the genetic stability of MV (33). On this regard, this project will use MVvac2 as the viral vector, which is the equivalent to the Moraten vaccine strain. The attenuated MV strain (Moraten) has been in use as a vaccine for almost fifty years with an excellent record of safety and efficacy. The Moraten vaccine strain was empirically attenuated from the Edmonston wild-type MV strain. It was passaged: 24 times in human kidney cells at 37ºC, 28 times in human amnion cells at 37ºC, 12 times in the intra-amniotic cavity of chick embryo at 37ºC, 16 times in chick embryo fibroblast at 37ºC, 8 times in chick embryo fibroblast at 36ºC, and then 40 times in chick embryo fibroblast in 32ºC (35, 36). The summation of these passaging resulted in many nucleotide substitutions in the MV genome which contributes to its attenuation; the specific substitution that caused the attenuation is still unknown (35).

After a single dose (Moraten strain) the vaccine confers life-long immunity, showing a robust immune response, both cell mediated and neutralizing antibodies (33, 37). In addition, a previous study on MV-vector vaccine expressing HIV envelope protein observed high-tittered antibodies against the expressed proteins in macaques previously immunized with MV. Thus, disputing the claim that revaccination with a MV-vectored vaccine in previously immunized individuals will reduce the immunogenicity of the recombinant vaccine. On the other hand, the long-term use of the vaccine strain makes a recombinant MV based on this vaccine vector to have an advantage economically; in which it can be produced on a large scale for a low cost using the production and distribution line for the current MV vaccine (33, 34). Various successful vaccines have been made through attenuated viruses, which include measles, mumps, polio, rubella, and yellow fever (37). It follows then that a recombinant MV as a viral vector will also be useful in presenting multiple viral antigens to the immune system. Therefore, we will use MV as a viral vector expressing our engineered hybrid DIII-HBsAg, to create the respective chimeric VLPs.

1.10 Research Project Objectives and Hypothesis. Since DIII correlates with the serotype-specific neutralizing epitopes, this research project centers on the development of a vaccine platform that will display this antigenic determinant on the surface of subviral HBsAg particles vectored by a recombinant MV. Thus, this chimeric VLP engineered from the hybrid DIII-HBsAg will be expressed as an additional transcription unit (ATU) inserted into the MV genome.

In this study we used HBsAg as a display scaffolding the DIII of DENV-2 that is only about 100 amino acids long. A previous study successfully expressed a repetitive

epitope from *Plasmodium falciparum* CS protein using an HBsAg as a carrier (38). Sixty-four amino acids of the CS protein were fused to the N-terminus of HBsAg, followed by 226 amino acids of the HBsAg itself. Our design followed this successful model by fusing the 101 amino acid of DIII of DENV-2 to the N-terminus of the 226 amino acid of HBsAg, using a 14 amino acid bridge (Fig. 9). Therefore, we only used the S domain of the HBV surface antigen fused with the DIII by the utilization of a disulfide bridge to generate DIII-HBsAg. This disulfide bridge will ensure the correct formation and independent folding for both DIII and HBsAg (Fig. 9). The generated artificial gene encoding for this protein was inserted into the MV genome so that the viral mechanism will drive the expression of this chimeric antigen. Following previous observation of HBsAg protein, the hybrid DIII-HBsAg is expected to self-assemble into a chimeric VLP. We hypothesize that the recombinant MVs will induce an immune response against MV, HBV, and DENV.

1.11 Specific Aims.

Aim 1: Generate a recombinant, infectious cDNA containing full-length measles virus genome with the co-linear HBsAg and hybrid DIII-HBsAg coding sequences. An overlapping PCR will be done in order to create the hybrid DIII-HBsAg coding sequence. The ATU containing this coding sequence will be inserted after the P cistron in MVvac2(DIII-S,S)P and after the N cistron in MVvac2(DIII-S)N. MVvac2(DIII-S,S)P will also have an ATU encoding HBsAg inserted after the N cistron. We expect that the insertion of the ATU will allow for the successful assembly of the hybrid VLP, with different quantity of DIII-S transcribed due to the location of the ATU in the MV genome; with or without the presence of native HBsAg.

Aim 2: Generation of recombinant viruses and analysis of their replication fitness. The infectious cDNA previously generated will be used to rescue the recombinant viruses following an established reverse genetic system. We expect to successfully rescue the recombinant MVs, which will follow a similar replication pattern as the parental strain, MVvac2.

Aim 3: Biochemical characterization of MVvac2(DIII-S,S)P and MVvac2(DIII-S)N. Biochemical tests done to characterize the recombinant MVs will analyze the expression of the hybrid DIII-S antigen and the correct formation of the hybrid VLP. We expect the DIII antigen to be displayed on the surface of the hybrid VLP, which will be successfully self-assembled, following the same characteristics as the native HBsAg VLP.

Aim 4: Immunogenicity study of recombinant viruses in measles-susceptible small animal model. Animal experiments will be done to analyze the immunogenicity of the recombinant MVs compared to the reference and parental strains. We hypothesize that the animal sera collected will contain neutralizing antibodies against measles, hepatitis B and dengue virus.

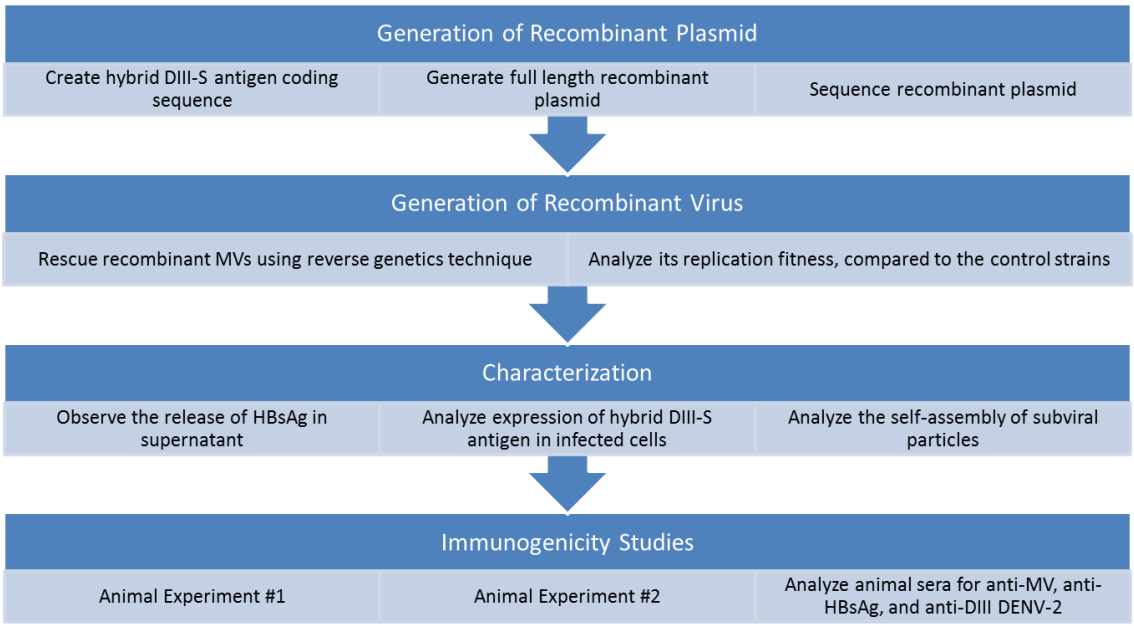

Table 2. Outline of the Specific Steps Completed to Reach the Research Aims.

Chapter 2. Materials and Methods

2.1 Cells and Viruses. Vero/hSLAM cell line (African green monkey cells which expresses the MV receptor, SLAM, on its surface) was used for experiments done with MV and recombinant MVs. These cells, courtesy of Yusuke Yanagi, were sustained with DMEM 5% FBS P/S, containing: Dulbecco's Modified Eagles's Medium with high glucose concentration (DMEM, Sigma-Aldrich, St. Louis, MO), 5% Fetal Bovine Serum (FBS, Atlanta Biologicals, Flowery Branch, GA), and 1% Penicillin-Streptomycin (P/S, Sigma-Aldrich, St. Louis, MO). To promote selection for the expression of SLAM, these cells were also supplemented with 0.5 mg/ml of G418 (Enzo Life Sciences, Farmingdale, NY). Helper 293-3-46 cell line, a derivative of HEK 293 cells, was used to rescue recombinant MVs. These cells, courtesy of Roberto Cattaneo, were sustained with DMEM 10% FBS P/S, and supplemented with 1.2 mg/ml of G418. BHK-21 cell line (Baby Hamster Kidney Fibroblast Cells) was used for experiments done with DENV-2. These cells were sustained with DMEM 10% FBS P/S when passaged for stock, but maintained in 50/50 DMEM 5% and DMEM 10% when seeded for experimental purposes.

Viral stocks for MV and recombinant MVs were prepared by infecting Vero/hSLAM cell monolayer with a multiplicity of infection (MOI) of 0.03 and incubated at 37^{o}C while being monitored for cytopathic effect. Infected cells were collected in Opti-MEM (Gibco by Life Technologies, Grand Island, NY) when it reached ~80% cytopathic effect. Cells collected were lysed by two cycles of freeze and thaw, and then centrifuged to clear the supernatants, of which small aliquots will be prepared from. Viral aliquots are titrated and stored at -80ºC. Viral stocks for DENV-2 (ATCC, Strain

16681) were prepared by infecting BHK-21 cell monolayer with a MOI of 1, and incubated at 37ºC for a specific amount of time, 5 days. Supernatant was then collected and cleared when necessary, small aliquots are made, which are then titrated and stored at -80ºC. Opti-MEM is used to replace the media during infection for MV as wells as DENV-2. Two viruses were used as controls: the parental strain, MVvac2 and the reference strain, MVvac2(HBsAg)N (39). The (HBsAg)N control strain contains an HBsAg ATU after the N cistron. In one animal experiment, DENV-2 was also used as a control.

2.2 Plasmid Design and Construction. Primers were designed for construction of an artificial gene expressing a fusion glycoprotein composed of the Domain III (Dengue 2) fused to HBsAg (Table 3). Primer 1 is designed as the forward primer to amplify DIII, it includes the coding sequence for the signal peptide of the light immunoglobulin chain. Primer 2 is the reverse primer to amplify DIII; it includes the partial formation of the coding sequence of an amino acid bridge composed of $(SG_3)_3$. Primer 3 is the forward primer to amplify HBsAg; it also includes a part of the aforementioned bridge. Primer 2 and primer 3 complementary sequence of the bridge aid in the overlapping PCR of the two gene sequences. Primer 4 is the reverse primer to amplify HBsAg and was also used as the reverse primer to amplify the final product DIII and HBsAg, in a similar way primer 5 is the forward primer that was used for the final amplification of the fused DIII-HBsAg coding sequence.

	Sequence	Gene Amplified
1	5’ATTCACGCGTATGGACATGCGGGCCCCCGCCCAGATCTTCGGCTTCCTCCTCCTCCTCTTCCCCGGCACCCGGTGCCAGCTCAAAGGAATGTCA-3’	**Domain III**
2	5’*TGATCCTCCTCCTGATCCTCCTCCTGAGCTAGC*CTTAAACCAGTTGAGCTTC3’	**Domain III-Bridge**
3	5’*GGAGGAGGATCAGGAGGAGGATCAGGAGGAGGA*ATGGAGAACATCACATCAG3’	**Bridge-HBsAg**
4	5’GACGTCTTAAATGTATACCCAAAGACA3’	**HBsAg**
5	5’ATTCACGCGTATGGACATGC3’	**Overlapping**

Table 3. Primer Sequences for Overlapping PCR to Construct the Hybrid DIII-HBsAg Coding Sequence. The italicized sequence primer 2 and primer 3 indicate the coding sequence for the bridge (15 amino acids) that was supplemented to link Domain III and HBsAg together.

Overlapping PCR was done to generate the hybrid DIII-S antigen coding sequence. First, the coding sequence of the DIII region of the envelope protein of DENV-2 (strain 16681, pD2/IC-30P-A) was amplified by RT-PCR. This amplicon is flanked by a MluI restriction site at the 5’ end and the coding sequence for the light immunoglobulin chain signal peptide as well as the coding sequence of a 15 amino acid bridge at the 3’ end. Second, the coding sequence of HBsAg from pB(+)MVvac2(HBsAg)N was amplified by PCR. This amplicon is flanked by a complementary region to the 15 amino acid bridge at the 5’ end and by an AatII restriction site at the 3’ end. Both fragments were spliced together by overlapping PCR and the product was cloned into the shuttle vector pJET 1.2.

In the first plasmid, pB(+)MVvac2(DIII-S,S)P, the DIII-S insert was cloned into the ATU located after the P protein (Fig. 14). The vector used as the backbone for this plasmid was a previously cloned pB(+)MVvac2(HBsAg)N (39). Therefore the first plasmid contains two ATUs, HBsAg coding sequence downstream of the N cistron and the hybrid DIII-HBsAg coding sequence downstream of the P cistron. To generate the

first plasmid, a shuttle vector pUC19 was digested at SbfI and KasI restriction site; producing a 2.4 kb vector. The insert was also digested with SbfI and KasI restriction enzymes, coming from pB(+)MVvac2(HBsAg)P, producing a 4.8 kb insert. The ligation of these products resulted in pUC(HBsAg)P, which was confirmed by a restriction digest with DraI. The HBsAg coding sequence cloned in the shuttle plasmid was then swapped by the hybrid DIII-HBsAg coding sequence using MluI and AatII digestion; producing a ~7 kb vector and ~600 bp insert. This ligation of this vector and insert resulted in pUC(DIII-S)P, which was confirmed by a restriction digest with PvuI. Then, the SbfI and KasI restriction fragment from pUC(DIII-S)P containing the hybrid DIII-S coding sequence downstream of the MV P cistron substituted the corresponding fragment in pB(+)MVvac2(HBsAg)N. This restriction digest produced a 17 kb vector and 4 kb insert (Fig. 16. A). The ligations of these digest products then resulted in the final full-length plasmid pB(+)MVvac2(DIII-S,S)P, which was confirmed by a restriction digest with HindIII (Fig. 16. B). In addition, the full-length plasmid was also sent for DNA sequencing, using the appropriate forward and reverse primers for MV sequencing the ligation boundaries, shown in table 4.

The second plasmid, pB(+)MVvac2(DIII-S)N was constructed using a vector that is pB(+)MVvac2(HBsAg)N. The insert of the HBsAg gene sequence at the N position is then swapped with the DIII-S insert. Therefore this plasmid only has one ATU where the HBsAg gene sequence is fused to DIII. The vector, pB(+)MVvac2(HBsAg)N and the insert from pUC(DIII-S)P were both digested with MluI and AatII, producing a 20 kb vector and a 1 kb insert. The ligations of these digest products then resulted in the final full-length plasmid pB(+)MVvac2(DIII-S)N, which was confirmed by a restriction digest

with HindIII (Fig. 15. A). This full-length plasmid was also sent for DNA sequencing, using the appropriate forward and reverse primers for MV, shown in table 4.

Plasmid Sequenced	DNA Sequencing Primers	Name
pB(+)MVvac2(DIII-S,S)P	5'-AGCTGCTGAAGGAATTTC-3'	**3101F**
pB(+)MVvac2(DIII-S,S)P	5'-AGCCTGCCATCACTGTA-3'	**3548R**
pB(+)MVvac2(DIII-S)N	5'-GGCAAGAGATGGTAAGG-3'	**1201F**
pB(+)MVvac2(DIII-S)N	5'-GTTGTCTGATATTTCTGAC-3'	**1939R**

Table 4. Primer Sequences Used in DNA Sequencing of Full-length Recombinant Plasmid. To sequence pB(+)MVvac2(DIII-S,S)P, we used 3101F forward primer and 3548R reverse primer, which means that the 3101-3578 nucleotides of the recombinant plasmid was sequenced. To sequence pB(+)MVvac2(DIII-S)N, we used 1201F forward primer and 1939R reverse primer, which means that the 1201-1939 nucleotides of the recombinant plasmid was sequenced.

2.3 MV Reverse Genetics System. A Measles Virus reverse genetics technique was used to rescue the recombinant MV (Fig. 17) following the method established by Radecke et al. modified by Parks et al. (36, 40). Day 1: Cell culture (293-3-46) was seeded in a T75. After the T75 flask has become confluent, cells were split into 2 plates (6-wells), using PBS and trypsin (Mediatech) in a 3:1 dilution. Done using 13 mL of DMEM-10%FBS and plating 1 mL of cell per well plus 1 mL of fresh DMEM-10%FBS. Incubated for 3-5 hours (until cells are attached). Transfection of cell line was done through calcium phosphate precipitation using a ProFection kit (Promega, Madison, WI) with two plasmids, the specific MV full length genome (Recombinant MV) and the pEMC-La (coding for the MV polymerase protein, courtesy of R. Cattaneo). Day 2: The transfected cells were heat-shocked for 3 hours at 42ºC. Day 3 and 4: Vero/hSLAM cells were seeded in P100 plates. After cell attachments, the rescue cells were overlaid on the Vero/hSLAM cells to recover MV infectivity on them. The appearance of cytopathic effects was monitored in the following days. Once syncytia are observed, single isolated syncytia can be picked and propagated on Vero/hSLAM cell line.

2.4 Multi-step Growth Kinetic Analysis of Recombinant MVs. Multiple-step growth kinetic was performed to document the replication fitness of recombinant MVs, MVvac2(DIII-S,S)P and MVvac2(DIII-S)N. Vero/hSLAM cells ($5x10^5$ cells/well) were infected with a multiplicity of infection (MOI) of 0.03, in a 6-wells plate incubated at 37ºC. Cells were scraped in 1 mL of Opti-MEM for the intracellular sample and supernatant for the extracellular sample was collected at 12, 24, 36, 48, 72, and 96 hours post-infection. Intracellular sample was lysed by a single freeze-thaw cycle at the 50% tissue culture infectious dose ($TCID_{50}$) was assessed in Vero/hSLAM cells using the Spearman-Kärber end-point dilution method to verify the efficiency of the production of MV recombinant particles (41). The controls used were the parental strain, MVvac2, and the reference strain, MVvac2(HBsAg)N.

2.5 Titration of Viruses. Titration of MV and recombinant MVs was done by assessing its $TCID_{50}$. Vero/hSLAM cells were seeded into 96-well plates with $2x10^4$ cells and 200 µl of DMEM 5% FBS P/S, per well. Plates were incubated at 37ºC for 1 hour. A ten-fold serial dilution is then done in a separate 96-well plate, by using 270 µl of Opti-MEM and 30 µl of the viral prep; the serial dilution is done 12 times. The dilution is then transferred by adding 30 µl of each dilution in 8 repeats (8 rows of the 96-well plate). The plate is then incubated at 37ºC and read 3 days later. The $TCID_{50}$ was calculated using the Spearman-Kärber end-point dilution method (41).

Titration of DENV-2 was done by a plaque assay using BHK-21 cells. A 24-well plate was seeded with $1x10^5$ cells and 500 µl of 250 µl DMEM 5% and 250 µl DMEM 10% FBS P/S. Plate was incubated at 37ºC for at least 4 hours to let the cells attach. Next, 8 series of ten-fold serial dilution is done by using 900 µl of Opti-MEM and 100 µl

of the viral prep. The dilutions is incubated at 37ºC for 1 hour and then 100 µl of each dilutions is added to the 24-well plate seeded with BHK-21 cells; this is done in duplicate. The 24-well plate is incubated at 37ºC for 4 hours, to allow for viral infection. Then 500 µl of overlay media is added to each well, which encourages the formation of isolate plaques. The overlay media contains: 3% carboxymethyl cellulose (CMC) made in 100 ml of Nanopure H_2o with the addition of 3 g of CMC (Sigma-Aldrich, St. Louis, MO), 20 ml of FBS, 2 ml of 200mM L-glutamine (Gibco by Life Technologies, Grand Island, NY), 200 ml of Modified Eagle's Medium 2x (MEM, Gibco by Life Technologies, Grand Island, NY), and P/S (Mediatech). Plates were then incubated at 37ºC for 5 days. Plates are developed by adding 500 µl of Naphtol Blue Black (NBB) to each well. NBB contains: 1 g of NBB (Sigma-Aldrich), 13.6 g of Sodium Acetate (Sigma-Aldrich), 60 ml of Glacial Acetic Acid (Sigma-Aldrich), and Nanopure H_2o up to 1000 ml. NBB is allowed to stain the cell for about 1 hour, then washed off by immersion of the plates in water.

2.6 Expression of HBsAg. A test using the Abnova HBsAg enzyme-linked immunosorbent assay (ELISA) kit (Novus Biologicals, Littleton, CO) was performed to observe the production of HBsAg by the recombinant MV. Using the supernatant collected from, 6-well plates seeded with $5x10^5$ Vero/hSLAM cells, infected with a MOI of 0.03 of the recombinant strain, MVvac2(DIII-S,S)P or the reference strain as a control, MVvac2(HBsAg)N. The supernatant was collected at 24, 48, 72 and 96 hours post-infection. These supernatants were used in the HBsAg ELISA compared to a commercial HBsAg standards and the included negative and positive control from the ELISA kit.

2.7 Preparation of Protein Extracts. To analyze the protein produced by the recombinant MVs, protein extracts must be prepared to analyze through a western blot. $1x10^6$ Vero/hSLAM cells were seeded in 100-mm-diameter (p100) dishes and incubated at 37ºC for 2-3 hours to attach. The cells were infected with a MOI of 0.3 with one of the viruses of mock-infected (Non-infected sample). During infection, media was aspirated, and virus was added mixed in 6 ml of Opti-MEM, then incubated at 37ºC. Infection was stopped 2 hours later by aspirating the Opti-MEM/virus and replacing the DMEM 5% FBS P/S. Cell monolayer was monitored and collected when about 80% cytopathic effect has been reached. To collect the protein extract, the media from the plate was aspirated and the plate was washed with phosphate-buffered saline (PBS) 3 times. Then cells are lysed by adding 500µL of RSB-NP40 buffer (1.5 mM of $MgCl_2$, 10 mM of Tris-HCl [pH 7.5], 10 mM of NaCl, and 1% Nonidet p-40, Sigma-Aldrich, St. Louis, MO) plus protease inhibitors (cOmplete Mini Protease Inhibitor Tablets, Roche Diagnostics, Manheim, Germany). The sample was then centrifuged at 4ºC for 20 minutes at 14,000 RPM. The supernatant containing the protein was then prepared by the addition of 6x SDS Page loading buffer (Laemmli buffer, Bio-Rad Laboratories, Hercules, CA) and denatured at 96ºC for 10 minutes. Samples stored in -30ºC if not directly used.

2.8 Analysis of Protein Expression. The samples of protein extracts were separate by SDS polyacrylamide gel electrophoresis (PAGE) in a 10% acrylamide gel. After the gel has been run, the protein were then transferred to nitrocellulose for immunoblotting using a 1:1000 dilution of a rabbit polyclonal anti-HBsAg antibody coupled to horseradish peroxidase (HRP, OBT0990P, AbD Serotec, Raleigh, North Carolina, USA), and a 1:500 dilution of a mouse monoclonal anti-DIII DENV-2

(GTX29202, GeneTex, Irvine, California, USA). The blot was incubated in the antibody, anti-HBsAg-HRP, for 2 hours; a secondary is not necessary because the primary antibody is already conjugated to HRP. This blot was developed using a chemiluminescence kit (SuperSignal West Pico Chemiluminescent Substrate, Pierce Biotechnology, Rockford, IL). When incubating with anti-DIII DENV-2, the blot was incubated for 3 hours with the primary antibody. Then incubated with the anti-mouse HRP-conjugated secondary antibody (GE Healthcare, Little Chalfont, United Kingdom) for 1 hour. Similarly, this blot was developed using the chemiluminescence kit. Membranes incubated with MV Anti-N were incubated with a primary antibody of rabbit polyclonal Anti-N 1:10,000 (provided by R. Cattaneo) for 1 hour and a secondary antibody of goat Anti-Rabbit-Alkaline Phosphatase conjugated (Anti-Rabbit-AP, Thermo Fisher Scientific, Anthem, AZ) 1:1000 for another 1 hour. The membrane was developed by visualizing the AP reactivity using Western Blue Stabilized Substrate for Alkaline Phosphatase Reagent (Promega, Madison, WI).

2.9 Particle Isolation and Determination of Density. HBsAg particles isolation was done using a discontinuous sucrose gradient of various concentrations: 60%, 50%, 40%, 30%, and 20% (Fig. 11). To purify the released particles, supernatants from $5x10^6$ Vero/hSLAM cells infected with either MVvac2, MVvac2(HBsAg)N, MVvac2(DIII-S,S)P or MVvac2(DIII-S)N were collected and clarified by centrifugation in 4ºC at 5,000 RPM for 15 min in an SX4750 rotor. 35 ml of the clarified supernatants were pelleted by ultracentrifugation in 4ºC at 45,000 RPM for 18 hours in an SW28 rotor. The supernatant was aspirated and the pellets were re-suspended in 1 ml of TNE buffer (10 mM Tris [pH

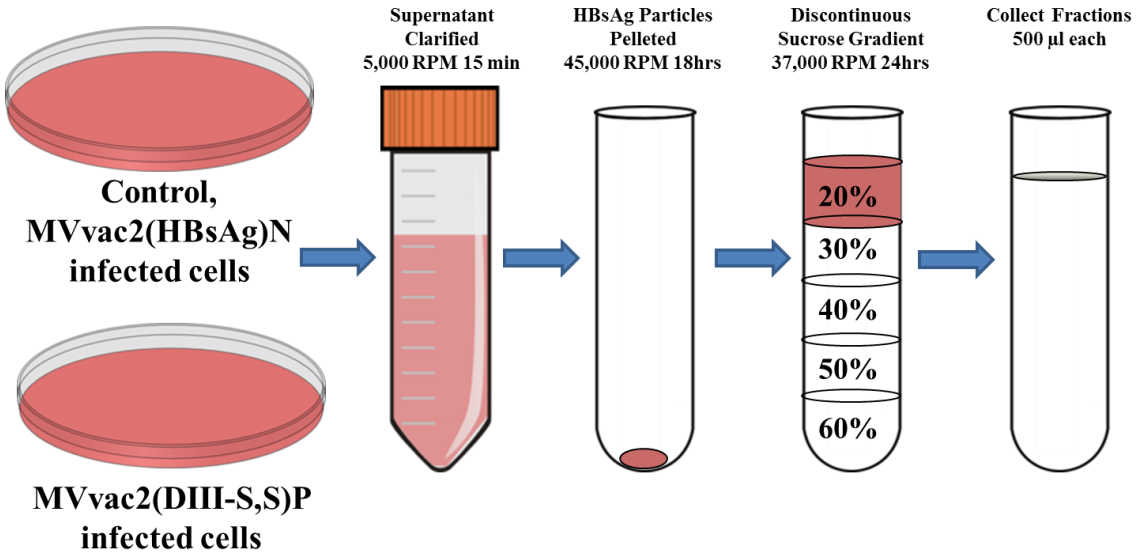

Figure 11. Diagram of the Ultracentrifugation of the Discontinuous Sucrose Gradient to Isolate VLPs. Monolayers of Vero/hSLAM cells were infected with recombinant MVs or controls. Supernatants from infected cells were collected and clarified in 4ºC at 5,000 RPM for 15 min. Clarified supernatant was collected and 35 ml was placed in an ultracentrifugation tubes. This was ultracentrifuged in 4ºC at 45,000 RPM for 18 hrs. Supernatant was aspirated and the pellet containing the VLPs was re-suspended with 1 ml of TNE buffer containing 20% of sucrose. 5ml ultracentrifugation tubes were prepared with the discontinuous sucrose gradient, including: 1 ml of each TNE buffer containing the sucrose concentration of 60%, 50%, 40%, and 30%, from the bottom to the top of the tube respectively. The 1 ml of re-suspended pellet is added on top of the 30% layer. This was ultracentrifugated in 4ºC at 37,000 RPM for 24 hrs. Then nine fractions of 500 µl, were collected from the top to bottom of the equilibrated tube.

7.8], 100 mM NaCl, 1mM EDTA) containing 20% sucrose. The TNE Buffer was used to create a 60% sucrose solution that was further diluted to produce 50%, 40%, 30% and the 20% sucrose solutions. Polyallomer centrifuge tubes (5 ml Centrifuge Tubes, Beckman Coulter, Brea, CA) were prepared with the discontinuous sucrose gradient, containing 60%, 50%, 40%, and 30% sucrose solution (1 ml each from the bottom respectively). The 1 ml of 20% sucrose solution containing the re-suspended pellet is added as the top layer. The tubes of fractions were ultracentrifugated in 4ºC at 37,000 RPM for 24 hours in a MLS50 rotor. To determine particle density and content, 500 µL fractions were taken from the top to bottom layers. Fractions were weighed and their density calculated. 25 µl aliquots of these fractions were taken and mixed 1:1 with SDS PAGE loading buffer. Samples were prepared to be run in a SDS PAGE, denatured at 96ºC for 10 min. Western blots containing these fractions were developed by various antibodies (Fig. 22).

2.10 Animal Experiments. Two animal experiments were performed for this project. The Arizona State University Institutional Animal Care and Use Committee sanctioned all experimental procedure. The first animal experiment was performed on HDD-SLAM-IFNar^{KO} transgenic mice. These mice express one of the human MV receptor, hSLAM, in a type I interferon deficient environment, which will allow for MV replication. These mice are also H2K^{b} and D^{b} knock out, which inhibits the mice MHC class I allele presentation restriction. In addition, these transgenic mice also presents MHC class I antigenic peptides with a HLA A2.1 restriction, the most common human MHC class I allele, using an artificial allele termed HHD, which contains the human β2 microglobulin fused to the HLA-A2.1 molecule where the α3 domain was substituted by the homologous region of murine origin. With this arrangement, the HHD molecule is

functionally independent of murine macroglobulin for expression and display and recognized by murine CD8+ T lymphocytes.

Groups of 2 to 5 transgenic mice were vaccinated by the intraperitoneal (i.p) route with three doses of $5x10^5$ $TCID_{50}$, including: 4 animals vaccinated with the parental strain, MVvac2, 5 animals vaccinated with the reference strain, MVvac2(HBsAg)N, and 5 animals vaccinated with the recombinant strain, MVvac2(DIII-S,S)P. Two animals had a vaccination schedule of three doses of $1x10^5$ PFU of the control, DENV-2. However, these 2 animals in actuality received only $5x10^2$ PFU doses. All of the animals were vaccinated at three different times, at day 0, 1 and 14 (Fig. 12). Then all of the animals were euthanized and exsanguinated 28 days after the last vaccination. Sera collected from immunized animals were separated and heat-inactivated at 56ºC for 1 hour. Aliquots of heat-inactivated sera were stored in -30ºC until analyzed.

The second animal experiment was performed on huCD46Ge-IFNar^{KO} transgenic mice. These mice express a different MV receptor, CD46, with human-like distribution.

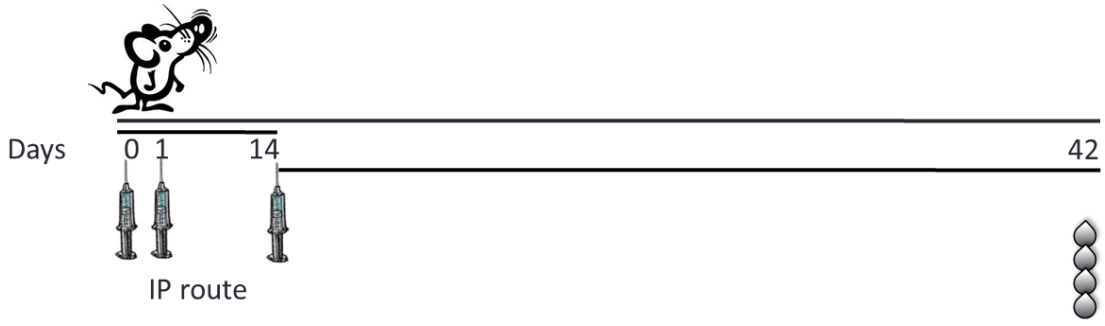

Figure 12. Diagram Representing the Vaccination Schedule Given to the HDD-SLAM-IFNar^{KO} Transgenic Mice in the First Animal Experiment. In the first animal experiment, the animals received three doses at day 0, 1, and 14. Day 0 was given to induce the expression of the MV receptor, SLAM, since it is only expressed in activated lymphocytes. Day 1 is the first dose given to the animals, followed by the booster second dose 14 days later. All animals were exsanguinated 28 days after their last vaccination. Animal sera were isolated and decomplemented. (Syringe – dose given, Blood – Mice

They also lacked of type I interferon pathway, which will allow for MV replication. These transgenic mice represent the gold standard for small animal models testing measles vaccine immunogenicity. These groups of mice were also vaccinated through the i.p. route, with one or two doses of $1x10^5$ $TCID_{50}$, including: 5 animals vaccinated with one dose of the parental strain, MVvac2 x1, 3 animals vaccinated with two doses of the reference strain, MVvac2(HBsAg)N x2, 4 animals vaccinated with one dose of the recombinant strain, MVvac2(DIII-S,S)P x1, 7 animals vaccinated with two doses of the same recombinant strain, MVvac2(DIII-S,S)P x2, and 3 animals vaccinated with two doses of the second recombinant strain, MVvac2(DIII-S)N x1. The first dose for all of the animals was given on day 0. Then animals scheduled to only receive one dose of their vaccine were exsanguinated 28 days after the first done, whereas the animals schedule to receive two doses gets their booster vaccine (Fig. 13). The rest of the animals were exsanguinated 14 days after the second dose was given. Sera collected from immunized

Figure 13. Diagram Representing the Vaccination Schedule Given to the huCD46Ge-IFNar^{KO} Transgenic Mice in the Second Animal Experiment. In the second animal experiment, the animals received either one or two doses of their respective vaccine. The first dose was given on day 0. Then, 28 days after the first dose, those animals with a vaccination schedule of only 1 dose were exsanguinated: MVvac2 x1 and MVvac2(DIII-S,S)P x1; whereas those animals with a vaccination schedule of two doses, received their second dose: MVvac2(HBsAg)N x2, MVvac2(DIII-S,S)P x2, and MVvac2(DIII-S)N x2. Then, 14 days after the second dose, the rest of the animals were exsanguinated. Animal sera were isolated and decomplemented. (Syringe – dose given, Blood – Mice Exsanguinated.

animals were separated and heat-inactivated at 56ºC for 1 hour. Aliquots of heat-inactivated sera were stored in -30ºC until analyzed.

2.11 Analysis of the Immune Response of Vaccinated Animals. The sera collected from each immunized mice were analyzed for its immunogenicity against all three viruses: measles, hepatitis B, and dengue 2. To determine MV neutralization titer, we performed the same experiment for sera collected from the first and second animal experiments. We performed serial two-fold dilutions of the sera in Opti-MEM and incubated with the addition of 100 $TCID_{50}$ of MVvac2 at 37ºC for 1 hour. To the sera-virus mixtures, $1x10^6$ Vero/hSLAM cells were added for every 96-well plate. These plates were then incubated at 37ºC for three days. Cytopathic effect of MV was monitored, and the neutralization titer was determined to be the highest dilution of serum that is still capable to cause complete neutralization of MVvac2 infectivity. Neutralization titer was recorded from an average of this experiment done in triplicates.

To determine the anti-HBsAg titer in both animal experiments, we used a commercial species-independent quantitative ELISA kit (Alpha Diagnostic Intl. Inc., San Antonio, Texas, USA). The anti-HBsAg titer was recorded in milli-international units per milliliter by comparison with the standards from the World Health Organization that was supplied by the manufacturer; these standards were included in the concentrations of: 20, 40, 80, and 160 mIU/ml. The anti-HBsAg antibody titer was recorded from an average of two readings for each sample done in this experiment.

The immunogenicity studies done against DENV-2 were done different in the first animal experiment compared to the second animal experiment. In the first animal experiment, a homemade ELISA was performed to test the reactivity of the collected sera

against DENV-2. This ELISA, however, was not quantitative. On a 96-well plate, whole DENV-2 was adsorbed to the bottom of the well overnight at 4ºC; each well contained 10^3 PFU of DENV-2 in 100 µl of PBS TWEEN 20 0.05%. Next, all mixture in each well is aspirated and 150 µl of the blocking solution, PBS TWEEN 20 0.05% containing 5% milk (Biorad, Bio-Rad Laboratories, Hercules, CA); then incubated at 37ºC for 1 hour. Wells were washed three times with PBS TWEEN 20 0.05%. Sera samples diluted 1:50 in 100 µl were added, and plates were again incubated at 37ºC for 1 hour; then wells were washed three times. An anti-mouse antibody added (1:2500), 100 µl to each well, then plates were incubated for at 37ºC for 30 min. Wells were again washed three times, and developed with HRP and 3M TMB-H_2SO_4 (tetramethylbenzemidine, Sigma-Aldrich, St. Louis, MO). The plates were then read at OD_{450}.

In the second animal experiment, the immunogenicity study against DENV-2 was done in two different approaches. In the first experiment, 10^5 PFU of DENV-2 were mixed with heat-inactivated sera that are dilution 1:5 in 200 µl of Opti-MEM, and incubated at 37ºC for 1 hour. Infectivity of DENV-2 remaining after sera neutralization was determined through a DENV-2 plaque assay in BHK-21 cells. In 96-well plate, ten-fold serial dilutions of the virus/sera mixtures were done using Opti-MEM. A 24-well plate seeded with $1x10^5$ cells per well in 500 µl of 50/50 DMEM 5% and DMEM 10% FBS P/S was incubated at 37ºC for 2-4 hours to allow the cells to attach. Dilutions done in Opti-MEM was then added to the 24-well plate, 100 µl of each dilution, and incubated at 37ºC for 4 hours. Next, 500 µl of the overlay media, 3% CMC, was added to each well, and these plates were incubated at 37ºC for 5 days. The plates were developed by aspirating each well and adding 500 µl of NBB, which fixed and stained the monolayer

of cells with acetic acid/napthol blue-black. The neutralization index was recorded as the logarithmic difference between the average DENV-2 titers when incubated with the control sera: sera collected from animals vaccinated with MVvac2, MVvac2(HBsAg)N, and previously obtained pre-immune sera; and the average DENV-2 titers when incubated with the experimental sera: sera collected from animals vaccinated with MVvac2(DIII-S,S)P and MVvac2(DIII-S)N. The determined anti-DENV-2 neutralization index is recorded on as $\log_{10}$NI. The second experiment performed to analyze the sera's immunogenicity against DENV-2 was the classis 50% plaque reduction neutralization assay ($PRNT_{50}$) using pooled sera from each experimental group of mice. 200 PFU of DENV-2 were mixed with a series of two-fold serial dilutions (1:5 to 1:320) of each pooled sera. This virus/sera mixture was incubated at 37ºC for 1 hour. Infectivity of DENV-2 remaining after sera neutralization was determined through a DENV-2 plaque assay in BHK-21 cells as described for the previous experiment. The neutralization titer was determined at the dilution that had a 50% reduction in the amount of plaques formed, compared to the control samples.

Chapter 3. Results

3.1 Plasmid Construction and MV Rescue. For this project, I aimed to generate two types of recombinant plasmids, the full length infectious cDNA plasmids are termed pB(+)MVvac2(DIII-S,S)P and pB(+)Mvvac2(DIII-S)N. The basic chimeric gene that all of these recombinants have in common is composed by the fusion of four elements (Fig. 14). At the amino terminus is the light immunoglobulin chain signal peptide, which is a stretch of 22 amino acids peptide (MDMRAPAQIFGFLLLLFPGTRC), to direct the synthesis of our chimeric protein to the endoplasmic reticulum. Then followed by 101 amino acids of the DIII of the DENV-2 envelope protein, a 14 amino acid bridge that is composed of the sequence $(SG_3)_3$ with a predicted α-helical structure. This bridge is important in allowing the independent folding of the DIII and HBsAg coding sequence that is located at the carboxyl terminus of this construction. The hybrid glycoprotein is

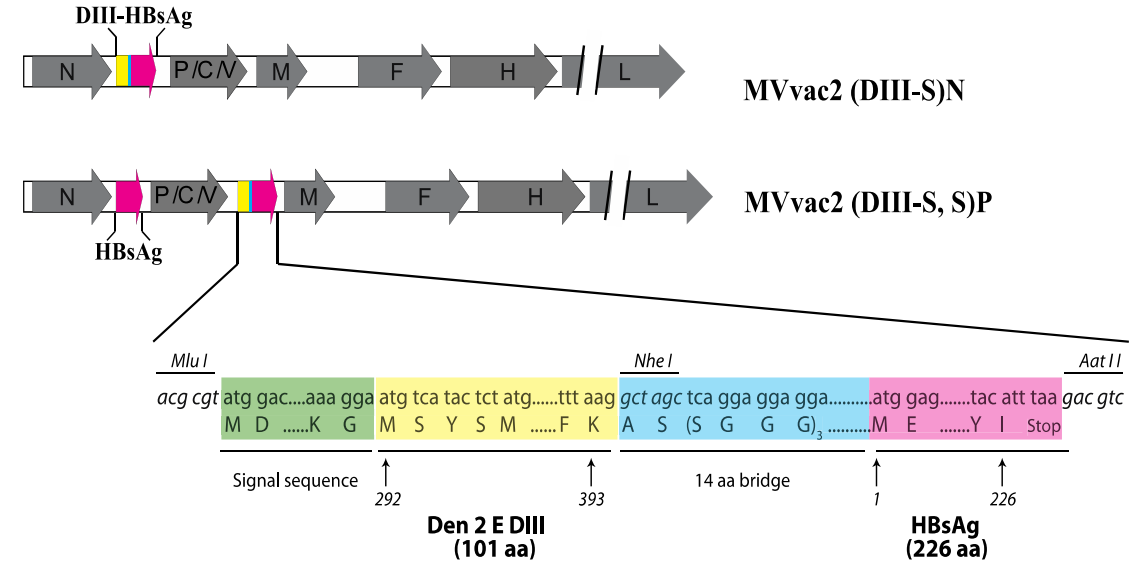

Figure 14. The Genomic Map of the Recombinant MV Generated. The top diagram represents pB(+)MVvac2(DIII-S,S)P containing the HBsAg ATU after the N cistron and the hybrid DIII-S ATU after the P cistron. The bottom diagram represents pB(+)MVvac2(DIII-S)N containing only the DIII-S ATU after the N cistron.

expressed from either of two different loci in MV, downstream the N gene and downstream the P gene, in addition to this insertion, pB(+)MVvac2(DIII-S,S)P contains a separate unmodified HBsAg coding sequence cloned downstream the N gene (Fig. 14). In figure 16 A, the insert and vector is obtained from the SbfI and KasI restriction fragments of pUC(DIII-S)P containing the hybrid DIII-S insert (4 kb), and of pB(+)MVvac2(HBsAg)N containing the vector (17 kb), which has the additional HBsAg ATU independently inserted after the N cistron. In Figure 15 and 16, we confirmed the identity of our candidate plasmids through a restriction digest using HindIII. We were able to observe the expected DNA band size in our experimental samples for both plasmids. In addition, we further corroborated the accuracy of our insertion by DNA sequencing of the gene boundaries.

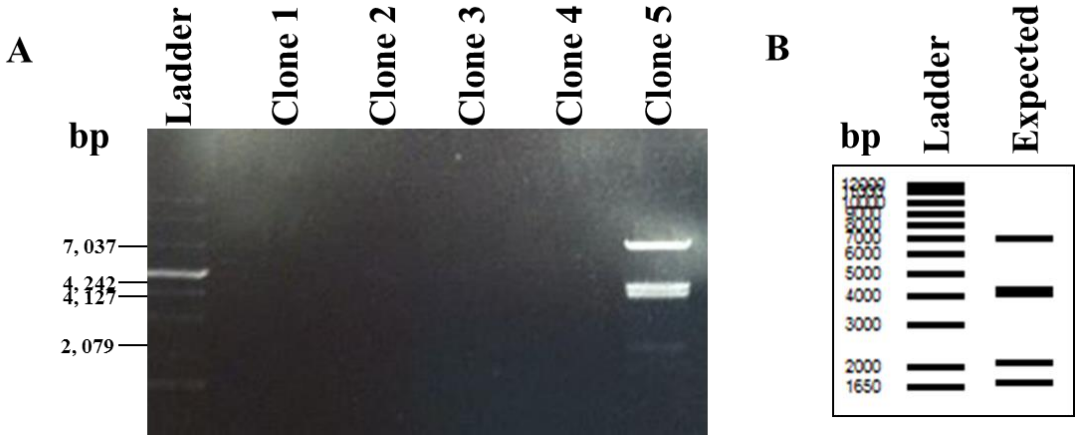

Figure 15. Diagram of the Confirmation Digest for the Recombinant Plasmid pB(+)MVvac2(DIII-S)N. A, a diagram of the agarose gel containing samples of the full length pB(+)MVvac2 (DIII-S)N digested by HindIII restriction enzyme. Clone 1-4 was too low in concentration that nothing can be seen. However, clone 5 shows the correct pattern of bands that are expected. **B,** shows the expected bands from a HindIII digestion of pB(+)MVvac2(DIII-S)N.

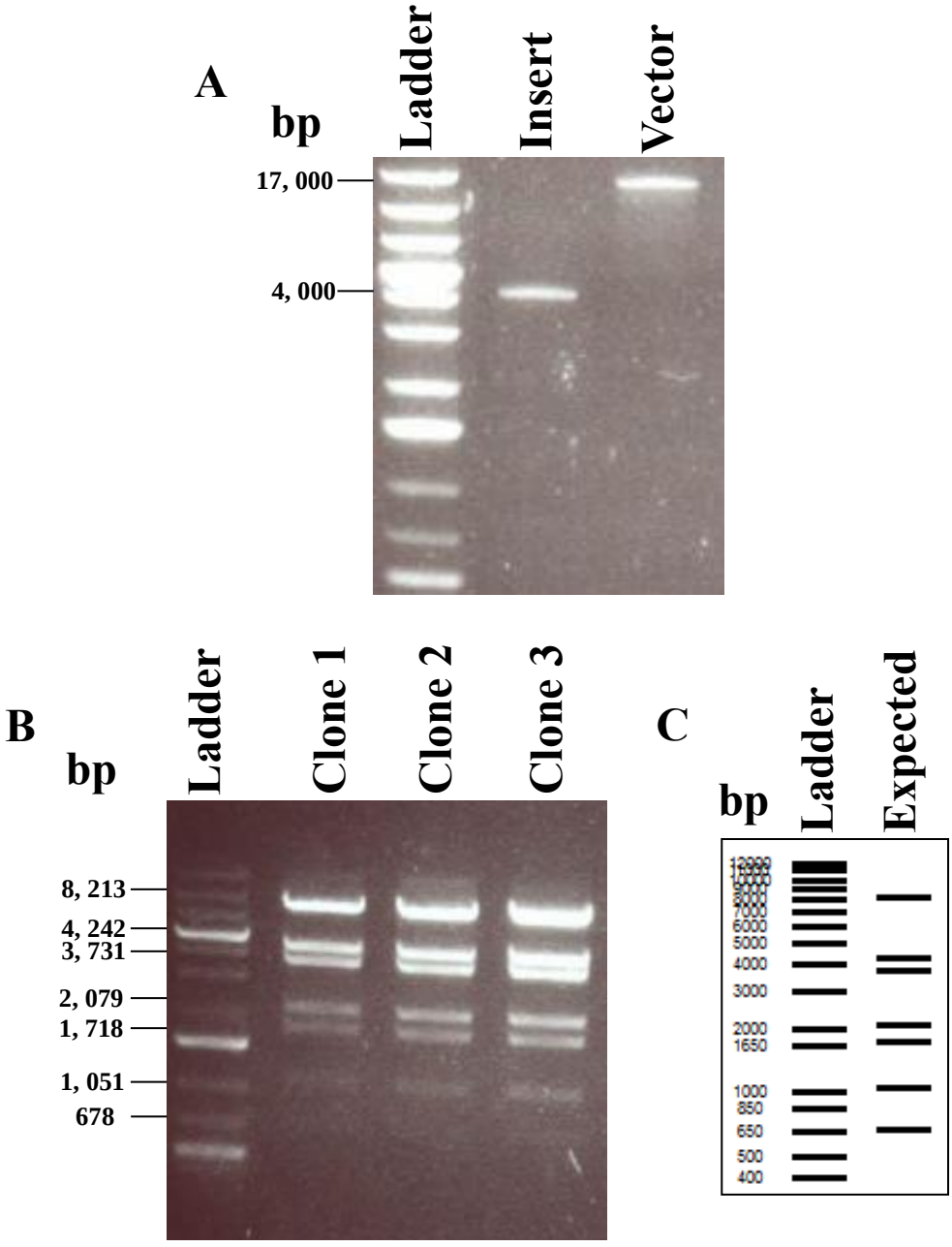

Figure 16. **Diagram of the Confirmation Digest for the Recombinant Plasmid pB(+)MVvac2(DIII-S,S)P. A,** a diagram of the agarose gel containing the digested and purified insert, hybrid DIII-HBsAg, and the vector, pB(+)MVvac2(HBsAg)N, at the expected size of 4,000bp and 17,000bp respectively. **B,** a diagram of the agarose gel containing samples of the full length pB(+)MVvac2 (DIII-S,S)P digested by HindIII restriction enzyme; showing all 3 clones. **C,** shows the expected bands from a HindIII digestion of pB(+)MVvac2(DIII-S,S)P.

3.2 Reverse Genetics System in MV Rescue. Using the full-length cDNA construction plasmids, we rescued the corresponding recombinant viruses, termed MVvac2(DIII-S,S)P and MVvac2(DIII-S)N, using an established reverse genetic system (Fig. 17). Helper cells (293-3-46), a strain from the HEK 293 cell line that is stably transfected with the N and P protein of MV as well as the T7 polymerase, were transfected with two plasmids. One plasmid encodes the full-length recombinant MVvac2, pB(+)MVvac2(DIII-S,S)P and pB(+)MVvac2(DIII-S)N, with a T7 promoter. The second plasmid, pEMC-LA, encodes the L protein of MV which is the viral RdRp; this construct also have a T7 promoter, as well as an internal ribosome entry site (IRES) before the L gene to ensure its translation. After transfection, the full-length MV genome will be transcribed by the T7 polymerase into full-length antigenomic RNA, and then the N and P protein expressed in the helper cell will bind to form the RNP complex. The MV RdRp produced from pEMC-LA will then bind to the RNP complex and replicate the MV genome. The MV genome in a RNP complex will be transcribed, which leads to the generation and expression of the viral mRNAs into viral proteins following its transcription gradient. This will result in virion assembly and release from the infected host cell (40, 42). The next step was to take helper cells and overlaid it on a monolayer of Vero/hSLAM cells, which was then monitored for syncytia. Plaque cloning was then performed by picking a single-isolated syncytia and infecting a new monolayer of Vero/hSLAM, ensuring a pure viral stock that originated from one clone of the virus. The virus was then amplified to generate viral prep made from multiple passages of the MV that was rescued.

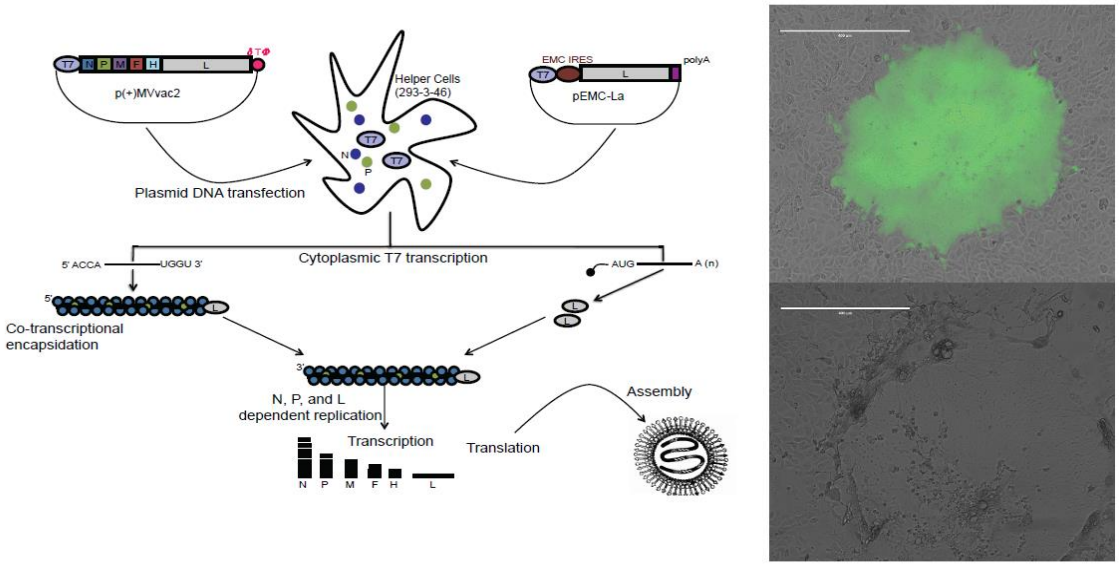

Figure 17. Generation of a Recombinant MV using the Reverse Genetics System. A helper cell line (293-3-46) was transfected with the recombinant MV cDNA and pEMC-La (codes for polymerase). Transcription and translation will progress in helper cells, which will result in the assembly of new virion. The helper cells and the virion were over-laid on vero/hSLAM cells (38). The growth of syncytia was observed. The top image depicts the syncytia formed by MVGreen, a positive control. The bottom image depicts the syncytia formed by pB(+)MVvac2(DIII-S,S)P. The microbars in the images are 400µm.

Measles infectivity generated after transfection of helper cells was amplified using plates of Vero/hSLAM cells with negative (non-transfected rescue cells) and positive controls (transfected with a control plasmid). Vero/hSLAM cells were monitored for cytopathic effects (CPE). Similar sizes of syncytia foci were observed for both the positive control and the recombinant MVs. This demonstrates that virus was indeed produced from the cDNA of the recombinant pB(+)MVvac2(DIII-S,S)P (Fig.17), initially. Hence, the insertion of two ATUs into MVvac2 did not disturb the replication of the virus. In addition, the green fluorescence marker on the positive control proves that the syncytium depicted on the bottom image in figure 17 is caused by the recombinant virus; in contrast to our recombinant, whose syncytia were not fluorescent. Hence, the second recombinant virus, MVvac2(DIII-S)N was rescued following the same procedure.

After the recovery of each recombinant virus was completed, they were amplified and titered. The stock of MVvac2(DIII-S,S)P reached a titer of $1.8x10^5$ $TCID_{50}$/ml, which is approximately ten times lower than the titer of the parental MV. Whereas the titer of MVvac2(DIII-S)N reached $5.6x10^7$ $TCID_{50}$/ml, which is comparable to the parental MV. Overall, the creation of the recombinant plasmids and the rescue of each recombinant virus were deemed successful, due to the generation of the viral stock with a viable titer for animal experimentation. However, the next step will be to observe the replication fitness of each virus.

3.3 Multi-step Growth Kinetics. To evaluate the replication fitness of the recombinant MV, a multi-step growth kinetics was performed in Vero/hSLAM cells using passage 1 of the viral stock. Cells in a monolayer ($5x10^5$ cells/well) were infected with a MOI of 0.03. Cell-associated viral progeny were collected at different time points from the intracellular fraction, which includes 12h, 24h, 36h, 48h, 72h, and 96h post-infection. These fractions were later quantified by its infectivity determined as $TCID_{50}$. The controls used in this experiment were the parental strain MVvac2, and the reference strain MVvac2(HBsAg)N, a virus that expresses the HBsAg protein from downstream the N position.

Shown in figure 18, the intracellular viral growth for MVvac2(DIII-S)N peaked at 36 hours post-infection and reached a titer of around 10^7 $TCID_{50}$/ml. Whereas MVvac2(DIII-S,S)P peaked at 48 hours post-infection and reached a titer of around $10^{5.15}$ $TCID_{50}$/ml, which is about 22 to 50 times lower than MVvac2(DIII-S)N. This indicated that the insertion of two ATUs in MVvac2(DIII-S,S)P had a significant impact on its

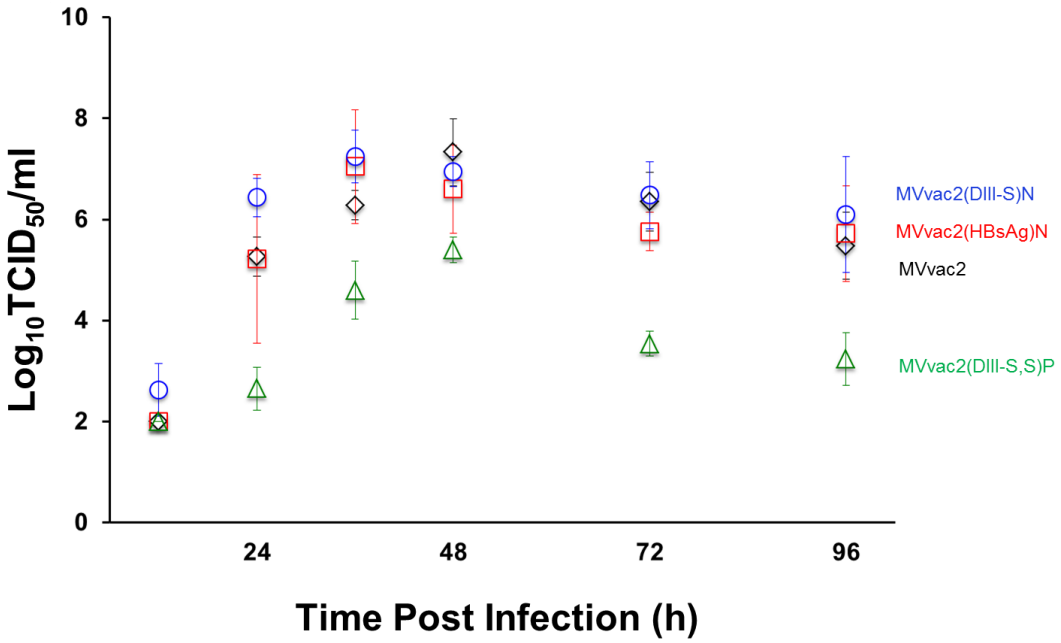

Figure 18. Multi-step Growth Kinetics of Recombinant MVs. Quantification of viral titers from cell-associated MV produced in Vero/hSLAM at specific time points post-infection (12h, 24h, 36h, 48h, 72h, and 96h). Cells were infected with either the parental strain MVvac2 represented by the black line or diamonds, the reference strain MVvac2(HBsAg)N represented by the red line or squares, the recombinant strain MVvac2(DIII-S)N represented by the blue line or circles or MVvac2(DIII-S,S)P represented by the green line or triangles. This data is an average of three independent experiments, with the error bars indicating the standard deviation for each average.

replication fitness; compared to MVvac2(DIII-S)N, which only contains one ATU, hence following a similar replication fitness as the parental strain, MVvac2, as well as the reference strain, MVvac2(HBsAg)N, which reached a titer of around 10^7 $TCID_{50}$/ml at its peak. However, despite the observed significant negative effect on viral fitness, we were able to create a stock of each of the recombinant viruses that reached a viable 10^6 $TCID_{50}$/ml, which was used for further experiments. The results shown in figure 18 represents the averages of three growth kinetic experiments performed independently of each other.

3.4 Production of HBsAg Detected by ELISA. An initial qualitative ELISA specific for HBsAg was performed to demonstrate the expression of HBsAg by the recombinant virus, MVvac2(DIII-S,S)P. Supernatants from infected cells were collected at different time-points (24h, 48h, 72h, and 96h post-infection) and analyzed. As a control, supernatant from cells infected with the reference strain MVvac2(HBsAg)N was

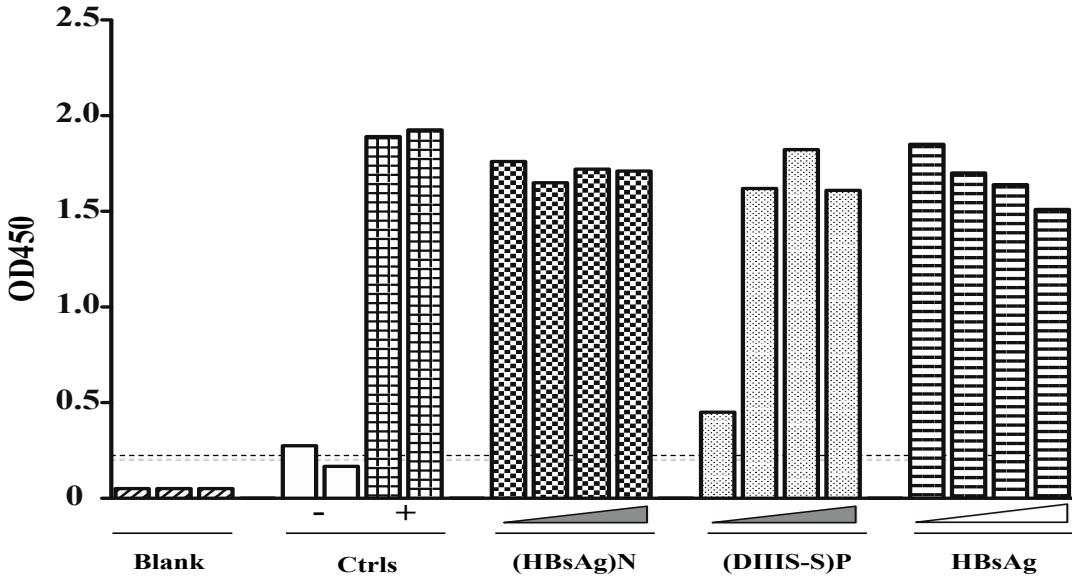

Figure 19. Identification of Secreted HBsAg by Recombinant MVs. MVvac2(DIIIS-S)P secretes HBsAg upon infection. An ELISA was performed to demonstrate the expression of the antigen used as platform to display Domain III. Positive (+) and negative (-) controls validate the experiment and set up the cut-off value (interrupted horizontal line) Supernatants from infected cells were obtained at different time points (the virus is indicated, solid triangles indicate 24h, 48h, 72h and 96h post-infection) and run in parallel with progressive dilutions of a HBsAg standards (empty triangle indicated a progressive concentration of 2.5, 5, 10 and 50 ng/ml) each sample was run in duplicate in one ELISA experiment, the average value is shown.

collected in parallel. A concentration curve using HBsAg standards was prepared for the ELISA. All of the samples from the infected media were positive for HBsAg since their readings were well above the background value (interrupted horizontal line) demonstrating that the antigen is being secreted upon infection (Fig. 19). Nevertheless,

readings of our standard curve demonstrated that this assay is only qualitative; we could not demonstrate any linearity of the OD450 readout. However it is important to note that the sensitivity of the assay is well below the lowest concentration tested (2.5 ng/ml). Therefore, after we were able to initially see the production of HBsAg from one of our recombinant viruses, MVvac2(DIII-S,S)P, we move forward in characterizing both of our recombinant viruses by performing more quantitative and detailed experiments.

3.5 Characterization of the Expression of Hybrid DIII-HBsAg Antigens from MVvac2(DIII-S,S)P. The intracellular viral particles from Vero/hSLAM cells infected with a MOI of 0.5 with either MVvac2, MVvac2(HBsAg)N, or MVvac2(DIII-S,S)P were collected. Each infected cell monolayer were lysed at 24h post-infection; non-infected cells grown in a monolayer under similar conditions as the infected cells was also lysed and used as a control.

Each cell extracts was analyzed for the incorporation of DIII into the HBsAg VLP by an anti-HBsAg western blot under non-reducing condition (Fig. 20). A doublet, two bands of HBsAg were observed by the anti-HBsAg antibodies in the cell extracts obtained from MVvac2(HBsAg)N, the positive control. The top and bottom bands are glycosylated and non-glycosylated isoforms of HBsAg, respectively, observed at theoretical molecular weight of 27 and 24 kDA as expected. The double bands are also seen in the cell extract collected from the recombinant virus MVvac2(DIII-S,S)P (Fig. 20). However, in addition to the expected doublet, we also observed another upwardly shifted double bands at 37 and 34 kDa; the corresponding theoretical molecular weight of the glycosylated and non-glycosylated isoforms of the hybrid DIII-S antigen. As expected, there were no observed binding of the anti-HBsAg antibodies in the samples

from the non-infected and MVvac2 cell extracts, used as negative controls. Overall, in figure 20, the upward shift of the double bands observed in the MVvac2(DIII-S,S)P cell extracts, shows the successful expression of DIII-HBsAg.

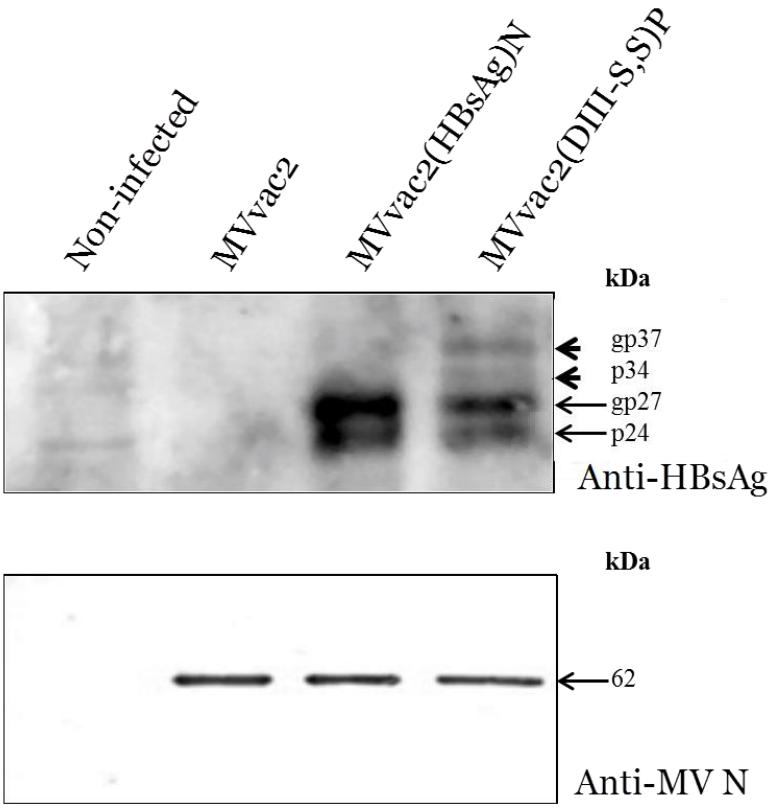

Figure 20. Western Blots Analyzing the Expression of Hybrid DIII-S. Western blot of a membrane containing 4 samples, the controls, non-infected, MVvac2, MVvac2(HBsAg)N and the recombinant virus, MVvac2(DIII-S,S)P . The top membrane was incubated with anti-HBsAg antibodies. The double bands observed in MVvac2(HBsAg)N shows the glycosylated (top) and non-glycosylated (bottom) bands, shown by the arrows. The upward shift of the double bands in MVvac2(DIII-S,S)P shown by the arrowheads, represent the expression of DIII by HBsAg. The bottom membrane was incubated with measles antibody against the N protein to corroborate equal amount of the samples are represented.

In order to corroborate that the same amount of sample was analyzed for each cell extracts, a second western blot was performed. Figure 20 shows a second diagram (bottom) which displays the western blot results using anti-N antibodies from MV; everything else other than the antibodies used, was run under the same conditions as the previous western blot. In this western blot, we observed the same amount of N protein present in each of the infected cell extracts.

3.6 Characterization of the Expression of Hybrid DIII-HBsAg Antigens from MVvac2(DIII-S)N and the Correct Display of DIII. After confirming the successful expression of the hybrid DIII-HBsAg antigen from the first recombinant virus, MVvac2(DIII-S,S)P, the experiment was repeated with the second recombinant virus, MVvac2(DIII-S)N. Lysates from Vero/hSLAM cells infected with a MOI of 0.5 with either MVvac2, MVvac2(HBsAg)N, MVvac2(DIII-S,S)P, or MVvac2(DIII-S)N were collected. Each infected cell monolayer was lysed at 24h post-infection. Each cell extracts was analyzed for the incorporation of DIII into the HBsAg VLP by an anti-HBsAg western blot under non-reducing condition (Fig. 21). A doublet was observed similar to the previous western blot, where two bands of HBsAg, the glycosylated isoform at 27 kDA and non-glycosylated isoform at 24 kDa, were observed by the anti-HBsAg antibodies in the cell extracts obtained from MVvac2(HBsAg)N, the positive control. In figure 21, the double bands and the upshifted doublet are also seen again in the sample containing MVvac2(DIII-S,S)P cell extract, as seen in the previous western blot (Fig. 20). Furthermore, between the two shifted double bands, the band corresponding to the non-glycosylated isoform, seen at 34 kDa, gives a much weaker signal than the band corresponding to the glycosylated isoform seen at 37 kDa. However, both of the bands

that were shifter upward does seem weaker than the two original doublet at 24 kDa and 27 kDa, possibly due to the amount of the hybrid DIII-HBsAg antigen that is actually being produced.

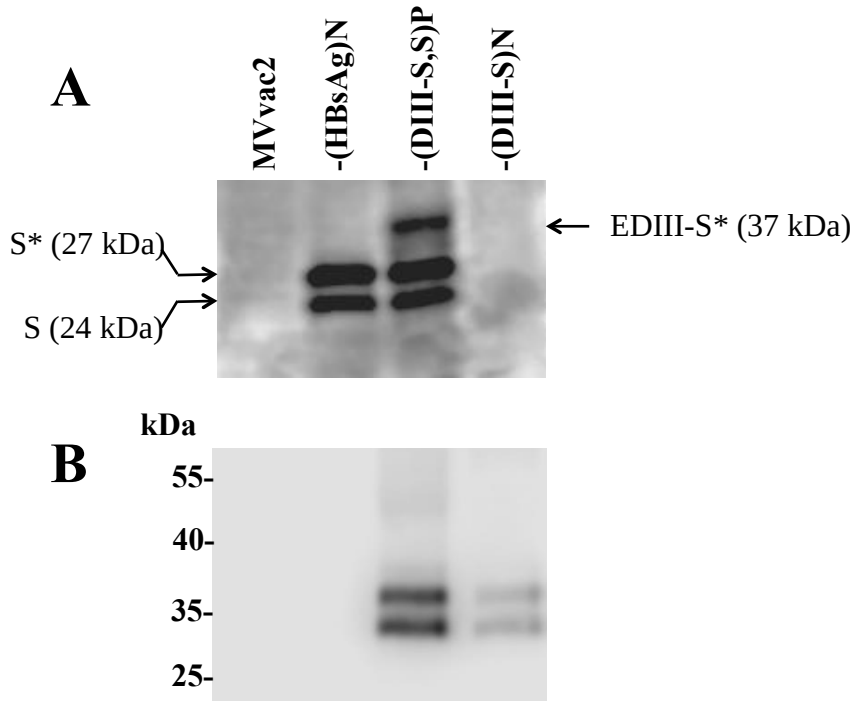

Figure 21. Analysis of Successful Expression of the Hybrid DIII-S Antigen in Cell Lysates from Controls and Recombinant MV Infected Cells. **A,** samples run under non-reducing conditions in a SDS-PAGE, western blot developed using a monoclonal antibody, anti-HBsAg (the S antigen); the molecular weight of the two forms of S are shown to the left and the molecular weight of the hybrid DIII-S to the right. The asterisk indicates glycosylated forms of HBsAg. **B,** samples run under non-reducing conditions in a SDS-PAGE, western blot developed using a monoclonal antibody specific for dengue envelope protein domain III. The double bands were observed at 37 kDa and 34 kDa

On the other hand, in the sample containing the MVvac2(DIII-S)N cell extract, we were not able to observe any binding of the monoclonal antibody. Since the western blot was incubated with anti-HBsAg antibody, this shows that the antibodies were not

able to detect the proteins present in the cell extract, possibly due to some changes that happened in the protein's folding pattern.

In order to analyze if the hybrid DIII-S antigen is displaying the correct DENV-2 epitopes present in DIII. We developed a western blot using antibodies specific for DENV-2 DIII. This was done under reducing conditions. We were able to observe two bands from both of our recombinant viruses. These two bands were seen at about 37 kDa and 34 kDa, corresponding to the glycosylation isoforms of the hybrid DIII-S previously seen in the western blot developed with anti-HBsAg (Fig. 21). However, the double bands observed from the sample containing cell extracts from MVvac2(DIII-S)N, were weaker than the bands observed from the samples containing cell extracts from MVvac2(DIII-S,S)P.

3.7 Particle Isolation and Characterization. The particle isolation experiment was performed to see the presence of hybrid VLPs in the supernatants of Vero/hSLAM cells infected with MVvac2(DIII-S,S)P. Figure 11 illustrate the procedure, where nine fractions were collected from the discontinuous sucrose gradient ultracentrifugation in order to confirm the expected density of the HBsAg particles, including the hybrid particles expressing DIII. These fractions were then loaded onto a SDS-PAGE under non-reducing condition. The western blot was developed with anti-HBsAg monoclonal antibodies. The HBsAg particles were observed in fractions 4 to 6 as shown by the double bands in both the control sample, fractions from the reference strain MVvac2(HBsAg)N and the experimental sample, fractions from the recombinant strain MVvac2(DIII-S,S)P (Fig. 22. B). In addition, we were able to observe again the upward shifted double bands (shown by arrows) that correspond to the glycosylated and non-

glycosylated isoforms of the hybrid DIII-S antigen (Fig. 22. B). These bands were detected in similar sucrose density (1.10-1.12g/mL, fraction 4 and 5) to the sucrose density of the native HBsAg secreted in the supernatants of MVvac2(HBsAg)N, the control (Fig. 22. A). Showing that the characteristics of the hybrid VLP secreted by MVvac2(DIII-S,S)P are similar to the native VLP secreted MVvac2(HBsAg)N.

Supernatant of Vero/hSLAM cells infected with MVvac2(DIII-S)N was analyzed in this experiment (not shown). Since we have observed a lack of binding of the anti-HBsAg monoclonal antibody (Fig. 21), we conclude that performing this western blot using the fractions from MVvac2(DIII-S)N will not be successful. We concluded that we might be able to detect the presence of hybrid DIII-S antigen by developing the western blot using the monoclonal antibody against DENV-2 DIII. However, we were not able to observe any binding of the antibodies in this western blot. Overall, we observed the presence of the hybrid DIII-S antigen in the fractions collected from supernatants of cells infected by MVvac(DIII-S,S)P.

The last western blot we performed using fractions collected from the particle isolation is developed using antibodies against the N protein of MV. This western blot used the fractions collected from the supernatant of cells infected with MVvac2(DIII-S,)P. We were able to observe that the MV particles migrated to fraction 6 to 9 based on the presence of the MV N protein shown on the bottom panel (Fig. 22). The fractions where the MV particles are located in are (fractions 6 to 9) clearly separate from the fractions where the native HBsAg VLP or hybrid DIII-S VLP are located in (mainly fractions 4 to 5). This demonstrated that the hybrid particle is incorporated separately from the MV particle, which migrates to the more dense fractions.

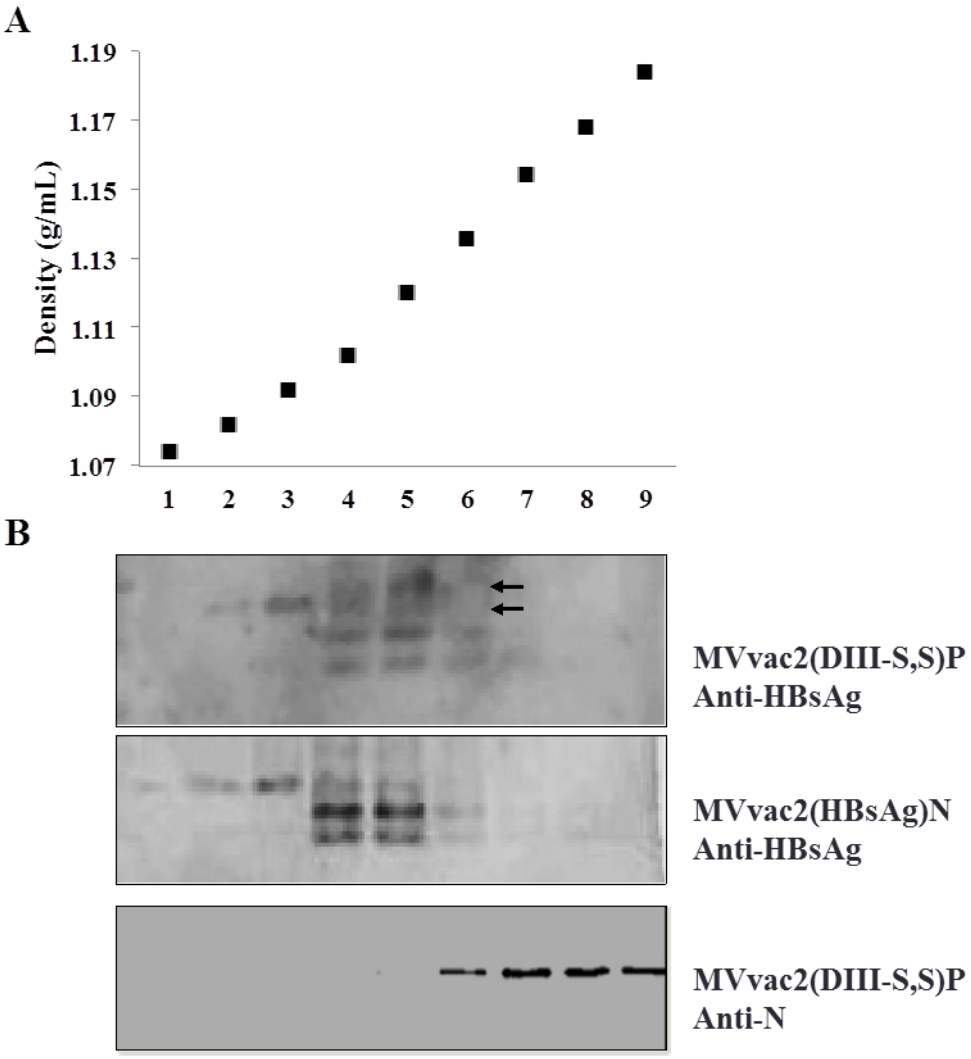

Figure 22. Particle Isolation by Discontinuous Sucrose Gradient and Analysis of Each Fractions Collected. Particle isolation diagrams from supernatants of Vero/hSLAM cells infected with either MVvac2(HBsAg)N or MVvac2(DIII-S,S)P. Supernatants were collected at 72h post-infection and clarified. Particulate material was then pelleted and resuspended in 20% sucrose solution, loaded onto the 30%-60% sucrose gradient, then centrifuged to equilibrium. Nine fractions were collected from the top (fraction 1) to the bottom (fraction 9) in 500µL aliquots. **A,** Each fractions were weighed to determine its density. The graphic plot shows a density gradient profile obtained from a representative experiment. The density of each fraction is shown by the graph at the top in g/mL. **B,** Fraction samples were run in a 12.5% SDS-PAGE. The antigenic reactivity of each fraction was analyzed by a western blot using an anti-HBsAg antibody (top two panels) and an anti-MV N antibody (lower panel). The top and bottom panels are depicting western blots using fractions from MVvac2(DIII-S,S)P infected cells. The middle panel is depicting a western blot using fractions from MVvac2(HBsAg)N infected cells. The hybrid DIII-S incorporate into the VLP is particle is shown by the arrows in the top panel. These double bands corresponding to the glycosylated and non-glycosylated isoforms of the hybrid DIII-S antigen can be seen at 37 kDa and 34 kDA, respectively.

Overall, we were able to confirm the incorporation of the hybrid DIII-S antigen on a scaffold of HBsAg, without affecting its density or the assembly of MV virion.

3.8 Immunogenicity Study. We performed two sets of immunogenicity study from two different animal experiments in order to analyze the immune response that will be induced by our recombinant viruses. The first animal experiment was done using MV susceptible transgenic mice HDD-SLAM-IFNar^{KO}. These mice express one of the human MV receptor, hSLAM, in a type I interferon inactivated environment, which will allow for MV replication. These mice are also H2Kb and Db knock out, which inhibits the mice MHC class I allele presentation restriction. In addition, these transgenic mice also presents MHC class I antigenic peptides with a HLA A2.1 restriction, the most common human MHC class I allele. Four viruses were used in vaccinating these mice, including: MVvac2, MVvac2(HBsAg)N, MVvac2(DIII-S,S)P, and DENV-2; for the initial animal experiment, we have decided to analyze only one of our recombinant viruses first. Groups of 2 to 5 animals received two doses through the intraperitoneal route. The doses given were 10^5 $TCID_{50}$ for MVvac2, MVvac2(HBsAg)N, and MVvac2(DIII-S,S)P. The doses given for DENV-2 were theoretically calculated to be 10^5 PFU, however the viral prep was later found to be of lower concentration; hence the dose given to the animal were only 10^2 PFU. All of the animals were vaccinated at three different times, at day 0, 1 and 14. Then all of the animals were euthanized and exsanguinated 28 days after the last vaccination. The vaccination at day 0 was given to induce expression of the MV receptor, SLAM, because it is only expressed in activated lymphocytes. Isolated and decomplemented sera were used in the immunogenicity experiments.

To analyze the measles immunogenicity, we performed a micro-neutralization assay, there the neutralization titer represents a 90% inhibition of MV cytopathic effect in Vero/hSLAM cells (Fig. 23. A). As shown in figure 23, all of the animals vaccinated with MVvac2, MVvac2(HBsAg)N, and MVvac2(DIII-S,S)P showed responsiveness of their immune system to the antigenic stimulus, present in the viral vector backbone for the reference strain and our recombinant strain. However, we observed a four-fold reduction in measles immunogenicity in MVvac2(DIII-S,S)P when it is compared to the parental strain, MVvac2. It is possible that the insertion of 2 ATUs, resulting in the reduced viral fitness observed *in vitro* may be responsible for the difference in immunogenicity. We also observed a smaller reduction in measles immunogenicity in mice vaccinated with the reference strain MVvac2(HBsAg)N, which have an insertion of only 1 ATU. We determined an anti-MV neutralization titer of about 1:1000 for MVvac2, 1:640 for MVvac2(HBsAg)N, and 1:250for MVvac2(DIII-S,S)P. As expected, measles immunogenicity in sera from animals vaccinated with DENV-2 was non-existent.

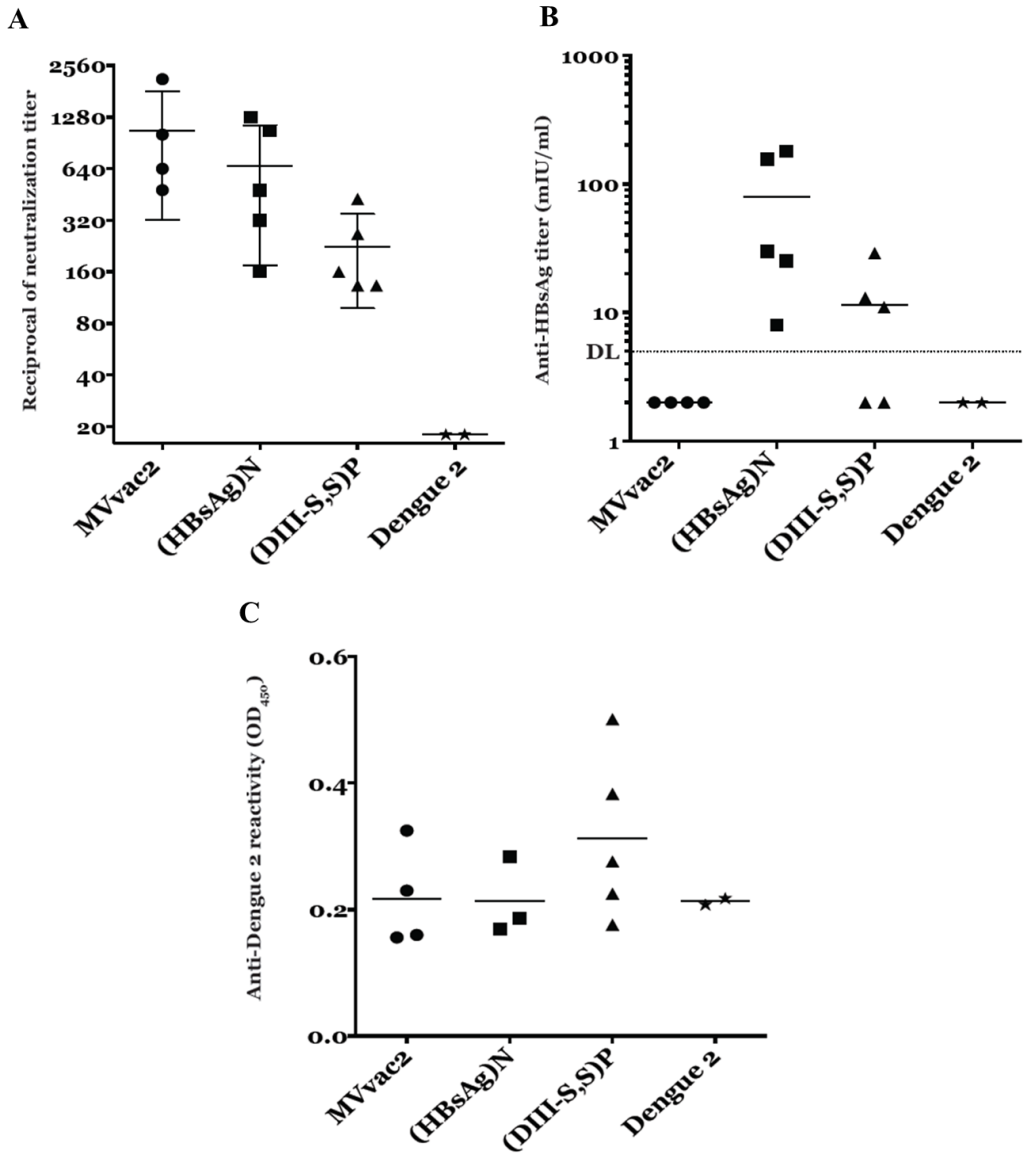

Figure 23. Immunogenicity Study against MV, Hepatitis B and DENV-2. Immunogenicity of recombinant MV vectors expressing hybrid DIII-HBsAg in HDD-SLAM-IFNar^{KO} genetically modified mice. Groups of animals received two doses of 10^5 $TCID_{50}$ or 10^2 PFU of DENV-2 through the intraperitoneal route (see material and methods for details). At 28 days after the last dose, all of the animals were exsanguinated. Serum was isolated and decomplemented, prepared to be used in neutralization assay and ELISA. Each mice is represented by one symbol and the group average is represented by the horizontal line. **A,** Results of the measles immunogenicity study, determining anti-MV neutralization. Y-axis scale represents the reciprocal of neutralization titer against MV. **B,** Results of the HBsAg ELISA, determining anti-HBsAg titer. Y-axis scale represents the anti-HBsAg titer in mIU/ml. The horizontal dashed line indicated detection level (DL) of the ELISA, this value was calculated from the reading of the standards and the control samples. The hepatitis b protective titer has been previously determined to be 10 mIU/ml. **C,** Results of the DENV-2 ELISA, determining anti-DENV-2 reactivity. Y-axis scale represents the anti-DENV-2 reactivity read under OD_{450}.

To analyze the anti-HBsAg reactivity of the sera samples, we used a commercial ELISA kit. We compared the readings from each sera collected from our animals, to a standard curve constructed with an international reference. The known hepatitis B protective titer is 10 mIU/ml and the detection level of our ELISA results is shown as DL (Fig. 23. B). As expected, there was no reactivity detected in sera collected from mice vaccinated with MVvac2 and DENV-2. We observed that the anti-HBsAg titer for both MVvac2(HBsAg)N and MVvac2(DIII-S,S)P passed the protective titer of 10 mIU/ml. However, the sera collected from mice vaccinated with MVvac2(DIII-S,S)P showed an eight-fold reduction in HBsAg immunogenicity when compared to MVvac2(HBsAg)N.

To analyze the anti-DENV-2 reactivity of our sera samples, we used an ELISA. Whole dengue 2 viruses was adsorbed to the bottom of the well overnight at 4ºC. After blocking, 1:50 serum dilutions were added and incubated. We used anti-mouse-HRP to bind to the left-over antibodies which reacted with DENV-2, and developed the plate with HRP and 3M TMB-H_2SO_4. Anti-DENV2 reactivity was only seen in two sera samples from animals vaccinated with MVvac2(DIII-S,S)P (Fig. 23. C). These two sera samples were also the samples that showed the highest measles and hepatitis B immunogenicity from the MVvac2(DIII-S,S)P vaccinated group. As expected, all of the mice vaccinated with MVvac2 and MVvac2(HBsAg)N did not show any reactivity to DENV-2. However, we were also not able to observe any reactivity in sera from animal vaccinated with DENV-2, which is supposed to be the positive control. This might have been caused by the error found in the doses given during vaccination of these mice, which happened to be much lower than previously determined. Overall, we saw reactivity against DENV-2 from sera collected from our vaccinated animals, but an ELISA does not

show protection. Protective titer will need to be determined by a neutralization assay for DENV-2.

In the second animal experiment that we completed, we used a different line of transgenic mice. Groups of huCD46Ge-IFNar^{KO} genetically modified mice were vaccinated with either one dose or two doses (shown by x1 and x2, respectively) of MVvac2, MVvac2(HBsAg)N, MVvac2(DIII-S,S)P, or MVvac2(DIII-S)N (Fig. 24). These mice express a different MV receptor, CD46, with human-like distribution. They also have inhibition of type I interferon, which will allow for MV replication. These transgenic mice represent the gold standard for small animal models testing measles vaccine immunogenicity. Groups of 3 to 7 animals received doses of 10^5 $TCID_{50}$ through the intraperitoneal route. At 28 days after the first dose were given, animals scheduled to receive one dose of vaccination were exsanguinated, whereas animals scheduled to receive two doses of vaccination were given their second dose. Then 14 days after the second dose, these animals were exsanguinated. Sera collected from these animals were isosolated and de-complemented as before.

To analyze the measles immunogenicity from the collected sera, we performed a micro-neutralization assay where the neutralization titer represents a 90% inhibition of MV cytopathic effect in Vero/hSLAM cells. All of the animals receiving the recombinant viruses in this experiment showed reactivity to the antigenic stimulus created by the viral vector backbone. The parental strain MVvac2 showed an average anti-MV neutralization titer of 1:1000, which is expected from the positive control. In addition, animal vaccinated with the reference strain, MVvac2(HBsAg)N, showed an average anti-MV neutralization titer of 1:2560. However, this neutralization titer is expectedly higher

because these mice received two doses. The sera sample from mice vaccinated with one dose of MVvac2(DIII-S,S)P showed an average anti-MV neutralization titer of 1:640, which is lower than the samples from the mice vaccinated with the parental strain. However, the sera from the mice that received two doses of MVvac2(DIII-S,S)P showed an average anti-MV neutralization titer of 1:1280, which reached a titer higher than those

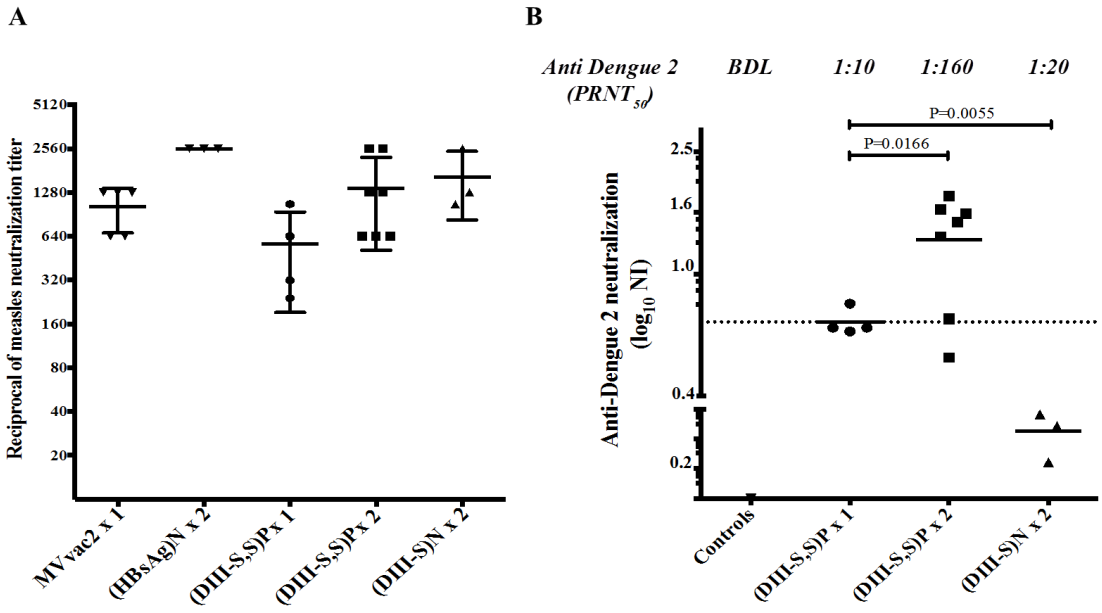

Figure 24. Immunogenicity Study against MV and DENV-2. Immunogenicity of recombinant MV vectors expressing hybrid DIII-HBsAg in huCD46Ge-IFNar^{KO} genetically modified mice. Groups of animals received one (x 1) or two (x 2)doses of 10^5 $TCID_{50}$ through the intraperitoneal route (see material and methods for details). At 28 days after the first dose, mice scheduled to receive only one dose were exsanguinated and mice scheduled to receive two doses were given their second dose. At 14 days after the second dose, the rest of the animals were exsanguinated. Serum was isolated and decomplemented, prepared to be used in neutralization assays and ELISA. Each mice is represented by one symbol and the group average is represented by the horizontal line. **A,** Results of the measles immunogenicity study, determining anti-MV neutralization. Y-axis scale represents the reciprocal of neutralization titer against MV. **B,** Results of the DENV-2 immunogenicity study, determining anti-DENV-2 neutralization. Y-axis scale represents the logarithmic neutralization index (LNI) against DENV-2. The controls were used to create the baseline value were sera collected from animal vaccinated with the parental strain, MVvac2, and reference strain, MVvac2(HBsAg)N. The horizontal dashed line shows the protective level of 0.7 LNI previously determined for other flaviviruses. Statistically significant differences are indicated by horizontal line above the experimental groups. Values from $PRNT_{50}$ neutralization assay is shown at the top of the graph.

of the mice vaccinated with MVvac2. In addition, the sera sample from mice vaccinated with two doses of MVvac2(DIII-S)N showed a higher average anti-MV neutralization titer of 1:1500. This measles immunogenicity study showed that receiving two doses of the recombinant viruses can induce similar amount of immune response against measles as vaccinating with only one dose of MVvac2. Therefore, the problem in reduction of neutralization titer against MV seen in mice vaccinated with the lower fitness recombinant virus can possibly be avoided by adding a second dose.

To analyze the reactivity of the collected sera against HBsAg, we used a commercial ELISA kit, comparing the readings from the sera from our vaccinated mice to a standard curve constructed with an international reference (data not shown). For this experiment, the sera of the animals were pooled for each group of mice. Pooled sera from mice vaccinated with two doses of the reference strain, MVvac2(HBsAg)N, reached a titer of 648 mIU/ml. Whereas pooled sera from mice vaccinated with two doses of the recombinant strain, MVvac2(DIII-S,S)P, reached a titer of 787 mIU/ml. In contrast, pooled sera from animals vaccinated with two doses of MVvac2(DIII-S)N, did not show significant reactivity. As expected, the pooled sera collected from mice vaccinated with MVvac2, did not show HBsAg reactivity.

To analyze the reactivity of the collected sera against DENV-2, we performed neutralization experiments through two different approaches. First, we analyzed the effect of a 1:5 serum dilution on the infection of BHK-21 cells with a 10^5 PFU/ml DENV-2 (Fig. 24. B). The reduction in DENV-2 infectivity caused by immune sera reactivity was recorded as the logarithmic neutralization index ($Log_{10}NI$). Sera samples collected from animal vaccinated with the parental strain, MVvac2, and the reference strain,

MVvac2(HBsAg)N, were used as the negative controls; their values is used to set the baseline of the graph (Fig. 24. B). Reactivity against DENV-2 was seen in sera collected from mice vaccinated with MVvac2(DIII-S,S)P. We observe that those of which received only one dose had an average of 0.7 LNI, which shows a reduction of 5 times in DENV-2 infection. In addition, those that received two doses of MVvac2(DIII-S,S)P had an average of 1.3 LNI, which shows a reduction of about 20 times in DENV-2 infection. Protective titer level against other flaviviruses that has been previously determined is at 0.7 LNI. Therefore, sera collected from the mice vaccinated with two doses of MVvac2(DIII-S,S)P reached a higher LNI than needed for the protective level. Whereas, those that only received one dose reach just at the protective level of LNI. However, sera collected MVvac2(DIII-S)N vaccinated mice did not reach a protective level.

The second neutralization experiment that was performed to analyze the ability of the mice sera to inhibit DENV-2 infection was the classic 50% plaque reduction neutralization assay (Fig. 24. B). Two-fold serum dilutions were incubated with 200 PFU of DENV-2 and the neutralization titer is determined by the dilution at which the sera have reduced the plaques by half, compared to a control. To determine the $PRNT_{50}$ through this experiment we used pooled mice sera for each group of mice following their vaccine schedule. We calculated the $PRNT_{50}$ from sera pooled from mice that were given one dose of MVvac2(DIII-S,S)P, two doses of MVvac2(DIII-S,S)P, and two doses of MVvac2(DIII-S)N to be 1:10, 1:160, and 1:20 respectively. We observed that the $PRNT_{50}$ of mice vaccinated with two doses of MVvac2(DIII-S)N is higher than those that received only one dose of MVvac2(DIII-S,S)P, this is the opposite of the result we received in the previous experiment. However, this may be due to the small amount of

DENV-2 epitopes that were accurately displayed causing borderline immunogenicity reaction against DENV-2 in this neutralization assay. Overall, the best PRNT50 titer that we observed through this neutralization assay resulted from the pooled mice sera from those receiving two doses of MVvac2(DIII-S,S)P.

Chapter 4. Discussion

VLP platforms are very attractive vaccine candidates since they offer the possibility of delivering antigenic material with a structure very similar to a full virion and are safe because there is no risk of infection. These platforms represent the current vaccines for hepatitis B virus and human papillomavirus. We decided to use HBsAg as a platform to display domain III of the envelope protein of DENV-2 because of the translational strategy used for the expression and assembly of the particulate antigen. During hepatitis B virus infection, the viral envelope is generated by the expression of three envelope glycoproteins, the small, medium and large surface antigens. Their coding sequences have in common a 226 amino acids scaffold towards the carboxyl terminus. Translation starts from in-frame initiation codons, giving origin to the expression of the preS1 gene for the large antigen, preS2 gene for the medium antigen, and S gene for the small antigen. These glycoproteins of 27, 36 and 42 kDa, have the potential to self-assemble into empty particles upon expression in infected cells with a proportion of 4:1:1. This project explored the possibility of swapping the preS1 and preS2 regions with the DIII of the DENV-2 envelope glycoprotein and a bridge, creating the hybrid DIII-HBsAg antigen. Two different recombinant viruses were then engineered using MV as the viral vector backbone. MV was selected to be used as the viral vector due to its safety and stability. We used MVvac2, which is the recombinant version of the attenuated strain, Moraten and Schwartz (genetically identical, despite the fact of being obtained separately), which is used in the current children's vaccination schedule. We take advantage of the MV expression gradient to strategically place our ATUs; where the amount of transcription is determined by the position of the cistron relative to the 3' end

of the genome. The cistron located towards the 3' end is transcribed at a higher frequency due to the graduate attenuation at gene boundaries. This knowledge of the transcription of MV genome is factored into the engineering of our recombinant viruses. These recombinant viruses are termed MVvac2(DIII-S,S)P with the insertion of two ATUs and MVvac2(DIII-S)N with the insertion of only one ATU.

In MVvac2(DIII-S,S)P we co-expressed from a different locus in the MV genome the HBsAg protein in addition to the hybrid DIII-S, in order to promote the native assembly of VLPs. The proportion of these two ATUs by MV transcription gradient will drive a proportion of expression close to 3:1. Therefore, the generation of unmodified HBsAg, from the cistron located after the N cistron, can be used as a scaffold for the hybrid DIII-S antigen. The ATU encoding the hybrid DIII-S antigen is inserted downstream of the P gene. The correct construction of this full-length plasmid, pB(+)MVvac2(DIII-S,S)P, was confirmed through HindIII digestion and DNA sequencing. In figure 16, we show the observed bands from samples of the full-length plasmid digested with HindIII. Compared to the expected band, we conclude that the correct full-length plasmid was created. In addition, the DNA sequence of the full-length plasmid was checked for errors, and we conclude that the sequence was accurate.

In MVvac2(DIII-S)N, we only inserted one ATU expressing the hybrid DIII-S to observe whether VLPs can assemble efficiently without an HBsAg scaffold. The ATU encoding the hybrid DIII-S antigen is inserted downstream of the N gene and as such we would have expected a robust expression of the antigen. The correct construction of this full-length plasmid, pB(+)MVvac2(DIII-S)N, was also confirmed through HindIII digestion and DNA sequencing. In figure 15, we show the observed bands from one clone

of the full-length plasmid. We conclude that the correct full-length plasmid was created after the result is compared to the expected bands. The DNA sequence also confirmed the accuracy of the full-length plasmid.

After confirmation of the correct sequences, we move on to rescue the recombinant viruses through the established reverse genetic techniques for MVs. In the bottom syncytia image in figure 17, we show the syncytia observed after we successfully rescued one of our recombinant viruses MVvac2(DIII-S,S)P. Then we continued to successfully rescue our second recombinant virus MVvac2(DIII-S,S)N (syncytia not shown). After the recombinant viruses were rescued, the viruses were passaged to create stock of viral prep. We were able to create a viral prep tittered at $1.8x10^5$ $TCID_{50}$/ml for MVvac2(DIII-S,S)P and $5.6x10^7$ $TCID_{50}$/ml for MVvac2(DIII-S)N. These titers are high enough for us to move forward with additional experiments, which also include our animal experiments.

The next step of the experiments was to analyze the replication fitness of the recombinant MVs compared to the parental strain, MVvac2, and the reference strain, MVvac2(HBsAg)N. The multiple-step growth kinetics that was performed showed a decrease in the replication fitness of MVvac2(DIII-S,S). The decrease of viral production compared to MVvac2 and MVvac2(HBsAg)N was concluded to be result of the two ATUs inserted into the MV genome. Although the two ATUs were inserted in non-sequential locus of the genome, the increased size of the genome may also account for the drop of MV infectivity progression. Furthermore, it makes sense to conclude that more insertions lead to a dilution of the genes downstream the insertions. Those genes are important for measles replication (like the polymerase, L gene) In addition, this negative

effect on the replication of recombinant MVs have been observed in other research utilizing MV vectors with multiple insertion of ATUs. On the other hand, this negative effect of the recombinant virus' replication fitness was not observed in MVvac2(DIII-S)N which makes us conclude that the gene DIII-SA is not by itself incompatible with measles replication.. Our second recombinant virus followed a similar replication fitness as the parental strain, MVvac2. In addition, it also follows the replication fitness of MVvac2(HBsAg)N, which also have one ATU encoding HBsAg. This supports the idea that only one ATU insertion into the MV genome was not able to affect its replication fitness, whereas the insertion of two ATUs was able to affect it negatively. The results from the multi-step growth kinetics is statistically valid, since it shows the corroborated average data from experiments repeated independent of each other.

Next, we performed an ELISA against HBsAg to confirm the production of HBsAg. This experiment was done as an initial assessment of the possible production of HBsAg from the recombinant MVs. Hence it was performed only using the supernatants collected from MVvac2(DIII-S,S)P infected cells, as well as the supernatants from MVvac2(HBsAg)N infected cells as the control. Samples of the supernatants were collected at different time-points post-infection. The result from the Anti-HBsAg ELISA showed the presence of HBsAg in the supernatant from MVvac2(DIII-S,S)P infected cells. Data above the dashed line (Fig. 19) showed the significant value, which represents the HBsAg present. However, we were not able to state any quantitative data from this experiment, because the assay shows only the possibility to discern positive from negative; due to the sensitivity of this specific ELISA kit. We plan to standardize this experiment in order to supplement the replication kinetics we collected. Therefore this

experiment was a supplement to the replication kinetics we collect. However, we characterized the production of HBsAg, as well as the hybrid DIII-HBsAg antigen, in a more precise approach, by protein immunoblots.

The results from the characterization of the expression of hybrid DIII-HBsAg antigen (Fig. 20) solidified that DIII-S hybrid glycoprotein was indeed expressed in infected cell extracts. We were able to corroborate that each sample contained the same amount of protein extract, as shown by the bottom western blot (Fig. 20) that is developed by the antibody against the N protein of MV. In the experimental sample, there is a clear upward shift of the double band in the sample collected from the protein extracts from cells infected with MVvac2(DIII-S,S)P representing the higher size of the protein with the addition of DIII from DENV-2. This can be easily compared to the control sample of protein extracts from cells infected with MVvac2(HBsAg)N, which only show one set of double bands at a lower size. However, the upwardly shifted double bands corresponding to the glycosylated (37 kDa) and non-glycosylated (34 kDa) isoforms of the hybrid antigen is shown to be at a lower quantity, by the intensity of the bands (Fig. 20). We concluded that this was due to the location of the ATU that is inserted encoding the hybrid DIII-S antigen, which is downstream of the P gene. Following the MV genome transcription gradient, this means that it was transcribed at a lower quantity due to its location. In addition, the amount of the native HBsAg glycosylated (27 kDa) and non-glycosylate (24 kDa) isoforms present in the sample from the MVvac2(DIII-S,S)P infected cells is similar to the same isoform in the sample collected from cells infected with the reference strain. Overall, from this initial characterization experiment, we were able to confirm from our western blots, the

successful expression of the hybrid DIII-HBsAg antigen from one of our recombinant MV, MVvac2(DIII-S,S)P. The next step we took was then to characterize both of our recombinant MVs for the production of the hybrid antigen.

The characterization for the expression of the hybrid DIII-HBsAg antigen was repeated with both recombinant MVs. The experiment was performed in the same way as the previous one. However, the western blots were containing the same samples, were developed with anti-HBsAg monoclonal antibody (Fig. 21. A) and anti-DIIII DENV-2 monoclonal antibody (Fig. 21. B). As expected, the upward shifted band that was seen in the sample from cells infected with MVvac2(DIII-S,S)P is present again in this western blot. We also see again the lower intensity of the bands at 37 kDa and 34 kDa. The bands corresponding to the native HBsAg can also be seen in the samples form cells infected with MVvac2(DIII-S,S)P and MVvac2(HBsAg)N. However, bands showing any reactivity were not seen in the protein extract from cells infected with MVvac2(DIII-S)N, concluding that the insertion of DIII into HBsAg, in the absence of HBsAg alone is not compatible with the recognition by our antibody. The reactivity of the anti-HBsAg antibodies highly depends on the conservation of the semi-native structure of the protein's epitopes, which is the reason why this western blot was run under non-reducing conditions. This monoclonal detects a region on HBsAg that is termed he "a" epitope that is essential for proper folding of HBsAg. We propose that the sole expression of DIII-S hybrid antigens leads to an unfolded product that is certainly tagged for degradation. Therefore, we suggest that the additional native HBsAg is needed to scaffold the DIII-S antigen, which will result in the correct protein structure that will be recognized by the antibody. In order to confirm that the expressed hybrid DIII-S antigen also presented the

DENV-2 epitopes shown in DIII, a western blot was developed with a monoclonal antibody specific to DIII of the DENV-2 envelope protein (Fig. 21. B). Here we observed two bands at 37 kDa and 34 kDa in both samples of protein extracts from cells infected with MVvac2(DIII-S,S)P and MVvac2(DIII-S)N. These bands correspond to the same bands in the western blot developed with anti-HBsAg antibodies, which represents the glycosylated and non-glycosylated isoforms of the hybrid antigen. The intensity of the bands was observed to be weak in the sample from cells infected with MVvac2(DIII-S)N, which supports the conclusion that the expressed hybrid antigen degraded and misfolded. In addition, a western blot containing the same samples was also developed with antibodies against the N protein of MV to corroborate that the same amount of each sample were used (data not shown). Therefore, we were able to confirm the expression of the correct DENV-2 epitope present in DIII from one of our recombinant MV, MVvac2(DIII-S,S)P. However, we suggest that MVvac2(DIII-S)N may be expressing an incorrectly folded hybrid DIII-HBsAg.

The next experiment that we performed was done to confirm the successful construction of hybrid viral-like particles. This was done by using a discontinuous sucrose gradient to fractionate by density samples collected from supernatants of cells infected with MVvac2(DIII-S,S)P of MVvac2(HBsAg)N. Due to the inability of the anti-HBsAg antibody to recognize the hybrid antigen produced by MVvac2(DIII-S)N (Fig. 21. B), this experiment was focused on the first recombinant virus MVvac2(DIII-S,S)P. MVvac2(HBsAg)N was used as a positive control, and cells were infected with the reference strain in parallel, because their ability to secrete VLPs following a specific sucrose density has been previously documented by other researchers. In the western blot

of the nine fractions collected from the supernatants of cells infected with MVvac2(DIII-S,S)P, developed with anti-HBsAg, we observed the upward shifted double bands corresponding to the hybrid DIII-HBsAg bands in fractions 4 to 5, shown with arrows (Fig. 22). We also observe the double bands corresponding to the native HBsAg in the western blots from both the recombinant strain and reference strain fractions; these are also sequestered in fraction 4 to 5 at a sucrose density of 1.10-1.12 g/ml. Therefore, we can conclude that the biophysical characteristics of the HBsAg subviral particle were not changed or affected by the incorporation of DIII, because the hybrid VLPs and the native HBsAg VLPs were sequestered in the same fractions and at the same density. In addition, the last western blot that was done (Fig. 22. B) was developed by antibodies against the N protein of MV, using fractions collected from the supernatants of cells infected with MVvac2(DIII-S,S)P. This western blot shows that the MV viral particles are sequestered at more dense fractions, fraction 6 to 9. This supports the notion that HBsAg- and hybrid-VLPs are secreted independently of the MV viral particles and that the production of the VLPs did not negatively affect the production of MV viral particles. Overall, we confirmed the successful incorporation of DIII in the hybrid VLPs, without affecting the antigenic characteristics of the VLPs.

After we have established that the hybrid antigen DIII-HBsAg can be expressed by our recombinant MVs and that viable hybrid VLPs can be produced, we moved forward with the most important experiments in our animal models. Two animal experiments were performed, using two different types of transgenic mice; sera collected from these animal experiments were then used for an immunogenicity study. The first animal experiment was done in HDD-SLAM-IFNar^{KO} transgenic mice. These mice were

vaccinated with the controls: parental strain, MVvac2, reference strain, MVvac2(HBsAg)N, and the positive control, DENV-2; as well as the experimental recombinant virus, MVvac2(DIII-S,S)P. In the immunogenicity study against measles, we observed a four-fold reduction of neutralization in sera from mice vaccinated with MVvac2(DIII-S,S)P compared to the parental strain, MVvac2 (Fig. 23). However, we also observed a two-fold reduction of neutralization in sera from mice vaccinated with MVvac2(HBsAg)N. This data would support the theory of the decrease in viral fitness based on the amount of ATUs inserted into the MV genome. Insertion of one ATU resulted in a two-fold reduction in neutralization and the insertion of two ATUs resulted in a four-fold reduction in neutralization. This will be a valuable knowledge for future construction of recombinant MVs.

In the immunogenicity study against hepatitis B and DENV-2, we observed reactivity for both in our experimental sample (Fig. 23). The result of the anti-HBsAg ELISA showed that the sera from mice vaccinated with MVvac2(DIII-S,S)P were able to reach the protective titer of 10 mIU/ml. However, the eight-fold reduction in its anti-HBsAg titer may be caused by the use of HBsAg in creating the hybrid VLP, which may cause a specific amount of the antigenic epitope of HBsAg to be hidden from the surface of the VLPs. The result of the homemade ELISA to analyze the reactivity of the sera to DENV-2, shows two sera samples from mice vaccinated with MVvac2(DIII-S,S)P that clearly reacted to DENV-2. The sera that came from these two mice were also the ones that had the highest measles neutralization titer at 1:426 and 1:266, as well as the highest anti-HBsAg titer at 54 mIU/ml and 13 mIU/ml, from the group of 5 mice vaccinated with MVvac2(DIII-S,S)P, which supports the notion that a robust anti-HBsAg immunity is

accompanied by Dengue 2 recognition. Although the mice vaccinated with DENV-2 was supposed to be the positive control. We realized at a later time, that these mice were given a dose of 10^2 PFU instead of 10^5 PFU, which may have resulted in the lack of immune response that was induced in these animals. Overall, the ELISA against DENV-2 only shows reactivity of the sera, not quantifying its neutralization capability. Therefore, a neutralization assay for DENV-2 was performed from the animal sera collected from the second animal experiment, to present a more accurate data.

In the second animal experiment, huCD46Ge-IFNar^{KO} transgenic mice were vaccinated with the controls: the parental strain, MVvac2, and the reference strain, MVvac2(HBsAg)N; as well as the experimental recombinant MVs: MVvac2(DIII-S,S)P and MVvac2(DIII-S)N. Different groups of animal received different doses as shown in figure 24. In the immunogenicity study against measles, we observed a similar reduction in anti-MV neutralization titer in the sera collected from animals vaccinated with one dose of MVvac2(DIII-S,S)P, as the previous animal experiment. Again, we believe that this is caused by the reduced viral replication fitness due to the insertion of two ATUs, as documented *in vitro* from our multi-step growth kinetics (Fig. 18). However, we observed that this negative effect from the weaker viral fitness can be solved by the administration of a booster dose. The anti-MV neutralization titer of the sera collected from mice which received two doses of MVvac2(DIII-S,S)P is comparable to that of the parental strain, MVvac2 (Fig. 23. A). In addition, the other groups of animal which also received two doses, MVvac2(HBsAg)N and MVvac2(DIII-S)N, also showed an anti-MV neutralization titer comparable to the animals that was vaccinated with MVvac2.

The immunogenicity study against hepatitis B was done through the use of a commercial ELISA. There were no significant reactivity seen in the pooled sera collected from animals that received one dose of MVvac2(DIII-S,S)P, two doses of MVvac2(DIII-S)N, or as expected, one dose of MVvac2 (data not shown). However, we did observed an anti-HBsAg titer of 787 mIU/ml from mice vaccinated with two doses of MVvac2(DIII-S,S)P and 648 mIU/ml from mice vaccinated with two doses of MVvac2(HBsAg)N. As previously witnessed, the administration of two doses of the recombinant virus highly increases the immune response induced. In the immunogenicity study against DENV-2, two different types of neutralization assays were performed. The result of the first approach, recorded in $Log_{10}NI$, shows a strong neutralization capability of sera collected from mice vaccinated with two doses of MVvac2(DIII-S,S)P; although the mice that received only one dose was able to reach the protective titer for reference flaviviruses (such as YFV and JEV) of 0.7 LNI. This result gave additional support to the theory that the administration of a booster shot may be needed to induce a completely protective immune response. On the other hand, the sera from mice vaccinated with two doses of MVvac2(DIII-S)N did not reach a protective titer. This may be caused by the improper folding of the hybrid DIII-HBsAg, resulting in the improper construction of the hybrid VLPs produced by this recombinant virus, as we have previously suggested. The second neutralization assay done against DENV-2, was the classic $PRNT_{50}$. Once more the result of the neutralization capability of the sera collected from the mice that received two doses of MVvac2(DIII-S,S)P was shown to be the strongest. However, the pooled sera from mice that received only one dose of MVvac2(DIII-S,S)P had a lower $PRNT_{50}$ titer than those that received two doses of MVvac2(DIII-S)N. It is possible that this

happened due to the presence of a few properly expressed DENV-2 epitopes that was previously seen in a western blot developed with anti-DIII DENV-2 (Fig. 21. B); this may have caused a minimal immunogenicity reaction against DENV-2 in this neutralization assay. Throughout the immunogenicity study, the animals that received two doses of MVvac2(DIII-S,S)P consistently showed higher immunogenicity against all three viruses.

The stability of the recombinant viruses could be observed through the presence of the bands in an immunoblot from samples of different passages. MVvac2(DIII-S,S)P have been amplified to reach passage 5, and the double bands corresponding to the DIII-S antigen from these samples can still be observed in an immunoblot. However, MVvac2(DIII-S)N have only been amplified to passage 3, and this was the passage used in all of the experiments concerning this recombinant MV. Further experiments are still needed to fully confirm the stability of the recombinant MVs, which include sequencing each passage of the virus as well as a serial passaging experiment. The result from these experiments will then be able to determine whether the viral genome remains unaffected through multiple passages.

Overall, we were able to confirm the successful construction of the recombinant MVs and analyzed their capability of eliciting immune response in the immunogenicity studies. In MVvac2(DIII-S,S)P, we demonstrated the correct assembly of our hybrid antigen and the release of the hybrid subviral particles in the supernatants of infected cells. In addition, this recombinant MV was able to induce a protective neutralizing immune response against all three viruses: MV, HBV, and DENV-2. Although we were not able to detect the successful secretion of the hybrid antigen from MVvac2(DIII-S)N

by anti-HBsAg, we confirmed that its replication fitness mirrored that of the parental strain, we were able to see it elicit immune response against measles, as well as express marginally some antigens that are recognized by the anti-DIII DENV-2. The result of our animal experiments also shows the importance of the vaccination dosage, as the animals that received two doses of MVvac2(DIII-S,S)P elicited the strongest immune response. Although we were not able to detect viable hybrid subviral particles secreted from MVvac2(DIII-S)N, we suggests that this is due to the lack of native HBsAg VLP that was available to scaffold the hybrid DIII-S antigen. In order to clarify this theory, we will perform a co-infection experiment using MVvac2(DIII-S)N and MVvac2(HBsAg)N

We decided to use HBsAg to create our hybrid VLP, because HBsAg independently creates its own VLP and it is also used in an approved human vaccine against hepatitis B. As shown in our results, the production of additional native HBsAg is necessary in the accurate assembly of the hybrid VLPs. In addition, our use of MV as the viral vector serves the purpose of a live adjuvant in the form of a live attenuated virus, which has been proven to be safe and stable.

Chapter 5. Conclusion

This is a proof-of concept for an ideal pediatric vaccine that would protect an individual against DENV, Hepatitis B virus, and MV. Using MV as a viral vector will make the production and distribution of this recombinant vaccine to be efficient and cost effective; mainly due to the system that is already in place for the production and distribution of MV based vaccines. In addition, this vaccine would be a safe option, since the Moraten/Schwartz vaccine strain used has been proven to have long-term safety. Our data confirms immunogenicity in MV susceptible animal models against all three: DENV, Hepatitis B virus and MV. The heightened immune response seen in animal who received two doses suggests that to achieve complete protection in humans, booster vaccination may be necessary.

Chapter 6. Future Perspective

In future experiments, we would like to look for possibilities of increasing the viral fitness of our recombinant MVs. As well as the possibility of avoiding the decrease in viral fitness by inserting only one ATU into the MV genome, but inoculating *in vivo*, with two recombinant MVs expressing the additional HBsAg needed. To circumvent the replication problem that having two ATUs originate, we propose to invert the order of insertion: DIII-S after N and HBsAg after P, which will increase the expression of the hybrid antigen and conserve a scaffold of HBsAg to construct the particle. Alternatively, we can infect cells with two separate vectors and collect the supernatant form co-infected cells. Arguably, if both antigens are expressed in the same syncytia, then hybrid VLPs will be secreted, it is tempting to think that MV virion with two genome can be assembled and propose and original solution to multiple insertion.

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