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LIPID RAFT FORMATION WITH DENGUE VIRUS PROTEIN NS1 INDUCES IL-8 IN INFECTED CELLS

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ABSTRACT

Dengue virus studies have shown that viral-encoded surface protein NS1G is linked to the host cell membrane via a GPI linkage and may exist in lipid rafts. Lipid raft formation may be required for the viral induction of host IL-8. This project investigated this hypothesis by expressing NS1 in both HEK293A and HeLa cells followed by treatment with methyl β cyclodextrin to block lipid raft formation. The levels of secreted IL-8 were assayed using a luciferase reporter under the control of an IL-8 promoter. The results confirm that lipid raft formation is required for IL-8 promoter activation.

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BACKGROUND

Dengue Incidence

Since its original isolation in 1943, dengue virus (DENV) has become an increasing concern worldwide (Mahy and van Regenmottel, 2007). Potentially 2.5 billion people, or two fifths of the world population, are now at risk for DENV worldwide, with 50 million cases annually and 20,000 deaths (WHO, 2009). Since 1970, there has been a tenfold increase of countries reporting DENV incidents, and it is now endemic in 100 countries, including tropical and subtropical regions (WHO, 2009). The most severe outbreaks used to occur in Southeast Asia and the Western Pacific, however recent studies show that Central and South America now report 70% of all cases worldwide (Teixeira and Barreto, 2009). In 2007, more than 890,000 cases were reported in the Americas (WHO, 2009). These figures are expected to rise, as the outbreaks spread deeper into the United States. Recently, reports of outbreaks have surfaced in Hawaii, the Texas-Mexico border, and Puerto Rico (Rothman and Mathew, 2008).

Dengue Symptoms and Classification

DENV can present symptoms either as dengue fever (DF) or dengue hemorrhagic fever (DHF). The majority of DENV infections are asymptomatic. DF, sometimes known as "breakbone fever", usually consists of an acute, self-limiting fever lasting 3-7 days (Rothman and Mathew, 2008). Other symptoms may include intense headache, painful joints and muscles, pain behind the eyes, and sometimes a rash (WHO, 2009). More severe symptoms may lead to vascular leakage, the hallmark of DHF. DHF symptoms include a fever lasting two to seven

days, hemorrhagic tendencies, plasma leakage and thrombocytopenia (Teixeira and Barreto, 2009).

The World Health Organization has classified dengue virus infection into four grades. Subjects with Grade I display a fever accompanied by other nonspecific symptoms. This is considered either DF or DHF. Grade II subjects have DHF, and suffer from spontaneous hemorrhagic manifestations. Grade III subjects show circulatory failure with a rapid yet weak pulse, and a diminished blood pressure (20 mmHg or less). Grade IV subjects suffer from profound shock and undetectable pulse and blood pressure (WHO, 2009). DHF may lead to Dengue Shock Syndrome (DSS). DSS is defined as circulatory failure represented by a rapid and weak pulse and hypotension, in addition to hemorrhagic tendencies (Teixeira and Barrito, 2009). Grades III and IV can be considered either DFH or DSS (WHO, 1999).

According to the WHO, DHF causes 500,000 hospitalizations annually, mostly in children. Of the 890,000 reported cases of dengue infection in 2007, 26,000 of those were classified with DHF. Although patients classified with DHF have a 2.5% mortality rate, this number can rise to approximately 20% in regions where proper medical treatment is not available (WHO, 2009).

Dengue Prevention and Treatments

Current disease prevention is limited to eradicating the mosquito vector via insecticides and by removing structures containing standing water (old tires, gutters, etc.) to decrease the spread of infected mosquitoes. Although some viral diseases can be prevented by vaccines, recent attempts to develop an effective, safe, economical dengue virus vaccine have been

unsuccessful. Current treatments for DHF patients include controlling febrile symptoms and replenishing plasma after plasma leakage.

Dengue Virus Serotypes

Four different serotypes of DENV have been isolated and studied. These serotypes are designated dengue-1 (DEN-1), dengue-2 (DEN-2), dengue-3 (DEN-3), and dengue-4 (DEN-4). Although the serotypes share 60-80% homology with each other, they remain structurally different (Mahy and van Regenmottel, 2007).

A primary infection with any one of the four serotypes by means of a mosquito bite leads to lifelong immunity to that serotype. Secondary infection requires productive infection by a different DENV serotype, heterologous from the primary infecting serotype, and leads to 90% of all severe cases, increasing the risk of DHF by 15-80 fold. Thus, one antibody type only provides partial short-term protection against other DENV serotypes (Sabin, 1952). A challenge for any vaccine under development is protection against DHF. Furthermore, immunizing against a single serotype, or incomplete vaccination against any single serotype, may lead to increased risk of a move severe infection (Rothman, 2004).

Dengue Prevention and Vaccines

Several attempts have been made to develop a vaccine that targets all four DENV serotypes. Live attenuated strains of all four DENV serotypes are now in Phase I and II clinical trials. These strains include all DENV antigens, replicate *in vivo*, elicit T and B cell memory, provide a strong durable immune response in mice, and provide protection in animal models. However, this attenuated virus approach has met difficulties without a fully valid animal model

that mimics all aspects of a human infection (Bhamarapravati and Sutee, 2000; Eckels et al., 2003).

Chimeric flaviviruses have also been studied as potential DENV vaccines. This approach includes inserting the pre-M and E genes of one of four DENV serotypes into a backbone derived from either Yellow Fever virus or an attenuated DENV strain. Like the live attenuated strains, these chimeric viruses provide *in vivo* replication, elicit T and B cell memory, provide a strong durable immune response, and provide protection in available animal models (Guirakhoo et al., 2002; Huang et al., 2003). This chimeric virus approach is currently in Phase I/II clinical trials.

A third approach at DENV vaccine development is using DNA plasmids expressing one or several DENV proteins from each serotype. This DNA vaccine approach is an excellent way to elicit T and B cell memory, does not replicate viruses *in vivo*, provides protection in animal models, and is anticipated to provide a durable immune response. However, this approach is only in preclinical trials (Chang et al., 2001; Simmons et al., 2001).

Cytokines and Chemokines Signaling in DENV Infection

Cytokines are small proteins (~25 kDa) released by various cells in the body as part of an immune response to an activating stimulus. Cytokine receptors are generally (but not always) heterodimers, which are categorized based on their shape and function. Cytokines can be chemo-attractants for immune cells (chemokines), can inhibit viral replication, induce the differentiation of T-cells, or mediate inflammation.

Chemokines are mediators of natural immunity and play a major role in the innate immune system. Their main function is to recruit leukocytes to site of infection and to mediate

lymphocyte trafficking. CXC chemokine receptors at their N-termini include two cysteines (C) separated by any amino acid (X). Seventeen mammalian CXC chemokines are currently known. CXC chemokines are further subdivided into glutamic acid-leucine-arginine positive (ELR) types, and ELR-negative types. Interleukin-8 (IL-8 or CXC-8) analyzed in this MQP project is an example of an ELR-positive chemokine. ELR-positive chemokines induce the migration of neutrophils, and interact with chemokine receptors CXCR1 and CXCR2. Monocytes, macrophages, fibroblasts, and endothelial cells produce IL-8, which then recruits neutrophils, basophils, and effector memory T cells to the site of infection through the CXCR1 and CXCR2 receptors.

Pathogenesis of DF and DHF

DENV predominantly targets dendritic cells and monocyte macrophages, but it is also known to infect B-cells and hepatocytes. When infecting the target cells, the E protein aids in the attachment of the virus to a target cell (Seema and Jain, 2005). Once attached to the host cell, viral infection occurs via endocytosis, then uncoating and expression of the viral genome (Marsh and Helenius, 1989). This is followed by the assembly of new virus in the cell, release of the virions, and attachment of the virus to receptors on another uninfected cell (Marsh and Helenius, 1989).

During primary DENV infection, inflammatory cytokines are released triggering an adaptive immune response including T cells, NK cells, and B cells responding to the virus. Naïve T cells that show specificity for the invading serotype expand and mount a response. As part of this response, T cells also release inflammatory mediators (Buchy et al., 2007). Primary

infection tends to last three to seven days, rarely requires hospitalization, and is not associated with severe symptoms (Halstead, 1980).

In the 1960's, a study concluded that greater than 85% of children suffering from DHF in Bangkok showed high dengue serotype cross-reactive antibody titers (Halstead et al., 1970). This started discussions that DHF may be related to a secondary infection. Subsequent research confirmed that DHF is substantially more common in secondary infections.

Antibody-Dependent Enhancement

Many theories contribute to the understanding of pathogenesis of secondary infection. One theory proposed is antibody-dependent enhancement (ADE). According to this theory, non-neutralizing antibodies bound to the DENV virus are taken up by cells containing the Fc receptor. This viral entry is mediated by antibodies specific for DENV from the primary infection (Gollins and Poterfield, 1969). Because antibody-bound virus attaches to the Fc receptors more efficiently than in a primary infection, there is an enhancement of penetration and fusion of the virion envelope with the membrane (Halstead, 1988). ADE increases the number of infected cells which then causes increased viral load and increased vascular permeability (Halstead, 1989). IgG type antibodies specific for E and NS1 antigens expressed on the surface of the infected cell are the primary antibody response to a secondary infection (Halstead, 1988). Results have shown evidence of ADE *in vitro*, however due to difficulties with animal models, *in vivo* results are limited (Rothman, 2009). Non-human primate models have been used to study antibody responses during secondary dengue infection. These studies have shown an increase of viremia in primates receiving passive immunity with DENV-specific antibody (Halstead, 1979;

Goncalvez et al., 2007). Although this provides *in vivo* evidence for ADE, the primate model is still controversial as it does not correspond with human pathogenesis.

T Cell Mediated Immunopathogenesis and Cytokine Storm

Another model to explain the observed plasma leakage during secondary infection is referred to as the T cell mediated immunopathogenesis. T-cell mediated immunopathogenesis causes a cytokine storm. According to this model, a more rapid reactivation of memory DENV-specific T cells due to an increased presentation of antigens on infected cells leads to a stronger secondary infection. The cross-reactive memory T cells still present from the primary infection have a lower affinity to the current serotype and become activated after the secondary infection. These memory T cells outcompete naïve T cells for the infecting serotype, and therefore alter the T cell response to result in a higher activation of T cells during a severe disease, a more rapid cytokine production, and an expansion of the lower avidity memory T cells.

The increased over-production of cytokines may lead to plasma leakage. A T cell response to DENV infection includes the secretion of Th1 cytokines, such as interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), lymphotoxin A (LTA), macrophage inflammatory protein 1β (MIP-1β), and IL-2. Small amounts of the Th2 cytokine, IL-4, are also secreted (Kurane and Ennis, 1989). IFN-γ activates monocytes and macrophages, and has been shown to upregulate the expression of Fcγ receptors and HLA (Gagnon et al., 1999; Goncalvez et al., 2007). IFN-γ is elevated in both DF and DHF patients (Kurane et al., 1991; Hober et al., 1993). Higher peak levels of IFN-γ were found in patients with DHF (Green et al., 1999).

TNF-α is produced by monocytes and macrophages, and has been shown to produce an increase in vascular permeability (Kurane and Ennis, 1988). IL-2 is a cytokine produced by Th1

cells which activate T and B cells, and NK (Natural Killer) cells. IL-2 has been associated with capillary leak syndrome and thrombocytopenia (He et al., 1995).

IL-2 is highly elevated in both DF and DHF patients. The soluble IL-2 receptor (sIL-2R) displayed much higher levels in DHF patients compared to DF patients (Boonpucknavig et al., 1979). IL-8 is also believed to play a role in DHF. Levels of IL-8 are elevated in DHF patients compared to DF patients (Raghupathy et al**.,** 1998). DENV has been shown to increase IFN-γ, TNF-α, LTA, MIP-1β, IL-2, and IL-8 secretion leading to an over-production of these cytokines and severe disease, particularly in DEN-2 and DEN-3 infections (Leitmeyer et al., 1999; Vaughn et al., 2000; Nogueira et al., 2002). Higher levels of IL-13, IL-18, IL-1β, IL-6, and IL-10 were also observed in DF and DHF patients (Hober et al., 1993; Green et al., 1999; Mustafa et al., 2001). IL-10 is an anti-inflammatory cytokine that displayed higher levels in DHF patients than DF patients (Green et al., 1999). The systemic over-production of cytokines during a secondary infection of DENV causing inflammation and plasma leakage results in a "cytokine storm".

In addition, this model also incorporates the previous model, as non-neutralizing antibodies present after a primary infection are thought to heighten uptake of the virus into antigen presenting cells. This causes a greater T cell activation, further increasing the likelihood of plasma leakage (Rothman and Mathew, 2008).

Dengue Virus

Structure

All flavivirus genomes consist of a single, positive-stranded RNA of 11 kb with a 5' type I cap, a methylated N2 residue, but lack a 3' polyadenylated tail. The genome (**Figure-1**) encodes a single long open reading frame surrounded by 5' and 3' noncoding regions (NCRs)

consisting of about 100 nucleotides, and 400 to 700 nucleotides, respectively. The 5'NCR consists of hairpin loops necessary for RNA translation and virus replication. The 3' NCR contains a 3' stem loop containing essential virus-specific and host-specific functional regions. The 3' NCR enhances the translation of a reporter mRNA containing this structure. Upstream from the 3' stem loop resides the CS1 region. The CS1 is a twenty-five nucleotide region which pairs with a complementary sequence in the 5'CS region (Lindenbach et al., 2007).

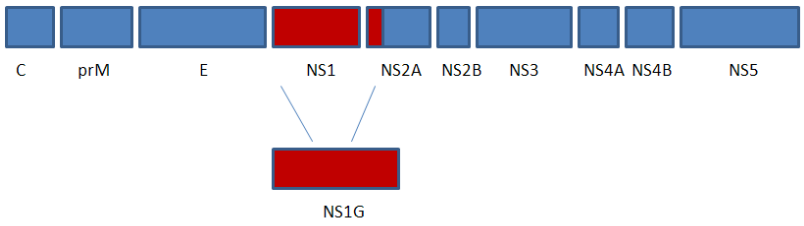

Figure 1: The Structural and Non-Structural Proteins of Dengue Virus. This project examined the NS1 protein (red in the diagram), in particularly the lipid rafts and GPI linkage on the NS1G protein. The NS1G protein is made up of NS1 and the first 26 amino acids of NS2A.

Translation of dengue virus RNA creates a single polyprotein that is co- and post-translationally processed by viral and cellular proteases. Cleavage of the polyprotein yields three structural proteins, and seven non-structural (NS) proteins in the order: (5') capsid (C), membrane (M), envelope (E), non structural (NS) proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5 (3'). Host cell enzymes (signal pepsidase or furin) or viral NS2B/NS3 cleave this polyprotein to yield the individual structural and nonstructural proteins (Standler et al., 1997; Jacobs et al., 2007).

DENV Classification

DENV belongs to the family *Flaviviridae,* genus *Flavivirus,* and is transmitted through the mosquito vector *Aedes aegypti*. Flaviviruses are enveloped, positive-stranded RNA viruses. DENV shares the *Flaviviridae* classification with eight other viruses with similarities in both structural and non-structural (NS) proteins. Other family members include: Aroa virus, Japanese encephalitis, Kolobera virus, West Nile virus, and Yellow fever virus. DENV also belongs to a larger heterogeneous group of viruses transmitted by insect vectors, called arboviruses. For these viruses, the transmission to vertebrate hosts is dependent on arthropod vectors, which for DENV is *Aedes aegypti*. Currently about two thirds of the world population live in areas with *Aedes aegypti* (Pinheiro and Corber, 1997).

The single-stranded RNA of flavivivurses encodes at least ten known proteins: nucleocapsid core protein (C), membrane associated protein (M), envelope protein (E), and non-structural proteins (NS) NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Mahy and van Regenmottel, 2007).

DENV Replication

The DENV virion is a spherical enveloped virus that has a diameter approximately 50 nm consisting of the three structure proteins (C), (M), (E), and the virus RNA. Virions enter the cell by means of receptor-mediated endocytosis (Clyde and Harris, 2006) through an unknown receptor. The E protein mediates the fusion of the viral membrane and plasma membranes. The RNA genome enters the cytoplasm and dissociates from the nucleocapsid protein to be translated as mRNA into one polyprotein which is both co- and post-translationally processed by viral and host proteases. The flavivirus genome is replicated on intracellular membranes. Newly

synthesized RNA enters the lumen of the endoplasmic reticulum (ER) where assembly occurs. After assembly in the ER, virions then enter the Golgi network (Whitehead et al., 2007). Mature infectious virions are created in the trans-Golgi network when the membrane precursor, prM, is cleaved by furin. At this point the M and E proteins rearrange on the virion surface yielding mature infections virions (Mukhopadhyay et al., 2005).

The mature DENV virion has a smooth surface, with E proteins laying in pairs parallel to the virion surface. The E glycoprotein can be divided into three structural domains which either aid in cell attachment, fusion, or targeting by protective antibodies. These are the central domain, the dimerization domain which presents a fusion peptide, and the receptor-binding domain. (Kuhn et al., 2002).

A. *NS1, NS1G, NS2A, and GPI Linkages*

Non structural protein 1 (NS1) is expressed and secreted on the surface of cells infected with DENV. It is hydrophobic, lacks a membrane spanning domain, and has been shown to be involved in viral RNA replication. It has been proposed that NS1 can activate the complement system (Kurso et al., 2007), and increase viral severity by NS1 antibody (Shu et al., 2000; Libraty et al., 2002). NS1 is initially translocated into the ER by a hydrophobic signal sequence (Falgout et al., 1989), NS1 goes through rapid dimerization before being transported through the Golgi apparatus where mannose carbohydrates are trimmed from the molecule (Winkler et al., 1989).

The NS2A protein is hydrophobic, and lies directly downstream from the 3' hydrophilic NS1 region. The beginning of this hydrophobic protein has a 26 amino acid sequence and displays similarities to the carboxyl terminal sequences present in eukaryotic proteins before being processed to attach a glycosylphosphatidylinositol (GPI) linkage (Ferguson et al., 1988).

A recent study showed that by reconstructing the NS1 protein to include the first twenty-six amino acids of the NS2A protein yields a possible GPI linkage. It is currently unknown whether NS2A is indeed GPI-linked, but if so, this would allow for signal transduction and viral replication (Jacobs, 2000).

The NS1G protein is a combination of the NS1 and NS2A. NS1G has been shown to be attached by a GPI linkage to the cell surface membrane that allows for signal transduction to occur in response to binding of NS1-specific antibody (Jacobs, 2000). The enzyme in the ER responsible for cleavage of the NS1/NS2A junction has not been identified (Falgout and Markoff, 1995).

The specific role of a GPI linkage is unknown. Many eukaryotic cell surface proteins have been observed anchored in the plasma membrane by a GPI linkage (Rothman and Ennis, 1999) (**Figure-2**).

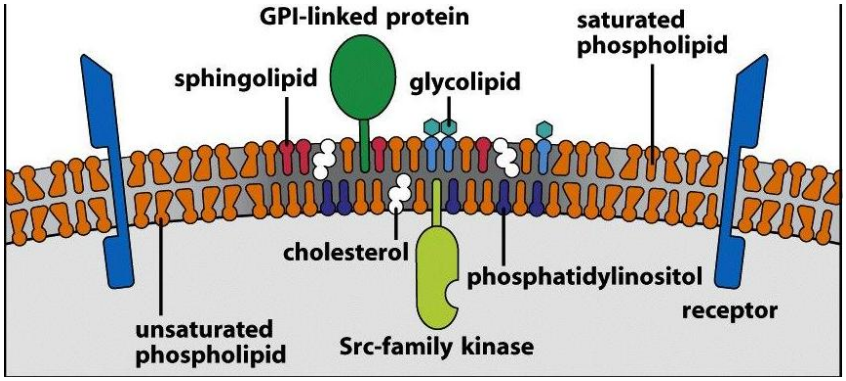

Figure 2: The Dengue NS1G Protein is anchored by a GPI Linkage on the Cell Surface. In this project, GPI linkages (similar to the protein shown in green) and lipid rafts were examined to understand signaling events causing IL-8 promoter activation (Murphey et al., 2008).

In order to add a GPI linkage, cleavage of a hydrophobic carboxyl-terminal signal sequence in the ER must first occur. This is followed by the covalent attachment of a preformed GPI

precursor. The complex is then targeted to the outer surface of the plasma membrane. GPI linkages may facilitate signal transduction or the transfer of proteins between the cell surfaces of different cells (Udenfriend and Kodukula, 1995).

Lipid rafts are a cluster of liquid phase microdomains in the plasma membrane, made up mostly of cholesterol and sphingolipids (**Figure-3**). Lipid rafts have been known to have several functions, including signal transduction, endocytosis, transcytosis across the endothelial membrane, and cholesterol homeostasis (Ikonen, 2001). Some viruses including DENV have been shown to interact with lipid rafts (Parton and Lindsay, 1999).

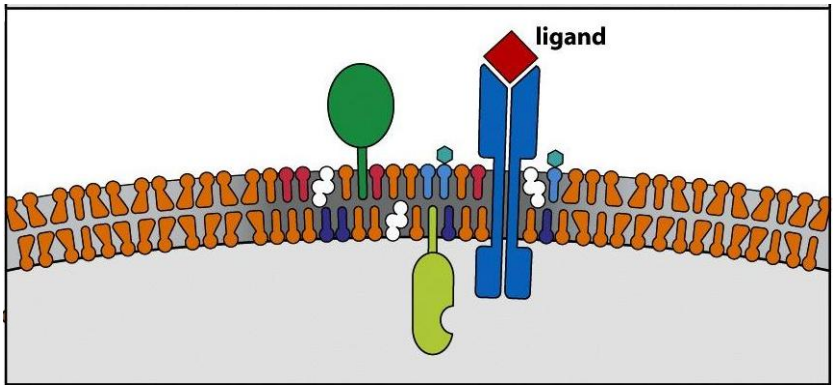

Figure 3: The Dengue NS1G Protein Associates with Lipid Rafts. Constructed mostly of cholesterol and sphingolipids localized to a clustered location, lipid rafts (diagram central) can be found in the plasma membrane. They are involved in many cellular events, including cell signaling, endocytosis, transcytosis, and cholesterol homeostasis. In this project, the relationship between lipid rafts in the Dengue NS1G protein and promoter activation of IL-8 were examined (Diagram from Murphey et al., 2008).

B. *Non-Structural Proteins (NS5) and IL-8*

Interleukin-8 (IL-8) is a small, secreted molecule alternatively called C-X-C motif chemokine 8 (CXCL-8). IL-8 is a proinflammatory chemokine responsible for the recruitment of basophils, neutrophils, T cells, and is a mediator of the inflammatory response. IL-8 functions as an activator of Beta-2 integrin for binding leukocytes. IL-8 can also up-regulate adhesion

molecules to increase membrane permeability to allow for the migration of monocytes and capillary leakage (Murphey et al., 2008). IL-8 can be induced in a variety of cell types such as leukocytes, tissue cells, and tumor cells. IL-8 can also be induced by several different stimuli, such as bacteria and viral products (Galkina-Taylor, 2010). IL-8 induction may lead to the enhanced replication of certain viruses, such as encephalomyocarditis virus, poliovirus, and human immunodeficiency viruses-1 and 2 (Muravama et al., 1994; Khabar et al., 1997; Polyak et al., 2001; Lane et al., 2001).

IL-8 induction by DENV NS5 provides a possible explanation for the production of chemokines in a DEN2V infection. NS5 is the viral polymerase, and functions as a methyl transferase. It is a large multifunctional protein that is located in both the cytosol and the nucleus. During late-stage DENV viral infection, NS5 dissociates from NS3, which exposes a nuclear localization signal. The secretion of IL-8 late in infection correlates with the movement of NS5 to the nucleus (Kapoor et al., 1995).

Endothelial cells infected with DENV have been shown to produce IL-8, activate complement, and may undergo apoptosis (Mangada et al., 2002). Recent studies have shown that the secretion of IL-8 is linked to disease severity (Rothman, 2002). Patients with acute DENV were reported to have elevated levels of IL-8 in their blood serum (Mangada and Rothman, 2005). IL-8 secretion induced by DEN2V NS5 protein is thought to be due to activation by the CAAT/enhancer binding protein (c/EBP) and NF-kB activity (Medin et al., 2005). The control of IL-8 expression at the transcriptional level by NF-kB is tightly linked to viral titres and viral antigens. However, the complete mechanism for IL-8 production during DENV is not yet known.

C. *NS1, NS1G, and IL-8*

NS1 glycoprotein is translocated into the ER during synthesis and is cleaved from the E protein by host signal peptidase. An unknown enzyme in the host ER cleaves the NS1/2A junction. Eight C-terminal residues of NS1 and more than 140 amino acids of NS2A are required for this cleavage.

NS1 has an important, yet unclear role in RNA replication. NS1 localizes to sites of RNA replication, and mutation of the N-linked glycosylation sites in NS1 leads to dramatic defects in both RNA and virus production. After about 30 minutes of synthesis, NS1 forms a very stable homodimer, and acquires affinity for membranes. Since its amino acid content is hydrophilic and lacks transmembrane domains, there are two possibilities for its membrane affinity. One possibility is that dimerization creates a hydrophobic surface for peripheral membrane association. The other possibility is it is linked to the cell membrane via a GPI-anchor. However, GPI-anchoring is currently reported only for DEN-2 NS1.

NS1G is a combination of NS1 and NS2A that has been shown to be attached by a GPI linkage to the cell surface membrane (Jacobs, 2000). Studies have shown an increase in promoter activation of IL-8 when human epithelial cell lines have been transfected with NS1G, but the exact mechanism for IL-8 induction has not been determined.

PROJECT PURPOSE

Recent studies have shown that protein NS5 of DEN2V induces a dose-dependent IL-8 activation via CAAT/enhancer binding protein (c/EBP) and the NF-κB signal pathway (Medin et al., 2005). Unpublished research has suggested that DENV NS1-transfected cells do not induce IL-8 production, while cells transfected with Dengue NS1G (containing a GPI anchor) display a dose-dependent induction of IL-8. The presence of a GPI linkage and an association with lipid rafts for the NS1G protein of DENV has been confirmed (Jacobs, 2000; Noisakran, 2008). It is hypothesized that the GPI linkage and association with lipid rafts for the NS1G protein causes signal transduction pathways to stimulate IL-8 promoter activation.

This MQP will confirm the induction of IL-8 by the DENV NS1G protein. Promoter activation of IL-8 will be assayed by a luciferase reporter assay under the control of an IL-8 promoter. pcDNA encoding no DENV protein will used a negative control, and MAL (MyD88-adapter-like) (Fitzgerald et al, 2001) will be used as a positive control. NS1, NS1G, NS2A, and NS2B proteins will be tested for IL-8 promoter activation. IL-8 promoter activation will also be tested in transfected cells treated with methyl-β-cyclodextrin to block lipid raft formation, and treated with phospholipase-C to remove GPI-anchors, to determine the effects on IL8 promoter activation.

METHODS

Cell Lines

Human embryonic kidney cells (HEK293A) were obtained from Invitrogen, and maintained in high glucose Dulbecco's Modified Eagle's Medium (DMEM) from Gibco. The base medium was supplemented with 10% fetal bovine serum, nonessential amino acids (0.01 mM), penicillin (100 µg/ml), and streptomycin (100 µg/ml) all from Gibco. The cells were incubated in an atmosphere of 5% carbon dioxide at 37°C. When cells approached 80-95% confluence, the cell line was passaged every 3-4 days in T-75 flasks. Spent medium was aspirated from the flask, and cells were gently washed with phosphate buffer saline (PBS) (Gibco). The cell layer was rinsed with a solution containing 0.25% (w/v) Trypsin, 0.53 mM EDTA (Gibco) to disrupt the monolayer. Complete DMEM was added and mixed to stop the trypsin reaction. Cells were diluted 1:15 for routine passaging. The cell line was cryopreserved at a concentration of 2×10^6 cells per ml in freezing medium. Cells were placed inside a passive freezer (Nalgene Mr. Frosty- Prod. No. C1562) with isopropyl alcohol overnight at -80°C. The cells were then stored the following day in liquid nitrogen.

Plasmids

The negative control for both the luciferase assay and FACS analysis was pcDNA3.1 plasmid (Invitrogen). The positive control plasmid used for the luciferase assay was Mal (MyD88-adapter-like) (Fitzgerald et al., 2001). The plasmids used throughout the experiments were NS140, NS1G40, NS2A, NS2B, prM, E, NS4A, NS4B, and NS5. All were cloned using a forward primer for homologous recombination into the Gateway entry vector pDONR201

(Invitrogen) (Medin et al., 2005). NS1G 40 was cloned using a reverse primer from 3' – 5' for homologous recombination into the Gateway entry vector pDONR201 starting at 26 amino acids downstream of NS1, the NH_2 terminus of NS2A (Invitrogen) (Falgout et al., 1989). The luciferase reporter plasmid for all experiments, IL-8, was provided by Naofumi Mukaida (Okamoto et al., 1994). The renilla reporter plasmid used in all experiments, TKPGL4 (Promega) was obtained by the renilla plasmid in the PGL4 vector driven by the Thymidine Kinase (TK) promoter.

Inducing plasmids for the FACS analysis were obtained by homologous recombination using the Gateway System (Invitrogen). The positive control was eGFP Amaxa which encodes the GFP for all cell proteins(Invitrogen). The negative control pceGFP, and plasmids NS1eGFP and NS1GeGFP, were all obtained by insertion of the expression vector eGFP by homologous recombination using the Gateway System (Invitrogen). The negative control expression vector eGFP is comprised of pcDNA6.2/C-EmGFP-DEST to express the control plasmid containing no Dengue gene. Plasmids NS1eGFP and NS1GeGFP were inserted with eGFP comprised of pcDNA6.2/C-EmGFP-DEST which targeted the gene of choice (Invitrogen).

Transfections

24-Well Plate

Cells were transfected in flat bottom 24-well plates. The transfection was prepared using Lipofectamine 2000 (Invitrogen). DNA transfections for a 24-well plate, with a 1:5 ratio of 100 µg/ml DNA: Lipofectamine-2000 (µl). Lipofectamine-2000 was diluted in Serum Free (SF) DMEM. After combining the Lipofectamine + SF media mix to a volume of 100 µl/tube, the tubes were incubated for 20 minutes at room temperature. During incubation, the cells were

washed with PBS and media without antibiotics. DMEM was added to each well (Gibco) followed by the DNA/plasmid solution. Incubation was at 37°C for 48 and 72 hours.

96-Well Plate

The DNA was aliquoted (with equal volumes and concentrations) into a round bottom 96-well plate. The transfection was prepared by adding SF DMEM + Genejuice (Novagen) mastermix to plasmids according to the manufacturer's protocol (Novagen). The transfection mixture was then added to cells in triplicate, and incubated for 24 hours.

To obtain accurate results using a 24-well plate in the FACS analysis, the DNA concentration was increased based on the well volume. Consequently, the DNA mass concentration used in a 96-well luciferase assay was significantly smaller. All plasmids were isolated using PureYield Maxipreps (Promega), and were used at a concentration of 100 ng/µl.

Dual Luciferase Reporter Assay

After a 24 hour transfection, passive lysis buffer (Promega) was added to each plate. Luciferin, the firefly luciferase substrate from *photinus pyralis* (Promega) and coelenterazine, the renilla substrate from *Renilla Reniformis* (Biotium) were used. Both substrates were added to each plate where luciferase activities were determined by a plate reader (Envision PerkinElmer) using the dual-luciferase reporter assay according to the instructions of the manufacturer (Promega). Luminescence was read on the white plate (Costar). All conditions were tested in triplicate, and at least two independent experiments were performed for each assay (Medin et al., 2005).

Methyl-β-cyclodextrin Addition

HeLa cells were treated with varying doses (10 mM, 30 mM, and 50 mM) of methyl-β-cyclodextrin initially dissolved in nuclease-free water at 6 and 12 hours post-infection. Methyl-B-dextrin was diluted in complete growth media. After treatment, cells were place at 37°C, and luciferase was read at 48 hours post-infection.

Phosphatidylinositol-specific phospholipase C (*PI-PLC*) Treatments

HEK293A cells were treated with vehicle, PI-PLC (0.1 U/ml), or PI-PLC (1 U/ml). The vehicle contained 60% Glycerol, FACS Buffer (0.01% Sodium azide), and PBS. The PI-PLC stock contained 20 mM Tris-HCL, pH 7.5, 1 mM EDTA; 0.01% Sodium Azide, and 50% glycerol. Treatments were performed in triplicate at 6, 12, 18, and 24 hours post-infection. Luciferase was read at 24 hours post-infection.

Single Stain FACS Analysis

After both a 48 and 72 hour transfection, cells were pipette from a 24-well plate to polystyrene FACS tubes. Cells were washed by centrifugation at 2500 RPM for 5 minutes, the supernatant was aspirated, and cells were washed with PBS. Cells were washed again with FACS wash buffer, 1% FBS, 0.1% Sodium Azide (Gibco). After another centrifugation, Cytofix/Cytoperm (BD Biosciences) was added and incubated at 4°C for 20 minutes. Cells were then washed with Perm Wash Buffer (BD Biosciences), followed by the addition of 7E11 antibody. After a 30 minute incubation at 4°C, cells were washed with PBS. FITC anti-mouse IgG Sigma Chemical Co., St. Louis, MO) secondary antibody was diluted to 100 µg/µl and

added to cells with a 30 minute incubation at 4°C. Cells were again washed, and resuspended in FACS Buffer. To avoid light, cells were covered with foil and stored at 4°C until reading.

RESULTS

The purpose of this project was to confirm the preliminary findings that Dengue protein NS1 does not activate the IL-8 promoter, while NS1G (containing a GPI anchor) activates the IL-8 promoter. The project's goal was also to determine whether lipid raft formation is required for the NS1G IL8 induction. The activation of the IL-8 promoter was assayed using a luciferase reporter under the control of an IL-8 promoter. Plasmid pcDNA, encoding no DENV protein, was used a negative control, and MAL (MyD88-adapter-like) was used as a positive control. Plasmids encoding Dengue proteins NS1, NS1G, NS2A, and NS2B will be tested for IL-8 promoter activation. IL-8 promoter activation will also be tested in transfected cells treated with methyl-β-cyclodextrin to block lipid raft formation, or treated with PI-PLC to remove GPI-anchors.

To measure promoter activation of IL-8, HEK293A cells were transfected with negative control pcDNA, positive control MAL, and plasmids encoding NS1, NS1G, NS2A, and NS2B. A luciferase assay was performed 24 hours post-transfection. Averages of seven assays were taken (**Figure-4**). Protein NS1G displayed a strong dose-dependent increase in IL-8 promoter activation (diagram center), while NS1, NS2A, and NS2B displayed overall low levels of promoter activation and no dose-dependent increases. This confirms the previous data of NS1G IL-8 promoter activation in our laboratory (Medin et al., 2005). The assay was repeated in HeLa cells to confirm the dose dependency (data not shown), where the results displayed IL8 promoter activation only for the NS1G protein.

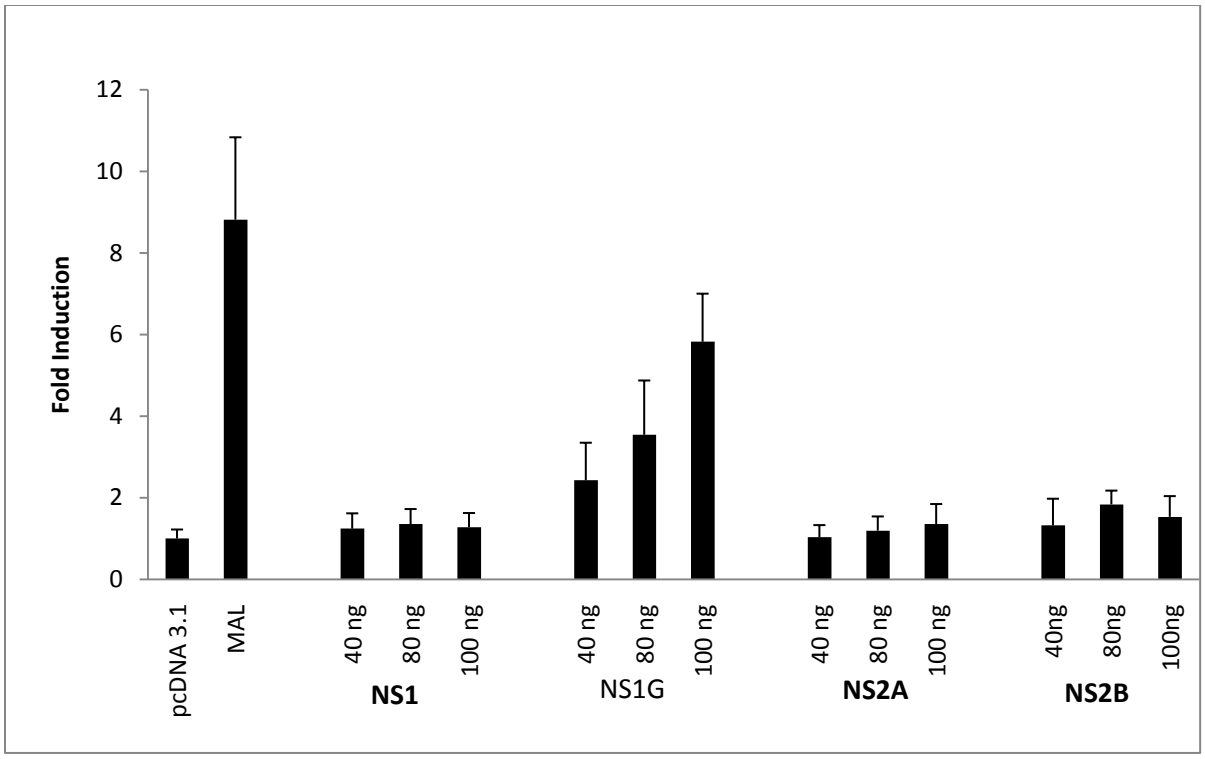

Figure 4. Assay of IL-8 Promoter Activation of Transfected HEK293A Cells. Each histobar represents the mean of thirty wells. Error bars denote standard error. Results are expressed as fold induction relative to cells transfected with pcDNA encoding no Dengue proteins.

Methyl Dextrin Experiments to Block Lipid Raft Formation

To test the effects of lipid raft formation on the ability of NS1G protein to activate the IL8 promoter, methyl-β-cyclodextrin was added to the HeLa cells at 6, 12, and 24 hours post transfection to disrupt cholesterol in the lipid bilayer (**Figures 5 and 6**). Concentrations of methyl-β-cyclodextrin used were 10, 25, 35, and 50 mM. After 48 hours incubation, a decrease in the IL-8 promoter activation was observed in the wells transfected with NS1G protein, indicating that lipid raft formation is needed for the NS1G-mediated IL8 activation. There was significantly less IL-8 promoter activation at the 6 hour cyclodextrin treatment (**Figure 5)** than for the 12 hour treatment **(Figure 6).** This data was strongly reproducible, as shown by two similar experiments in **Appendix-1**.

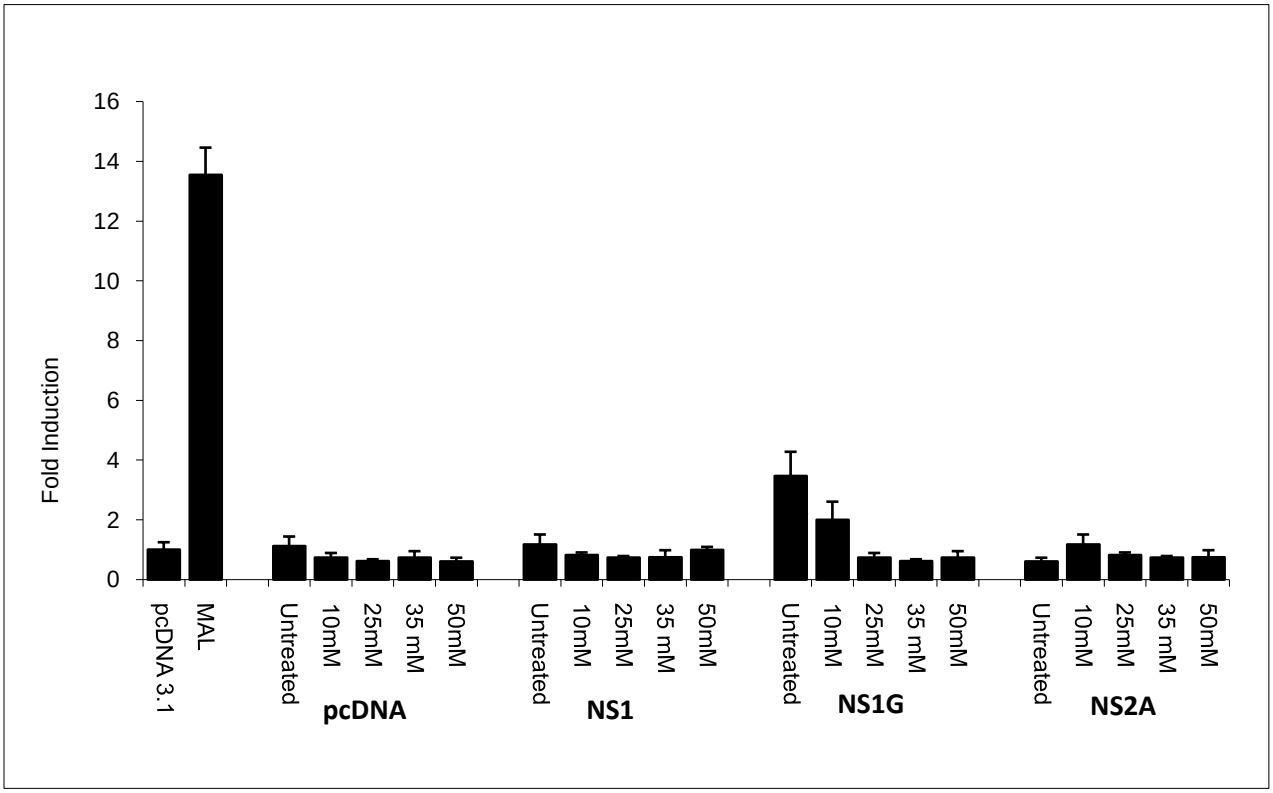

Figure 5. The Effect of Methyl-β-Dextrin Treatment on IL-8 Promoter Activation in HeLa Cells. Cells were treated with cyclodextrin at 6 hours post-transfection, and were read for luciferase at 48 hours post-transfection. Histobars represent the means of three wells. Error bars denote standard error. Data are shown as fold induction relative to cells transfected with pcDNA negative control plasmid.

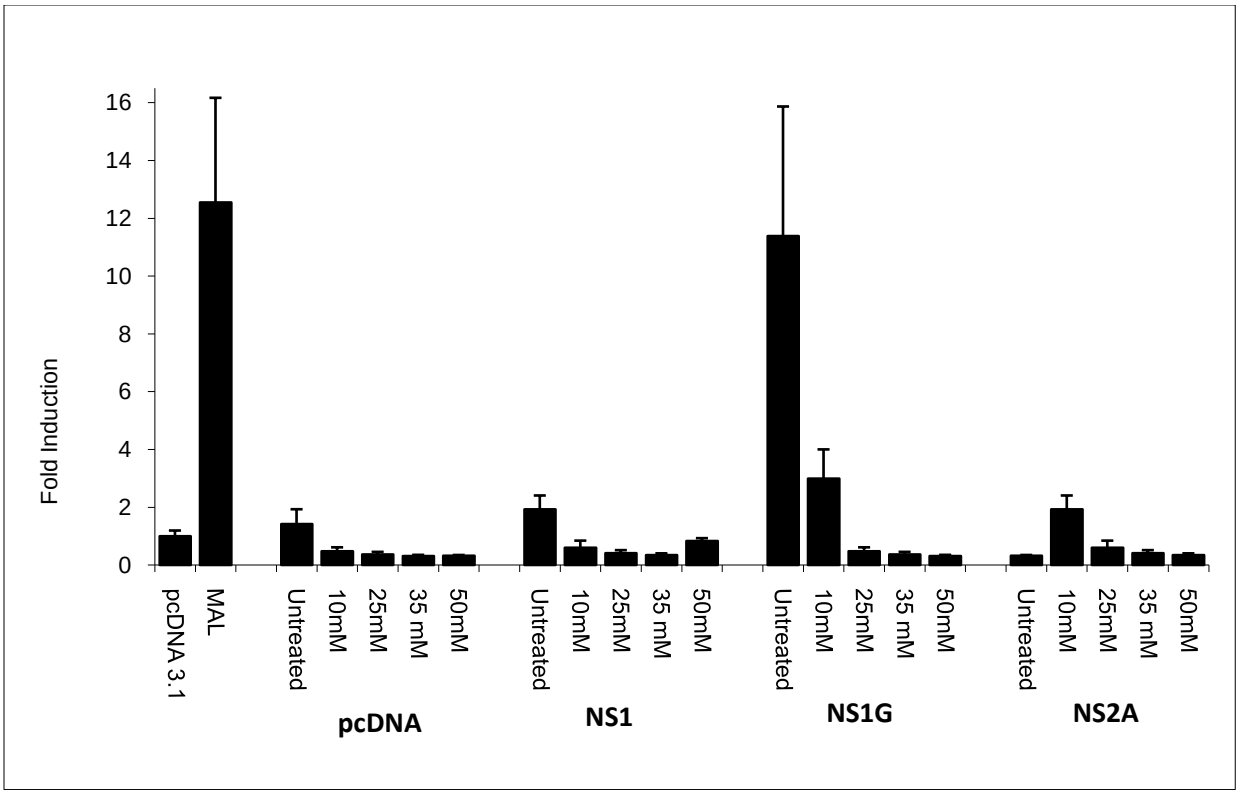

Figure 6. The Effect of Methyl-β-Dextrin Treatment on IL-8 Promoter Activation in HeLa Cells. Cells were treated with cyclodextrin at 12 hours post-transfection to decrease lipid raft formation, then read for luciferase activity at 48 hours post-transfection. Histobars represent the means of three wells. Error bars denote standard error. Data are shown as fold induction relative to cells transfected with pcDNA negative control plasmid.

PI-PLC Experiments to Degrade GPI Linkages

To test the possibility that a GPI linkage in the Dengue NS1G protein helps activates the IL-8 promoter, the enzyme Phosphatidylinositol-Specific Phospholipase-C (PI-PLC) was added to the cell growth medium at 18 hours and 22 hours post-infection, then the luciferase activity was measured at 48 hours post-infection (**Figures-7 and 8**). PI-PLC is an enzyme that cleaves most GPI linkages present in the cell membrane, which should disrupt the GPI linkage of NS1G to the cell membrane decreasing NS1G's function. The PLC treatment was added to cells transfected with pcDNA (negative control), MAL (positive control), NS1 (without a GPI anchor), NS1G (containing a GPI anchor), and NS2A. The results of the first experiment in

which the PLC treatment was performed 18 hrs post-transfection (**Figure-7**) unfortunately showed no strong increase in luciferase activity in the NS1G sample not treated with PLC (first histobar, fourth set), so this experiment is difficult to interpret. Further complicating the data is the fact that the PLC vehicle alone caused a strong increase in luciferase activity (middle histobar, fourth set). The PLC-treated sample showed a slight decrease from the vehicle sample, indicating the PLC treatment *might* have decreased IL8 promoter activation.

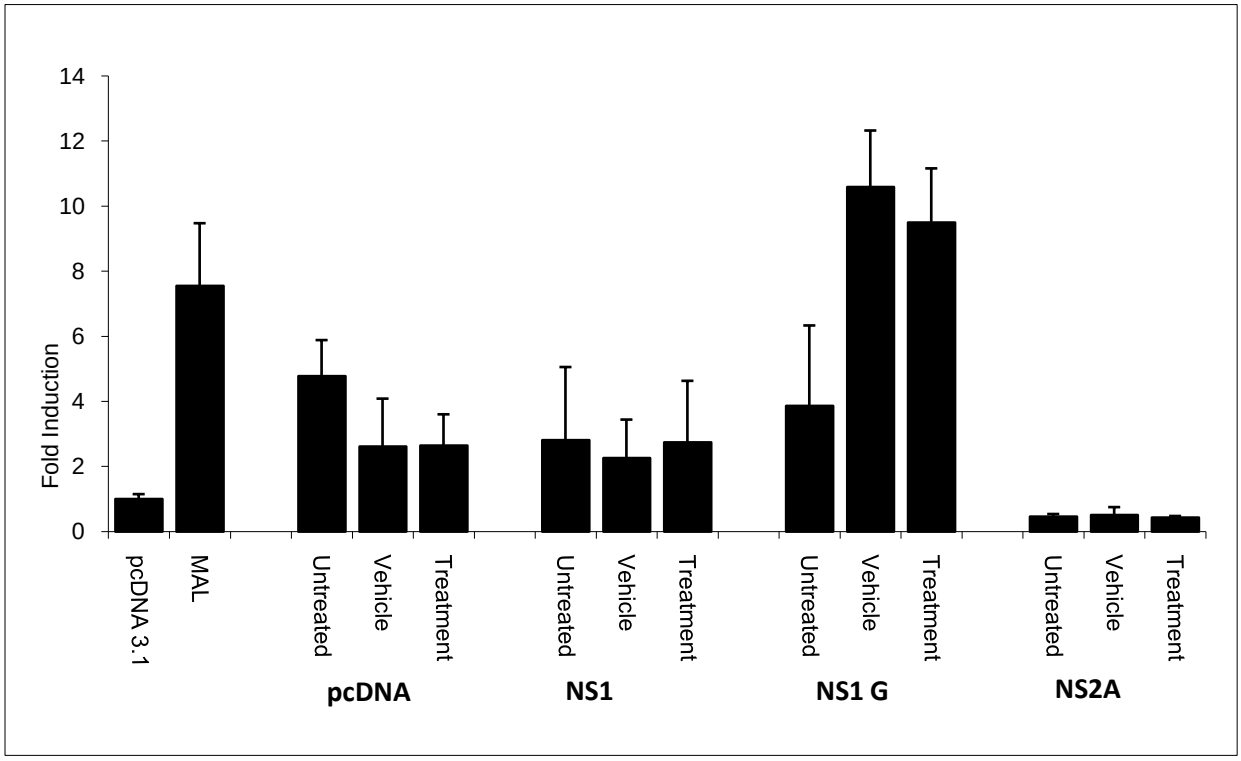

Figure 7. The Effect of PI-PLC Treatment of Transfected HEK293 Cells on IL-8 Promoter Activation . Cells were treated with PI-PLC at 18 hours post-transfection (histobars labeled "treatment"), then luciferase activity was read at 24 hours post-transfection. Histobars represent the means of three wells. Error bars denote standard error. Data are shown as fold induction relative to cells transfected with pcDNA negative control plasmid.

The experiment was repeated with the PLC treatment at 22 hours post-transfection (**Figure-8**), however the same overall trend in the data was observed, with a strong vehicle induced activation of luciferase activity, and a slight decrease in activity with the PLC treatment.

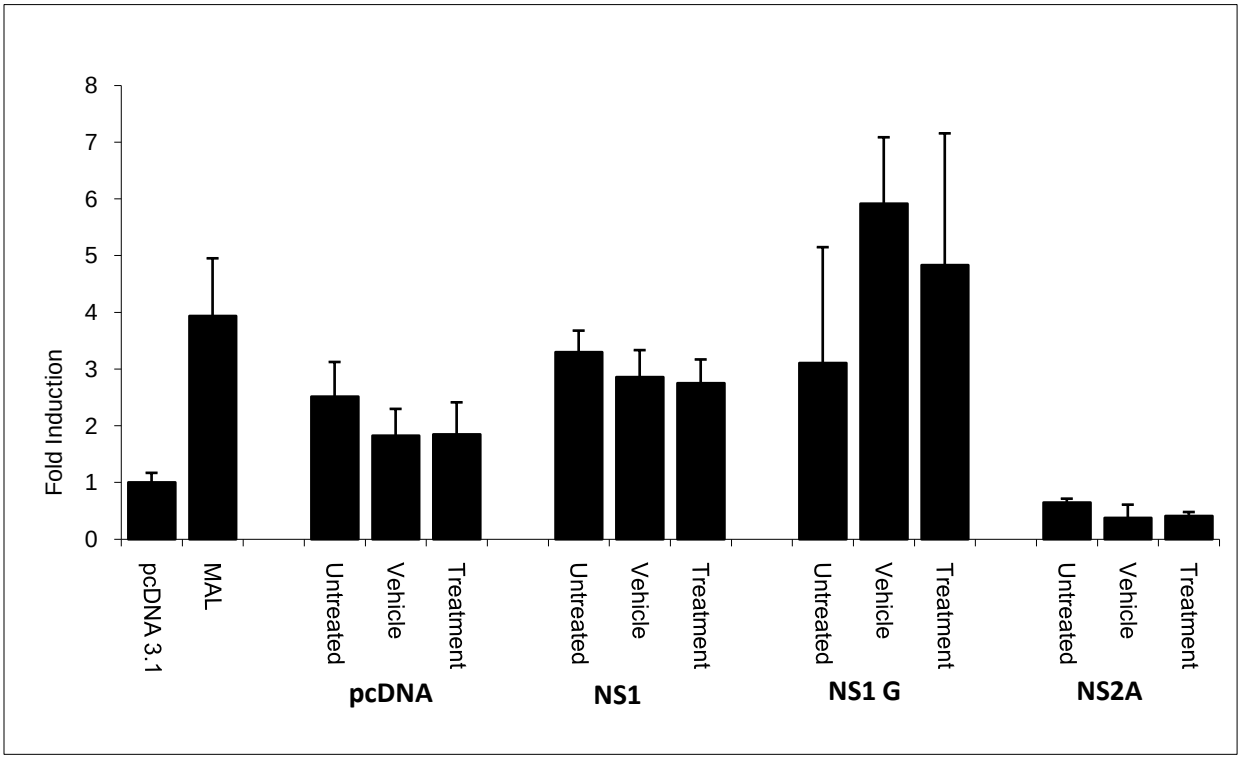

Figure 8. The Effect of PI-PLC Treatment 22 Hrs Post-Transfection on IL-8 Promoter Activation in HEK293A Cells. Experimental details are as described in the previous figure, except cells were treated with PLC at 22 hours post-transfection.

DISCUSSION

Our results demonstrate that DENV NS1G protein can help activate the IL-8 promoter in HeLa or HEK293 cells *in vitro*. The promoter activation is decreased in cells treated with methyl-β-cyclodextrin to decrease lipid raft formation, thus lipid raft formation is important for the activation. The effects of the NS1G GPI linkage on promoter activation is still not understood, as the PLC vehicle by itself strongly induced promoter activation (although the samples containing PLC did show a slight reduction in activity relative to the vehicle-treated samples).

Lipid rafts are cholesterol- and sphingolipid-rich congregations in the cell lipid bilayer that are resistant to solubilization by mild detergents. Rafts have been shown to help recruit signaling proteins to the plasma membrane (Murphey et al., 2008). Lipid rafts have also been observed as a platform for membrane traffic and protein sorting (Simons, 1997). Thy-1 proteins, GPI-linked proteins, and other acylated proteins such as Src kinases, are typically found in lipid rafts (Murphey et al., 2008).

The luciferase assays performed on HEK293A and HeLa cells transfected with NS1G encoding plasmid confirms a plasmid dose-dependency on IL-8 promoter activation. There is no plasmid dose-dependency for NS1, NS2A, or NS2B transfected cells. Thus, NS1G and the first twenty six amino acids downstream in NS2A appear to be responsible for the IL-8 promoter activation. Furthermore, recent studies have shown that NS1G associates with lipid rafts (Noisakran et al., 2008) and is anchored by a GPI linkage (Jacobs, 2000). This association suggests that NS1G protein may function in cellular signaling on the cell surface. In this project, NS1G was found to associate with lipid rafts causing promoter activation of IL-8.

The exact mechanism for DENV pathogenesis is still uncertain. The T cell mediated immunopathogenesis and cytokine storm model demonstrates a possibility for a severe secondary infection in dengue hemorrhagic fever. According to this model, cross reactive memory T cells present from a primary infection leads to a rapid reactivation due to increased antigen presentation. These higher affinity memory T cells out compete naïve T cells and are responsible for a systemic over-production of cytokines. These cytokines include IFN-γ, TNF-α, LTA, MIP-1β, IL-2, IL-4 and IL-8. The secretion of these cytokines leads to a cytokine storm. By finding a way to stop the induction of certain cytokines, it may be possible to stop the cytokine storm during secondary infection (Rothman, 2008). In this project, with the addition of methyl-β-cyclodextrin, a compound known to decrease lipid raft formation, a lowering of the NS1G-induced promoter activation of IL-8 was observed. This suggests that lipid rafts impact the promoter activation induced by the NS1G protein.

Our attempts to test the effects of PI-PLC treatment on the IL8 promoter activation were inconclusive. If a GPI linkage anchoring the NS1G protein to the cell membrane facilitates the signal transduction that results in the activation of the IL-8 promoter, cleaving the GPI linkage may decrease the activation. This GPI linkage could aid in the ability of NS1G to induce cytokines during secondary infection. In this study, the addition of the PI-PLC treatment at 6, 12, 18, and 22 hours post transfection resulted in background noise induced by the vehicle used to deliver the treatment. The vehicle included 60% Glycerol, FACS Buffer (0.01% Sodium azide) and PBS, so perhaps one of these components stressed the cell to activate the promoter.

This research shows a possible impact on the cytokine storm in dengue hemorrhagic fever. NS1G's association with lipid rafts provides one possible mechanism by which this viral protein can induce cytokines during secondary infection. Further studies on the contribution of

lipid rafts on NS1G function, and a further understanding of the pathogenesis of dengue virus will lead to other preventative techniques. The disruption of lipid rafts on the cell surface may lead to a new vaccination technique. Future research with PI-PLC might be effective by altering the vehicle. Also, a comparison of PI-PLC results with other known GPI-linked viral proteins could be informative.

APPENDIX 1

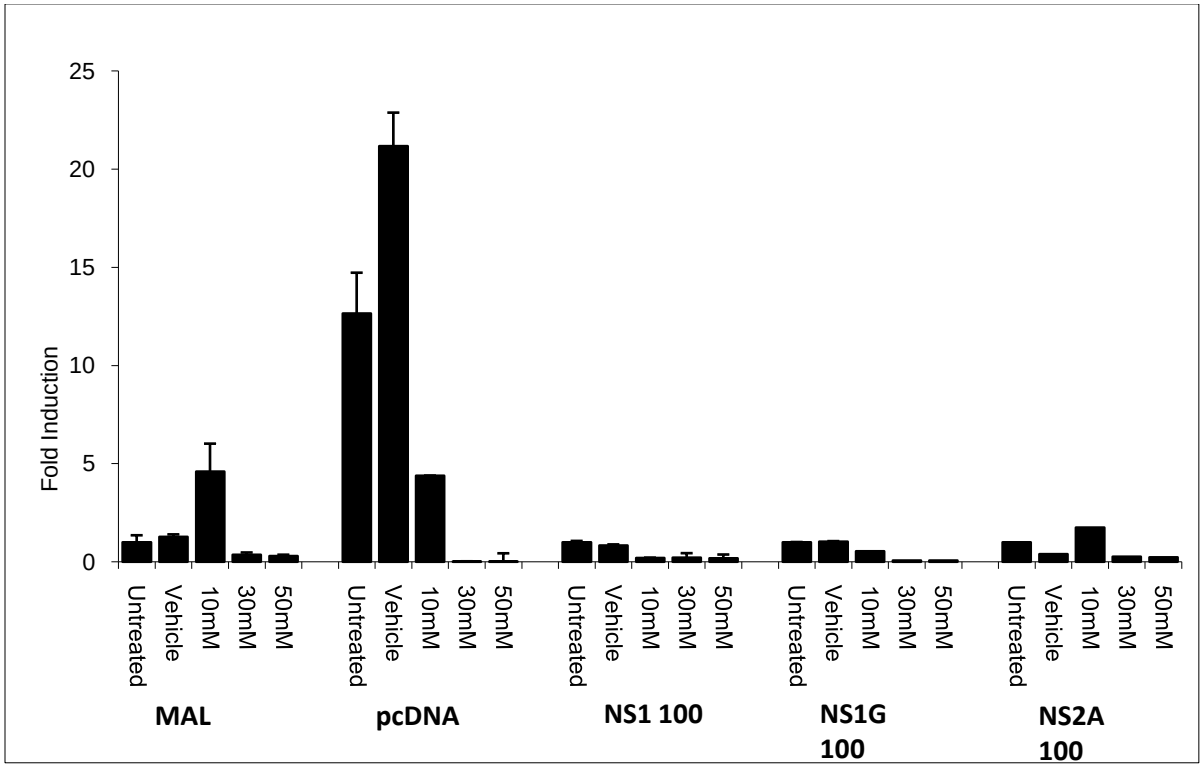

Figure 9. The Effect of Methyl-β-Dextrin Treatment on IL-8 Promoter Activation in HEK293A Cells. Cells were treated at 12 hours post-transfection and read at 24 hours post-transfection.

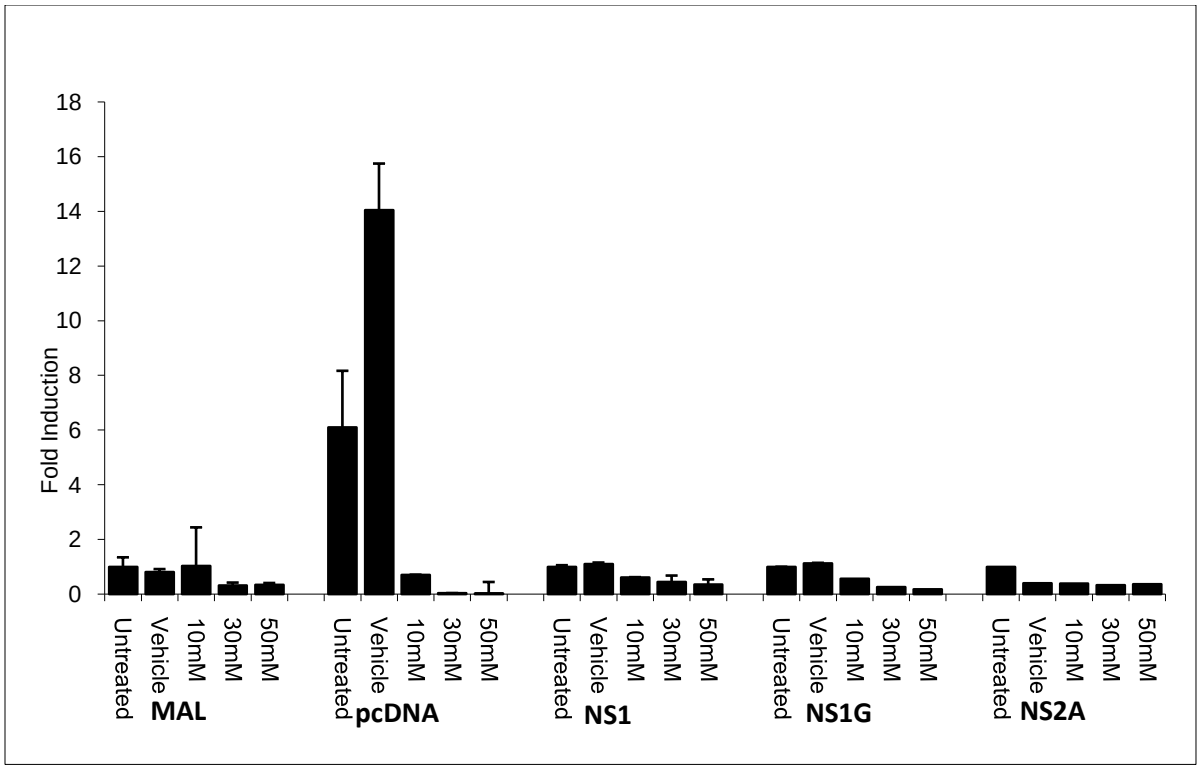

Figure 10. The Effect of Methyl-β-Dextrin Treatment on IL-8 Promoter Activation in HEK293A Cells. Cells were treated at 6 hours post-transfection and read at 24 hours post-transfection.

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