Defining the innate immune consequences of Dengue virus infection: Antibody dependent enhancement and the plasmacytoid dendritic cell interferon response.

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Abstract

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Dengue virus circulates as 4 distinct serotypes that are transmitted to humans by the bite of an infected *Aedes* mosquito. Infection usually results in mild or asymptomatic febrile illness that resolves. In a minority of cases dengue infection can lead to “severe” life-threatening illness characterized by extravascular leakage of fluid, thrombocytopenia, hypovolemia, and shock. Risk factors for acquiring severe disease include pre-existing humoral immunity, age, type I interferon levels (IFN), the level of viraemia, and the frequency of plasmacytoid dendritic cells in the bloodstream during acute infection. During secondary infection pre-existing antibodies against a different serotype are thought to bind virus and enhance infection through FCyR mediated endocytosis in a phenomenon known as antibody dependent enhancement of infection (ADE). However the cellular mechanism during ADE and cell intrinsic consequences that lead to reduced type I IFN or reduced plasmacytoid dendritic cell (pDCs) frequency are not known. Therefore, we studied the innate immune consequences of Dengue virus infection during ADE infection of myeloid cells and investigated the role of pDCs during flavivirus infection. Our results indicate that during ADE, dengue virus infects predominately monocyte cells in a manner that enables virus to transiently overcome a pre-existing interferon response. We also found that flaviviruses efficiently infect pDCS resulting in robust type I IFN production that is dependent on the RIG-I like receptors with no impairment on cell viability. However, virus replication persisted despite robust IFN induction. Collectively these data highlight Dengue virus's promiscuity for infecting relevant innate immune cell types and demonstrate the virus's adept ability to persist during a productive anti-viral response.

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Chapter 1. INTRODUCTION

Flavivirus family

The positive sense RNA virus family flaviviradae of the genus flavivirus encompasses multiple arthropod borne viruses that can cause significant and severe disease in humans (McKenzie et al. 2009, Gould and Soloman, 2008). Viruses in this family of public health importance include West Nile virus (WNV), Japanese encephalitis virus (JEV), Tick-borne Encephalitis virus (TBEV), Yellow Fever virus (YFV), and Dengue Virus (DenV). All flaviviruses contain a positive sense RNA genome that consists of three structural and seven nonstructural proteins. Transmission occurs from the bite of an infected arthropod vector (mosquitos or ticks). For most of the viruses in this family humans represent dead-end hosts that do not support virus replication to high enough levels for transmission (WNV, JEV, TBEV). However, DenV and YFV are unique in that humans are thought to be the primary host for virus amplification and transmission (although non-human primates do support infection, it is unclear whether they serve as a reservoir for transmission).

Dengue disease

Infection with flaviviruses usually results in asymptomatic illness or a mild self-limiting infection with flu-like symptoms and fever. A subset of patients progress to severe illness characterized by viral breach of the blood brain barrier and the initiation of immune mediated encephalitis that can cause severe morbidity and mortality. Dengue virus however, has only rarely been shown to cause encephalitis, instead “severe dengue” (formally called Dengue Hemorrhagic Fever or Dengue Shock Syndrome) results in a more systemic severe disease leading to extravascular leakage of serum and blood, severe fever, petechiae/bruising, and thrombocytopenia (WHO 2015, CDC 2015). This spectrum of symptoms can lead to hypovolemia and circulatory failure/vascular collapse (shock) resulting in organ failure and potentially death. Another interesting distinction between Dengue virus and the other flaviviruses is the fact that the immune system has usually cleared the virus from the body prior to the onset of “Severe Dengue” symptoms (Whitehead et al. 2007). Therefore, dengue related disease sequallae are considered an immune pathology resulting from an over exuberant or poorly regulated immune response.

Dengue epidemiology

Dengue virus is considered an emerging infection. Globally, 2.5-3.5 billion people are at risk of being infected every year. Each year dengue is responsible for between 50 and 100 million apparent infections (as high as 400 million total infections) and between 250,000-500,000 cases of "severe dengue" (Bhatt S et al., 2013, Murray et al. 2013, Thomas et al. 2003). Severe infection is most common in children and infants. Disease risk is greatest in areas of high urban density and low income communities resulting in a disproportionate burden among vulnerable populations and individuals with poor access to healthcare. Dengue virus circulates as 5 closely related serotypes (serotypes 1-5) and is transmitted via the bite of an infected *Aedes aegypti* or *Aedes albopictus* mosquito. Over the last 70 years at least serotypes 1-4 have been disseminated globally with all hyper-endemic regions now experiencing co-circulation of these serotypes (Murray et al. 2013, Zhang et al. 2005, Thomas et al. 2003). Hyper-endemic regions experience shifts in the predominant serotype profile in 2-4 year cycles resulting in large epidemics (Zhang et al. 2013, Thomas et al. 2003). Since the 1950s, case counts of dengue have increased over 30-fold and the incidence of "severe" disease has increased over 4-fold (Murray et. al 2013).There are currently no available treatments or vaccines for dengue infection therefore vector control and supportive care are the only available options.

Predictors of severe disease

Investigations into predictors of severe disease have consistently demonstrated that pre-existing heterotypic humoral immunity is associated with severe disease through a phenomenon known as "antibody dependent enhancement" (Halstead 1977, Guzman 2002, 1990, OhAinle 2011, Dejnirattisai, 2010, etc.). A few studies have suggested that cross-reactive cellular immunity also contributes to severe disease (Mongkolsapaya et al. 2003, Rothman 2011, Duangchinda 2010). Numerous studies have demonstrated that the levels of type I interferon during the febrile stage of illness inversely correlate with severe disease progression. However an association for severe disease and the level of viraemia has been less (this may illuminate fundamental areas of research for understanding disease pathology, or may be due to measurement error/bias of blood sampling during the febrile stages of illness) (Simmons et al. 2007, Pichyangkul 2003, Vaughn et al. 2000). Similarly, well-defined cohort studies in Nicaragua have demonstrated that viral fitness is also attributable to an additional risk of severe disease above pre-existing humoral immunity (Oh'ainle et al. 2011). Finally, a few studies have reported genetic susceptibility factors such as IL-10 and MHC class I polypeptide-related sequence B (MICB) polymorphisms (Khor et al. 2013, Boonnak et al. 2011). Therefore, a wide range of factors are thought to contribute to disease severity.

Cellular infection and viral replication

Following the bite of an infected mosquito, DenV attaches to tissue resident myeloid cells such as Langerhans cells, macrophages, and immature dendritic cells via interactions between the viral envelop proteins and cellular attachment factors with heparin-sulfate binding domains and C-type lectins such as DC-SIGN (Tassaneetrithep 2003, reviewed in Fischl and Bartenschlager, 2011) . Upon recognition by unknown receptors DenV is internalized via receptor mediated endocytosis within clathrin coated pits. Acidification of the endosome results in conformational changes in the viral envelope proteins, promoting fusion and release of the 7-methyl capped + sense strand of RNA into the cytosol. Immediately upon release of the viral genome, the transcript is translated as a single polyprotein that is cleaved by the viral protease NS2/B3 and a - sense RNA template strand is created to allow for genome amplification (Paranjape and Harris 2010).

During the course of infection DenV sets up "replication factories" predominately within fatty acid and endoplasmic reticulum containing membranes (reviewed in Fischl and Bartenschlager, 2011 and Paranjape and Harris 2010). Immature viral particles bud into the ER and are trafficked and secreted by exocytosis through the trans-golgi network (TGN). The immature virus envelope fusion loop is obscured by the viral pre-membrane protein (prM) preventing viral fusion during trafficking. During exocytosis through the TGN, the host enzyme furin cleaves the prM molecule exposing the fusion loop and generating "mature" infectious virus. However, furin cleavage efficiency is low therefore, it is estimated that up to 40% of viral particles have incomplete prM cleavage and are still immature/non-infectious (Dejnirattisai et al. 2010).

During the virus replicative cycle, infected cells are thought to eventually recognize DenV pathogen associated molecular patterns (PAMPs) promoting cellular activation and maturation into antigen-presenting cells (APCs). Activated APCs traffic to the lymph node providing the virus with an expanded population of susceptible myeloid cells such as other APCs and monocytes to infect, as well as initiating the adaptive immune response.

Immune response to dengue infection (viral restriction factors)

Given that DenV infects myeloid sentinel cells such as dendritic cells, macrophages, and monocytes, multiple pathogen recognition receptors (PRRs) have the opportunity to recognize the virus and mount a response. Reports have indicated that the endosomal toll like receptors (TLRs) 7 and 3 recognize DenV leading to an NF-kB dependent pro-inflammatory response and interferon regulatory factor 3 (IRF3) nuclear translocation leading to type I interferon release (Rodriguez-Madoz et al. 2010, Tsai et al. 2010).

Similarly, data primarily from mouse derived dendritic cells and macrophages, indicate that both of the primary Rig-I like receptors (RLRs), RIG-I and MDA5, recognize cytosolic DenV genomic RNA resulting in IRF3 activation (Loo et al. 2008, Pichlmair et al. 2006, Hornung et al. 2006). Following IRF3 activation, type I interferons are secreted and the released interferon binds to the type I interferon receptor (IFNAR) resulting in localized JAK/STAT activation and the expression of a wide range of antiviral factors collectively called interferon stimulated genes (ISGs) (Schoggins et al. 2011, Wong and Chen 2014) . The ISG response serves to limit viral replication among infected cells and also creates a more hostile environment through paracrine stimulation of neighboring cells to limit viral infection.

TLR7 and TLR3 are endosomal pathogen recognition receptors. TLR7 recognizes the single stranded RNA from incoming viral particles or ruptured viral particles that are “sampled” by resident macrophages (Kato et al. 2006). TLR3 on the other hand recognizes double stranded RNA that is present during viral replication. Therefore TLR3 likely can only recognize DenV replication complexes in the context of cell death or via autophagic mechanisms. The RIG-I like receptors (RLRs) scan cytosolic RNA for foreign motifs and can recognize replicating viral RNA (Loo et al. 2011). However, it is thought that Flavivirus replication within membranes limits the amount of cytosolic viral RNA that is available for RLR mediated pathogen recognition (reviewed in Suthar et al. 2011). Finally, multiple studies have reported that STING, a cellular DNA pathogen recognition receptor, recognizes DenV and mounts an IRF3 mediated interferon response analogous to those of the RLRs (Aguirre et al. 2012, Yu et al. 2012). However, it is unclear how STING recognizes DenV since the virus does not create a DNA intermediate during its replicative cycle.

Vaccines using live attenuated viral vectors are available for both YFV and JEV with reportedly high rates of vaccine efficacy with only moderate vaccine associated adverse events. However, prophylactic vaccine development for Dengue has sought to elicit a broadly neutralizing antibody response (Capeding et al. 2014, Whitehead et al. 2007). These vaccination attempts have been largely unsuccessful and complicated by the fact that an effective Dengue vaccine must completely protect against all 5 serotypes to be efficacious. Antibodies to Dengue virus primarily target the structural proteins E and prM (Sukupolvi-Petty et al. 2010, Perison et al. 2007). This is particularly problematic due to the phenomenon of antibody dependent enhancement (ADE, discussed in the next chapter) where serotype specific (heterotypic) neutralizing antibodies against one virus strain are non-neutralizing/sub neutralizing against heterologous serotypes and can actually increase viral infection efficiency and potentially bias the immune response. Similar findings were reported for cellular immunity where cross-reactive T-cells with epitopes against primarily non-structural proteins (NS3, NS1) were thought to confer increased risk of disease severity (Mongkolsapaya et al. 2003, Rothman 2011, Duangchinda 2010). However, more rigorous studies and recent data suggest that cellular immunity is surprisingly cross-reactive between strains and can control viral replication and dissemination (Weiskopf et al. 2011, Zellweger et al. 2013).

Viral evasion and antagonism of the innate immune response.

A perplexing phenomenon is that DenV is the slowest replicating of all the related Flaviviruses, yet the levels of viraemia observed in patients are orders of magnitude higher compared to WNV and JEV infections. Therefore, DenV must have evolved a number of mechanisms to evade immune detection and antagonize the innate immune response to delay pathogen recognition. Indeed a number of known mechanisms have been identified. As discussed previously, it is thought that viral replication within membranes restricts access of viral nucleic acids to RLRs and limit the ability of cytosolic autophagy mechanisms to shuffle viral PAMPs to endosomal TLRS.

DenV uses multiple nonstructural proteins to shut down the interferon response. Specifically, the viral RNA-dependent RNA polymerase (NS5a) has been shown to target STAT2 for proteosomal degradation resulting in blockade of Type I IFN (IFN-α/β) signaling and the ISG response (Ashour et al. 2005, 2009, Jones et al. 2010, Morrison et al. 2013, Perry et al. 2011). Additionally, various reports have demonstrated a role for NS2a, NS4a, and NS4b for the inhibition of STAT1 phosphorylation resulting in a blockade of Type I IFN response as well as the Type II IFN-gamma response (Munoz-Jordan et al. 2003, 2005, Ho et al. 2007). Two parallel recent studies have indicated that the NS2/b3 viral protease cleaves STING resulting in an inhibition of the IRF3 mediated interferon response (Aguirre et al. 2012, Yu et al. 2012). However, it is important to note that most of these observations have been made using overexpression systems and highly susceptible artificial cell lines such as 293t and HepG2 cells. Finally, DenV non-coding viral RNA (sfRNA) has been shown shut down the G3BP1, G3BP1, CAPRIN1 complexes role in interferon stimulated gene RNA translation into antiviral effectors (Bidet et al. 2013).

The relative importance DenV's ability to antagonize and control the interferon response has come from a number of observations. First multiple *in vitro* studies have shown that pre-treatment of cells with physiological levels of type I interferon (alpha/beta) is sufficient to completely block infection (Diamond et al. 2002, 2003). Conversely, type I interferon treatment following infection is unable to completely restrict viral replication or clear infection. Second, mice possess STAT1 and STAT2 as well as STING proteins with divergent target site sequences for the viral proteins NS5a, 4b, and NS2/b3 respectively compared to humans (Ashour et al. 2009, Aquirre et al. 2012). Therefore, mice are completely protected from dengue infection unless they are deficient in type I interferon receptor (IFNAR) pathway.

Thesis overview

Therefore, the subsequent data contained herein describe efforts to better understand the innate immune consequences of DenV infection among relevant cell types. Specifically the innate immune consequences of DenV infection are investigated as they relate to known predictors of human disease: 1) In the context of antibody dependent enhancement (ADE) 2) Investigation of the interferon response following infection of plasmacytoid dendritic cells with relevant flaviviruses.

Chapter 2: DEFINING THE INNATE IMMUNE CONSEQUENCES OF ANTIBODY DEPENDENT ENHANCEMENT DURING DENGUE VIRUS INFECTION

2.1 Background

History

Dengue virus or related viruses have infected humans for hundreds of years and are thought to have originated in tropical Africa (Halstead 2015, Murray et al. 2013). Virus dissemination likely occurred through the spice trade to Asia and via the slave trade into the Americas. In 1907, Dengue virus was identified as a virus that was carried by *Aedes* mosquitoes. Due to the increased globalization following World War II, Dengue virus serotypes became disseminated throughout tropical and subtropical regions of the world. However, it was not until the 1950's that the first clinical cases of "severe" hemorrhagic Dengue were identified. In the following decade massive epidemics of Dengue occurred in Southeast Asia (Halstead 2015, 1970).

In 1962 in Thailand a large outbreak with DenV serotype 1 occurred with low rates of severe disease reported. An outbreak of serotype 2 occurred in 1964 in the same region resulting in high proportions of cases with severe dengue (Halsted 1970). Dr. Scott Halsted made the surprising observation that severe disease was 15-80 times more likely to occur among children previously infected with serotype 1 in 1962. An important exception was that a relatively high proportion of severe disease was observed among infants. Similar observations were made during dengue epidemics of serotype 1 and 2 in Latin America during 1977 and 1981 (Guzman et al. 1990). Epidemiologists in Cuba identified over 300,000 cases in 1981 with over 10,000 cases of "severe dengue." Among cases of severe dengue 98% had been previously infected with serotype 1 in 1977. The remaining 2% of cases primarily comprised infants, while children ages 1-2 years old were protected from severe disease. Testing of sera from mothers of infants that developed severe dengue revealed pre-existing immunity to serotype 1 from 1977 (Guzman 1990). Subsequent studies during outbreaks in Southeast Asia and Latin/South America have consistently demonstrated increased risk of severe disease among individuals with pre-existing heterotypic immunity (Guzman et al. 2000, OhAinle 2011, etc.).

These findings led Dr. Halstead to hypothesize that pre-existing humoral immunity was non-neutralizing against a heterotypic infection and contributed to increased diseased severity. The term to explain this phenomenon was coined "antibody dependent enhancement (ADE)". To test this, serum from macaques infected with serotype 1 was transferred into Dengue naive macaques that were subsequently infected with dengue serotype 3. Those animals that were transferred serum had viraemia that was over a log higher than control animals (Halstead and O'Rourke 1977). These results suggested that heterotypic antibodies resulted in increased levels of virus.

Since these primary discoveries were first made, considerable research has been performed to understand the phenomenon of ADE, the cells types affected, and the effects that ADE has on infected cells as well as the human host. Research efforts have been hindered by the fact that no relevant animal models currently exist for severe dengue. Primates support high levels of viraemia, yet fail to develop the hallmark symptoms of severe disease.

Mouse models of ADE

Immunocompetent mice completely restrict viral replication due the divergent target site sequences for DenV virulence factors preventing DenV-mediated disruption of the interferon mediated antiviral response (Aguirre et al. 2012, Ashour et al. 2000). However, to study the adaptive immune response to DenV numerous groups have used the AG129 mouse model that is deficient in the type I and II interferon receptors (IFNAR) preventing the interferon mediated ISG antiviral response. Interestingly, AG129 mice that are parenterally transferred heterotypic antibodies or passively immunized against a heterotypic viral strain demonstrate increased viremia and disease severity including extravascular leakage and shock (Zellweger et al. 2010, 2014, Balsitis et al. 2010, Ng et al. 2014). A recent study immunized pregnant AG129 mice and then infected the pups with a heterotypic strain of virus. Consistent with human observations, they found that infected pups experienced increased disease severity and viraemia that disappeared after placental antibody titers waned, demonstrating that the presence of placental antibodies alone was sufficient to cause severe dengue symptoms (Ng et al. 2014). Finally, mouse studies have demonstrated that the FC region (factors crystalizable/constant) of heterotypic antibodies are necessary for disease symptoms since antibodies with only the Fab epitope specific variable region are able to neutralize virus and prevent severe disease in mice (Balsitis et al. 2010). Therefore, this finding demonstrates that the effect of enhancement requires the FC region which is known to be associated with complement activation (rare for viruses) and cellular attachment and clearance by FC receptor bearing cells.

Enhancing antibody characteristics

Research has demonstrated that enhancing antibodies are usually IgG1 and IgG2 subclasses (Sukupolvi-Petty et al. 2000, Rodrigo et al. 2009, Walhala et al. 2011, De Alwis et al. 2014). The dengue virion "breathes" sampling different confirmations resulting in altered epitope availability (Lok et al. 2008, Murkherjee et al. 2014, Nelson et al. 2008, Vanblargarn et al. 2013). In vitro studies have demonstrated that an occupancy rate of 30 binding antibodies per virus particle is necessary for neutralization (Nelson et al. 2008, Peirson et al. 2007). The majority of neutralizing antibodies target domain I of the envelope protein. However, domain I is poorly conserved across dengue serotypes, therefore these antibodies are poorly cross reactive. Enhancing antibodies have been shown to target domain III of the envelop protein fusion loop (Mukherjee et al. 2014, Sukupolvi-Petty et al. 2010, Peirson et al. 2007). The fusion loop is highly conserved across dengue virus strains therefore it is highly cross reactive. Unfortunately, fusion loop epitopes are cryptic (buried deep within the virion) and are of low abundance resulting in reduced antibody binding and promoting enhancement instead of neutralization (Mukherjee et al. 2014, VanBlargan et al. 2013, Sukulpovi-Petti et al. 2010, Goncalvez et al. 2007). Another interesting finding is that the viral chaperone protein prM, that must be cleaved for virus infectivity, also has highly conserved domains between serotypes. Therefore, studies have shown that prM specific antibodies are capable of enhancing cellular infection and can actually rescue infectivity of immature virions with incomplete prM cleavage (Dejnirattisai et al. 2010, Lok et al. 2008, De Alwis et al. 2014 Goncalvez et al. 2007).

ADE FC receptors usage

Mammalian cells can express a number of FC receptors that bind to the FC region of the antibodies. Numerous studies have demonstrated that the activating FC gamma receptors RIIa and RI are able to enhance infection (Boonnak et al. 2011, 2010, Chan et al. 2011, 2014). Interestingly, enhancing antibodies at high enough titers can actually become neutralizing because antibody saturation on virions has been shown to result in the ligation of the inhibitory FC receptor, FC gamma RIIb (Chan et al. 2014). Ligation to the inhibitory FC gamma receptor IIb does not result in virus internalization, thereby preventing infection (Steevels and Maayard 2011). These findings explain why individuals have a protective period following a heterotypic primary infection where they have dramatically reduced risk of developing apparent or severe dengue until antibody titers from their primary infection wane(Chau et al. 2008, Ng et al. 2013). Conversely, ligation of activating FC gamma receptors results in endocytosis and internalization of the immune complex, promoting acidification of the resulting endosome and supporting viral fusion as well as providing opportunities for DenV antigen presentation and pathogen recognition by endosomal TLRs (Steevels and Maayard 201). Multiple studies have demonstrated that ab-virus immune complexes undergo internalization and upon acidification of the endosome, viral fusion and infection still take place (Chan et al. 2014, 2011, Peirson et al. 2007, Dejnirattisai et al. 2010).

Infected Cell Types

Dengue infection is most efficient among myeloid lineage cells such as monocytes, macrophages, and dendritic cells. However during infection and ADE the primary targets for replication are thought to be monocytes and mature dendritic cells. Indeed, ex-vivo peripheral blood mononuclear cell (PBMC) profiling studies from cases with acute infection demonstrated high rates of monocyte infection (Durbin et al. 2008, Kou et al. 2008, Sun et al. 2011, Halstead 1977). In vitro studies have shown that cells expressing high levels of DC-SIGN are efficiently infected and the presence of enhancing antibodies does not increase the overall rates of infection among these cells (Boonnak et al. 2008, Sun et al. 2011, Tassaneetrithep et al. 2003). These findings suggest that DC-SIGN has greater affinity for the virion than the FC-Ab interaction and that the DC-SIGN mediated entry pathway tends to predominate. Among cells such as monocytes and mature DCs DC-SIGN, expression is low and rates of infection are only high in the presence of enhancing antibodies.

Mechanisms of ADE induced severe disease

While there has been clear evidence that heterotypic antibodies and infection enhancement predict disease outcome for many cases, the mechanisms whereby infection enhancement contributes to immune pathology has been far less clear. The fact that peak viraemia is not an ideal predictor of disease progression further complicates understanding of the severe disease pathology (Libraty et al. 2002, Chau et al. 2008, Simmons et al. 2007). Thus, a key question is whether ADE drives greater levels of viraemia promoting systemic immune activation (as previously stated severe disease onset occurs during the defervescent phase after the virus has been cleared from the body), or whether enhancement of infection biases the overall immune response. Studies have investigated whether the antibody response drives endothelial cell destruction or whether dengue specific antibodies cross react with platelets resulting in complement mediated platelet destruction and thrombocytopenia. However recent data and biological plausibility have suggested that such a mechanism is unlikely (reviewed in Halstead 2012, Eugenio et al. 2013).

In addition to the broad systemic consequences of ADE, little is known about the mechanisms of entry in concert with FC gamma receptor ligation and whether there is a cell intrinsic bias in the innate immune response. Multiple ADE studies have reported increased virus output per infected cell either indicating that ADE concentrates virus in susceptible cells or that ADE imparts a cell intrinsic response (termed intrinsic ADE) that provides a more favorable environment for viral replication (Boonnak et al. 2008, 2011, Kou et al. 2011, Sun et al. 2011). Several studies from a group in Thailand reported rapid shutdown of the interferon response, TLR signaling "collapse", and IL-10 mediated immune suppression (Ubol et al. 2010, Modhiran et al. 2010). A study from a related group in the US reported reduced interferon response following ADE and pronounced IL-10 production among monocyte derived mature dendritic cells for individuals that were homozygous for the CC mutation in the IL-10 promoter (Boonnak et al. 2011). However, the IL-10 promoter polymorphism is not present in the majority of cases of severe dengue, so this phenomenon cannot explain the overall disease pathology. In contrast other studies have reported no loss of innate immune signaling and even profound IFN-beta production following infection under enhancing conditions (Kou et al. 2011). Interestingly, a recent study reported that during attachment of antibody bound virus to activating FC-receptors an inhibitory co-receptor LILR1B is co-ligated that disrupts the JAK/STAT signaling pathway and suppresses the PRR response (Chan et al. 2013).

Intrinsic ADE

If intrinsic ADE does take place a number of potential mechanisms could explain this phenomenon. For example, downstream signaling following FCγ receptor ligation could polarize infected cells making them more permissive for viral replication. Similarly, receptor mediated endocytosis (RME) through FCy receptors could result in altered opportunities for PRR recognition due to differential endosomal entry pathways and potentially affording the virus with different replicative niches/subcellular localization. Altered interactions with extracellular receptors or co-factors during ADE could also limit the cells ability to identify and restrict viral replication. Alternatively, viral entry in the context of ADE may allow for improved entry kinetics or increased rates of viral infection per cell, enabling more rapid viral takeover and control of cellular processes and the interferon response. Alternatively, cellular tropism shifts imparted by ADE may allow for altered pathogen recognition and changes in rates of viral replication.

Therefore, I hypothesized that dengue virus infection during ADE biases the innate immune response to a phenotype that is more permissive for viral replication. To study this, I developed an in vitro ADE system utilizing lab adapted strains of Dengue virus serotypes 2 and 4, the immortalized myeloid cell lines U937 and THP1, and perform enhancement using monoclonal enhancing antibodies and DenV patient convalescent sera. I define the innate immune signature between ADE and non-ADE conditions using low multiplicities of infection in both monocyte and macrophage-like cells types.

2.2 Methods

Cell lines and viruses

Vero African green monkey kidney cells and the *Aedes albopictus* C6/36 mosquito cells were grown in minimum essential medium supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT). The human monocytic cell lines U937 and THP1 were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 ug/ml streptomycin, and 100 U/ml penicillin. THP-1 and U937 cells were differentiated into macrophage-like cells by treatment with 20 nM PMA overnight at 37 C. DenV2 New Guinea C (NGC) strain and DENV4 H241 (Kindly provided by Dr. Jay Nelson, Portland, OR) were propagated from viral reference stocks by single passage in C6/36 cells grown at 28 C for 7-10 days. Upon visual identification of cytopathic effect, viral supernatants were harvested and subjected to centrifugation at 1500 g for 20 minutes, and clarification through a 0.2uM filter. Viral titers were quantified using the Vero plaque assay. Sendai virus, Cantell strain, was purchased from the Charles River Laboratory. Cells were infected with 100 HA units per ml of Sendai virus and harvested at the indicated time-points post infection.

Vero cell plaque assay

The Vero cell plaque assay was performed as described previously (Pinto et al. 2014). Six log dilutions of virus stock were made from each supernatant sample and inoculated into six-well tissue culture plates containing confluent Vero cell monolayers. Virus adsorption was allowed to occur for 2 hr. Following adsorption the Vero monolayer was overlaid with complete minimal essential medium, containing 1% low-melting-point agarose (Invitrogen, Carlsbad, CA). Infection plates were incubated for 6 days at 37°C for and overlaid with the vital stain neutral red (Sigma, St. Louis, MO). Plaques were counted by visual inspection at 12-16 hr. following neutral red staining for determination of the number of infectious viral progeny as approximated by the number of plaque forming units (PFU) of Dengue virus per milliliter of supernatant.

Monoclonal antibodies and Dengue virus immune serum

Intracellular DV infection was measured using the E206 monoclonal antibody (MAb) (kindly provided by Dr. Michael Diamond, St. Louis, MO), a mouse-specific anti-E immunoglobulin G1 (IgG1) that is conserved for serotypes 1 to 4. Infection enhancement was performed with the broadly enhancing IgG1 domain III fusion loop specific (DIII-fl) monoclonal antibodies E60 and E18 (kindly provided by Dr.

Michael Diamond, St. Louis, MO, Sukolpovi-Petty et al. 2008). We also accessed small aliquots of a DV1 patient immune serum collection (kindly provided by Dr. Robert Tesh, Galveston, TX). An IgG1 GAPDH specific MAb (IgG1) was used as an isotype control (Invitrogen, Carslbad, CA).

ADE assay

DV MAbs E60, E18, IgG1, or DV immune sera were serially diluted from stocks in a volume of 100ul. For infection DENV2 NGC or DENV4 H241 at a multiplicity of infection (MOI) of 1 were used unless otherwise indicated. Diluted antibodies and virus were co-incubated in 1% heat inactivated FBS RPMI serum for 1hr at 37% with 5% CO2 to allow steady state binding and immune complex formation. Following immune complex formation 200ul of the immune complex solution was added to 0.5×10^6 cells and incubated for 3hrs at 37C with 5% CO2. Following infection the cells were washed 3 times with complete medium and re-suspended in 500ul complete RMPI supplemented with an equivalent dilution of antibodies to allow for subsequent rounds of viral entry. After initial determination of enhancement stoichiometry, optimal enhancing conditions were used for each MAb/Human sera condition and antibody concentration matched isotype controls were included for each experimental condition.

Flow cytometry

FACSCalibur Canto2 instruments (BD Biosciences, San Jose, CA) were used to monitor cell surface staining of U937 cells. The following surface marker antibodies were utilized: anti-CD64-PerCP-Cy5.5 (FCyR1), anti-CD32-PE-Cy7 (FCyRIIa), and anti-CD209-APC (DC-SIGN) (BD Biosciences, San Jose, CA). For detection of cell viability a cellular permeability Live/Dead pacific blue stain was utilized (Invitrogen, Carlsbad CA). For detection of intracellular viral protein, cells were fixed and permeabilized with Cytofix/Cytoperm according to the manufacturers protocol (BD, Biosciences), and stained with E206 (anti-DV Env MAb) conjugated to AlexaFluor-488 (Invitrogen, Carlsbad, CA) at 24 and 48hr post infection.

Western blotting

Western blot analysis was carried out as previously described (Loo et al. 2008). Briefly, cells were either mock infected or infected with Dengue virus 2 (NGC) or West Nile Virus TX in the presence or absence of enhancing antibodies at an MOI of 1 or 5 as indicated. Cells were harvested post-infection at defined time intervals and cellular lysates were extracted for total protein content. Harvested cells were lysed and protein was extracting using in extraction buffer (150 mM NaCl 50, mM Tris HCl [pH 7.5], 0.5%

sodium deoxycholate, 1% NP-40, 1 mM EDTA, and 0.1% sodium dodecyl sulfate, supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotonin, leupeptin, and pepstatin). Following lysis extracts were centrifuged at 15,000g for 10 min at 4°C.Protein extracts were quantified using the Bradford assay (Bio-Rad) and 20ug of protein lysate were loaded per well. Cellular proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed via Western blot using targeted protein-specific antibodies. Mock-infected samples collected at each time point were also analyzed with each of the antibodies tested. The membranes were probed with mono-clonal and polyclonal antibodies specific for total IRF-3, phospho-IRF3 (p-IRF3, Ser396), MAVS (cardif AT107), ISG56 (IFIT-1), total IRF7, total STAT1, phospho-STAT1 (Tyr701), MDA5, RIG-I, and GAPDH (Santa Cruz Biotechnology), or WNV. All secondary antibodies were purchased from Jackson Immunoresearch. Immunoreactive bands were detected with Amersham ECL Plus reagent (GE Healthcare).

RNA extraction and analysis

Cells were harvested and lysed using the QiaShredder (Qiagen) kit per the manufacturer's recommendations. Total cellular RNA from experimental cultures was extracted for quantitative reverse transcription-PCR (qRT-PCR) using the RNAeasy kit (Qiagen) according to the manufacturers protocol. The extracted cellular RNA was then reverse transcribed using the iScript select cDNA synthesis kit using both oligo (dT) and random primers according to the manufacturers protocol (Bio-Rad). RNA amplification was conducted from 10 ng cDNA at a 10 µl reaction mixture in 384-well opitical plates. Amplification was performed under the following conditions: 30 min at 48 C, 1 cycle at 95 C for 10 min, 30 cycles at 95 C for 15 seconds and 1 min at 60 C and dissociation curve (95 C 1min, 65 C 2 min and 65C–95C at 2/sec). Cellular mRNA and viral RNA expression levels were determined by SYBR green qRT-PCR using gene- or virus-specific primers on a ViiA7 thermocycler (Applied Biosystems). Data was analyzed by the ΔΔCT method. Specific primer sets are as follows:

hIFITM1, forward: TACTCCGTGAAGTCTAGGGACAG; hIFITM1, Reverse: AACAGGATGAATCCAATGGTCA; hIFIT-1, forward: TTGATGACGATGAAATGCCTGA; hIFIT-1, reverse: CAGGTCACCAGACTCCTCAC; hIFN-beta, forward: ATGACCAACAAGTGTCTCCTCC; hIFN-beta, reverse: GGAATCCAAGCAAGTTGTAGCTC; hTFN-alpha, forward: AAGCACACTGGTTTCCACACT; hTNF-alpha, reverse: TGGGTCCCTGCATATCCGTT; DENV, forward: TTAGAGGAGACCCCTCCC; DENV, reverse: TCTCCTCTAACCTCTAGTCC;

ELISA

Enzyme linked immunosorbent assays (ELISA) was performed for absolute quantitation of TNF-alpha, and IL-6 (eBiosciences) per the manufacturer's instructions. Supernatants were diluted 1/10 or 1/100 prior to analysis.

Statistical analysis

All data were analyzed using Prism software using the unpaired student's *t-test* (GraphPad Prism). P-values significant at the 0.05 alpha level were considered significant.

2.3 Results

Determination of optimal ADE conditions:

I first sought to assess ADE efficiency and the innate immune response between macrophages and monocytes. These cells types were chosen based on observations that infection most readily occurs among monocytes during enhancement due to their lack of DC-SIGN expression whereas under non-enhancing conditions, primary infection takes place in macrophages and immature dendritic cells (Durbin et al. 2008, Boonnak et al. 2011). To develop an in vitro ADE system I utilized the human monocytic cell line U937 to represent monocyte infection and PMA treated THP1 cells to represent macrophage infection.

U937 cells were infected with DenV serotype 2 New Guinea C (DenV2 NGC) at variable MOIs (0.01-5) in the presence or absence of monoclonal enhancing antibodies (E18, E60) or dengue patient convalescent sera. Enhancement of infection resulted in a 100 fold increase in cellular levels of viral RNA and a similar 100 fold increase in the levels of infectious progeny released under optimal ADE conditions for all antibodies and sera tested. The optimal concentrations of antibodies were robust across a range of MOIs (0.01-5, data not shown). Enhancement in PMA treated THP1 cells was far less efficient resulting in only a 3-5 fold increase in viral RNA levels and progeny. As expected, the optimal concentration of enhancing antibodies were the same as those identified in the U937 cells. The E60 antibody provided the best overall enhancement with the lowest necessary antibody concentration; therefore this antibody was used at 0.01ug/ml for all subsequent experiments.

Comparison of viral output between the monocytic and macrophage-like cells revealed that peak viral output under enhancing conditions was equivalent between cell types (Figure 2.1). However, virus output under non-ADE conditions was roughly 100 fold greater in PMA treated THP1 cells compared to the U937 cells. To assess the cell viability and infected cell frequency across conditions, flow cytometry analysis was performed and cells were stained for viability (Live/Dead) and intracellular levels of viral protein using an Alexa-Fluro488 conjugated E206 Dengue virus 2 Env specific MAb. Across conditions and between cells types there were no detectable differences in cell viability (data not shown).

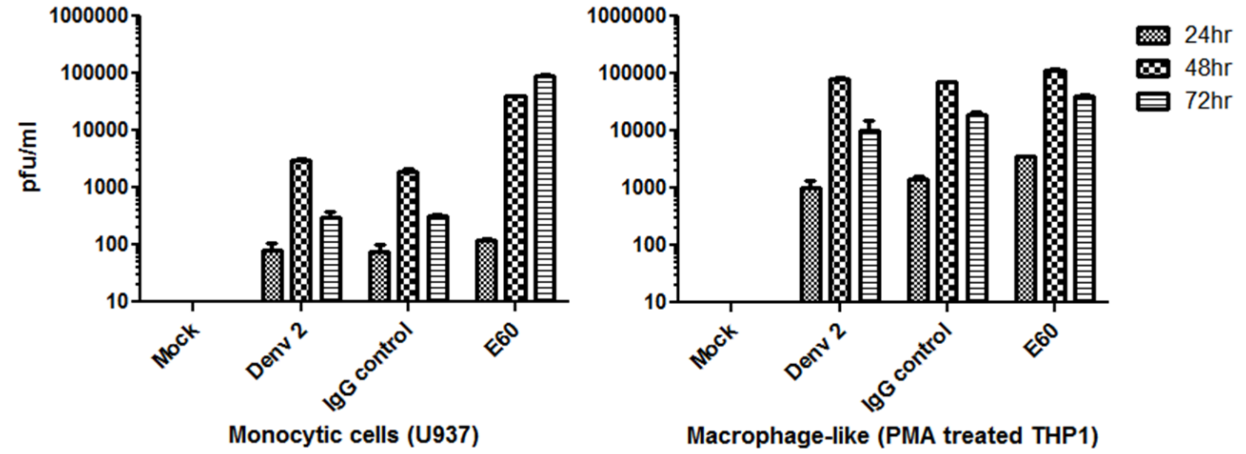


Figure 2.1. E60 Mediated Enhancement of DenV2 infection. Cells were infected at an MOI of 1 in the presence or absence of 0.01ug/ml of the indicated antibodies and harvested at the indicated time-points post-infection. Results are representative of at least 5 independent experiments. Error bars represent the standard deviation.

Within each cell type, enhancement resulted in elevated levels of infected cells that increased between 24 and 48hrs, whereas infection in the absence of enhancing antibodies resulted in very low levels of infection (Figure 2.2). Therefore the findings under non-ADE conditions suggest that either the infection is transient due to viral replication kinetics or there is innate immune restriction of virus taking place that ADE is either circumventing or overcoming.

Collectively, the plaque data and the flow analyses suggest that macrophage-like cells undergo reduced enhancement, yet support over 100 fold greater virus production per infected cell. Interestingly, under ADE conditions monocytic cells are infected at roughly a 10 fold higher rate, yet virus output is over 100 fold higher than monocytic cells infected under non-enhancing conditions. This finding suggests that during ADE monocytic cells are both infected at a higher rate and infectious virus production per infected cell increases. No significant differences in known receptor/adapter expression (DC-SIGN, FCyRIIA, FCyRI) were detected between cells types, therefore differential expression of these receptors does not explain these findings (A1). These findings suggested that fundamental differences in virus replication and potentially the innate immune response existed between cell types and across enhancement conditions.

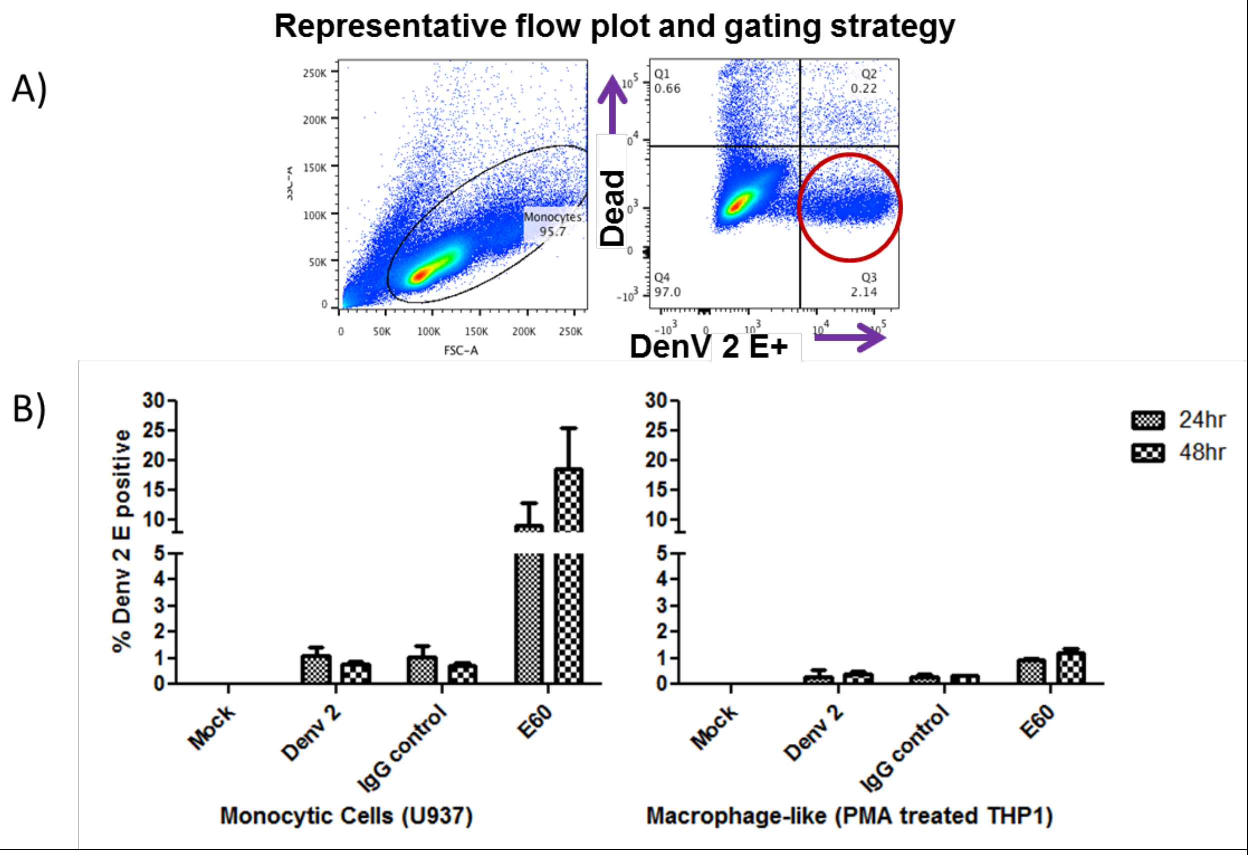


Figure 2.2 ADE increases infected cell frequency of monocytes and macrophages. Cells were infected at an MOI of 1 in the presence or absence of 0.01ug/ml of the indicated antibodies. Cells were fixed and intracellularly stained with an Alexa-Fluor 488 conjugated DenV 2 monoclonal ab (E206) for flow cytometry analysis. A) Representative flow plot and gating strategy. B) Percentages of infected cells at indicated time points post-infection.

Limited innate immune signature following DenV infection.

To assess the innate immune consequences of infection, the innate immune response was assessed between enhancing and non-enhancing conditions across the representative cell types. In order to approximate the pathways involved, a representative panel of innate immune genes were analyzed by qRT-PCR. Specifically, TNF-alpha was chosen as this approximates NF-kB mediated pro-inflammatory activity, IFN-b was chosen to represent the antiviral response, and IFIT-1 is indicative of IRF3 activation and/or JAK/STAT mediated transcription of interferon stimulated genes (ISGs). Finally, IFITM1 was chosen as this ISG has been shown to be exclusively dependent on JAK/STAT mediated signaling. As a positive control the RLR activating virus Sendai Virus (SeV) was used at 100 HAU. Where indicated 100IU IFN-b and WNV TX at an MOI of 3 were also utilized as positive controls.

Infection results revealed only low level innate immune message among monocytic cells, despite high levels of infection under ADE conditions (Figure 2.3). However, the transcript levels were elevated across enhancing conditions only in the macrophage-like THP1 cells, suggesting that these cells were actively recognizing and responding to the virus at later time-points.

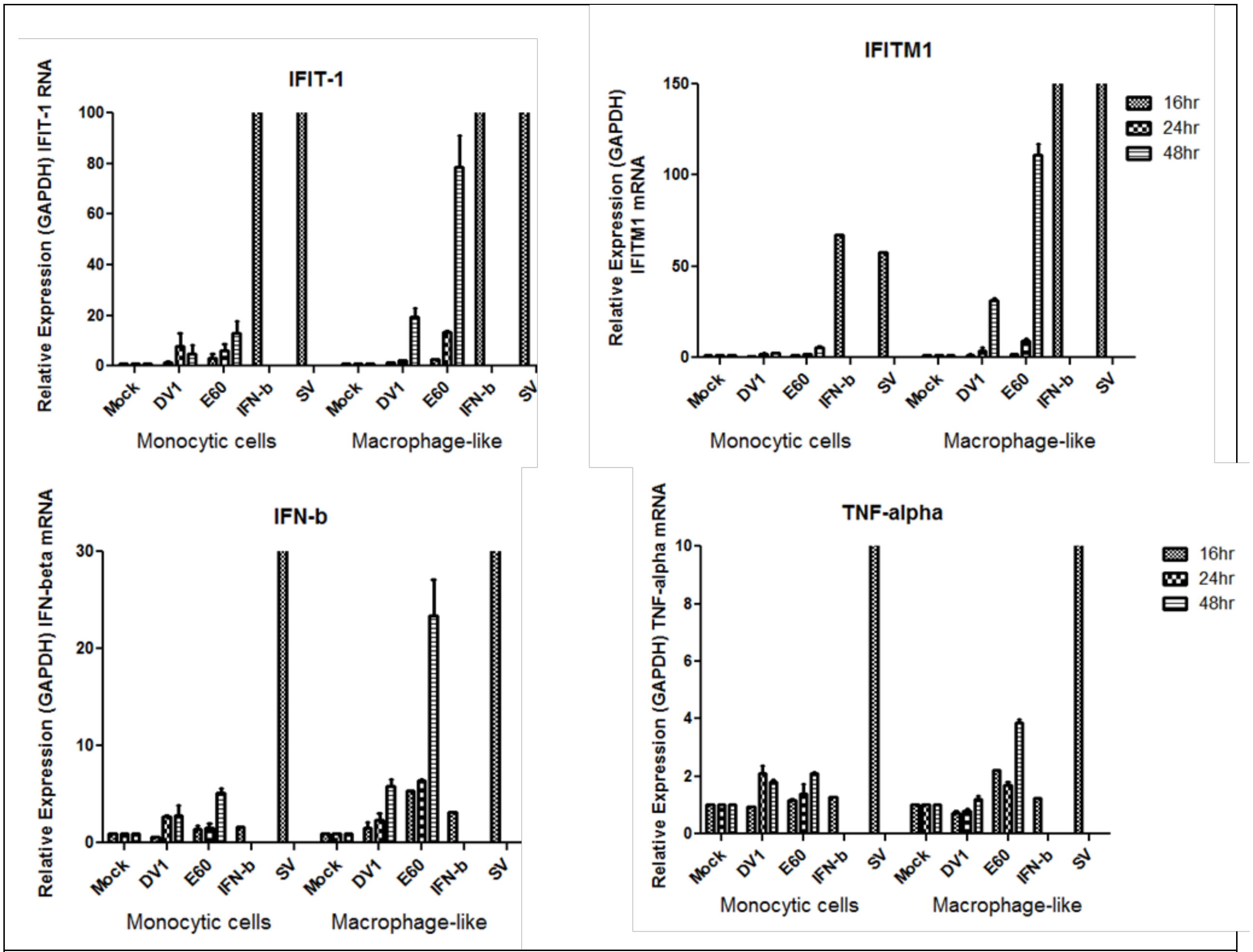


Figure 2.3. Dengue infection results in low level innate immune gene induction. Innate immune gene induction was measured by qRT-PCR analysis for IFN-β, IFIT-1, IFITM1, and TNF-alpha mRNA, and relative fold induction after normalization to GAPDH was compared to that of mock infected cells. Results are representative of 3 independent experiments with at least duplicate samples. Error bars represent the standard deviation.

Cellular protein levels were measured by western blotting and blots were probed for pathogen recognition receptors RIG-I and MDA5 as well as key innate immune genes including STAT-1, IRF3, phospho-STAT-1, phospho-IRF3, and IFIT-1 (ISG56). GAPDH was also probed as a loading control. Protein analysis revealed no detectable innate immune signature following infection among the monocytic cells (Figure 2.4a). Similarly, little immune activation was detected at the protein level among macrophage-like THP1 cells (Figure 2.4b). However, WNV and the E60 enhanced cells revealed low level STAT-1 phosphorylation.

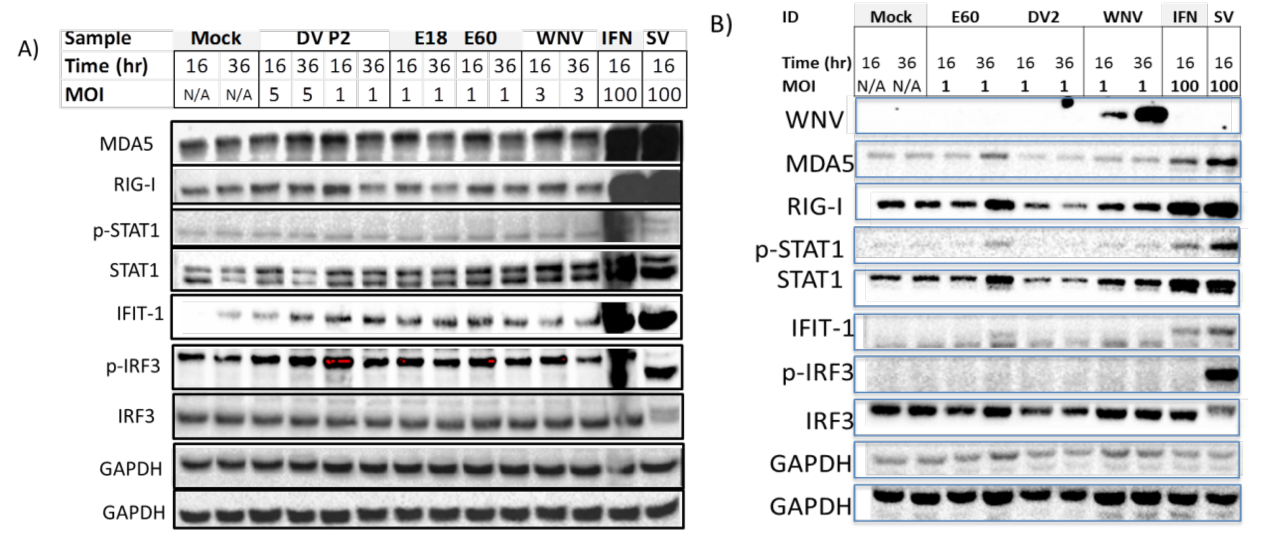


Figure 2.4 Dengue virus infection results in low level innate immune protein expression. Analysis of innate immune protein levels in cells infected with DENV2 NGC under enhancing or non-enhancing conditions and harvested at 16 and 36hrs. A) U937 cells B) PMA treated THP1 cells. WNV TX also included as a reference. Infection enhancement was confirmed via plaque assay. Sendai virus (SV) and IFN-beta (IFN) were used at 100 HAU and 100 IU respectively as positive controls.

For protein level analysis of pro-inflammatory/NF-κb mediated innate immune gene induction enzyme linked immunosorbent assays (ELISA) were performed for TNF-alpha and IL-6. These assays indicated that no detectable pro-inflammatory cytokines were released into the supernatant following DenV infection (Data not shown).

These data suggest that DenV infection is largely immunologically silent. Increasing the MOI to 5 did not significantly increase the dynamic range of the response either (data not shown). However, given the low rates of infection (1% under non-ADE conditions up to 20% under ADE conditions in U937 cells) it was unclear whether a low level innate immune signature was present but was being diluted out by the bystander cell populations, or whether there was no immune detection until later time-points.

ADE overcomes the IFN induced antiviral state

Given that there was only a low level innate immune signature following DenV infection, options for studying the innate immune consequences of ADE were limited. I hypothesized that ADE may allow for sustained rounds of infection and viral replication following initiation of the interferon mediated antiviral response through: 1) FCγR/co-receptor mediated signaling 2) FCγR entry/localization in immunologically favorable compartments 3) Bombarding permissive cells with enough virus to overcome the ISG mediated barriers to infection.

To test this, monocytic U937 or THP1 cells were pre-treated with 50IU of IFN-β and were subsequently infected following expression of known ISGs. Pilot experiments demonstrated that ISGs were upregulated and translated into protein within 12 hours following IFN-beta treatment (A2). Following a 12hr pre-treatment with 50IU of IFN-beta, infection resulted in a pronounced and increasingly time-dependent blockade of viral replication under non-enhancing conditions (A3). Surprisingly, under optimal ADE conditions, cellular levels of viral RNA increased in the presence of ISG response, but overall levels were 2 logs lower than untreated cells (A3). Importantly, plaque titers for ADE conditions in the presence of IFN-β, were higher than those observed during primary viral infection in the absence of interferon treatment. Using a dose response including 10IU and 100IU IFN-β pre-treatment, demonstrated consistent reductions in virus output across all conditions. However, the cellular RNA levels among ADE conditions increased significantly between 24 and 48 hrs consistent with viral replication (Figure 2.5 A,B). Similarly, infectious virus output following resulted in consistent increases in virus between 24 and 48hrs post-infection during ADE. Importantly, the level of infectious virus during ADE following IFN pre-treatment, were higher than those obtained from untreated cells infected under primary infection conditions (Figure 2.5c). Similar results were obtained using monocytic THP1 cells and DenV4 H241 (Data not shown).

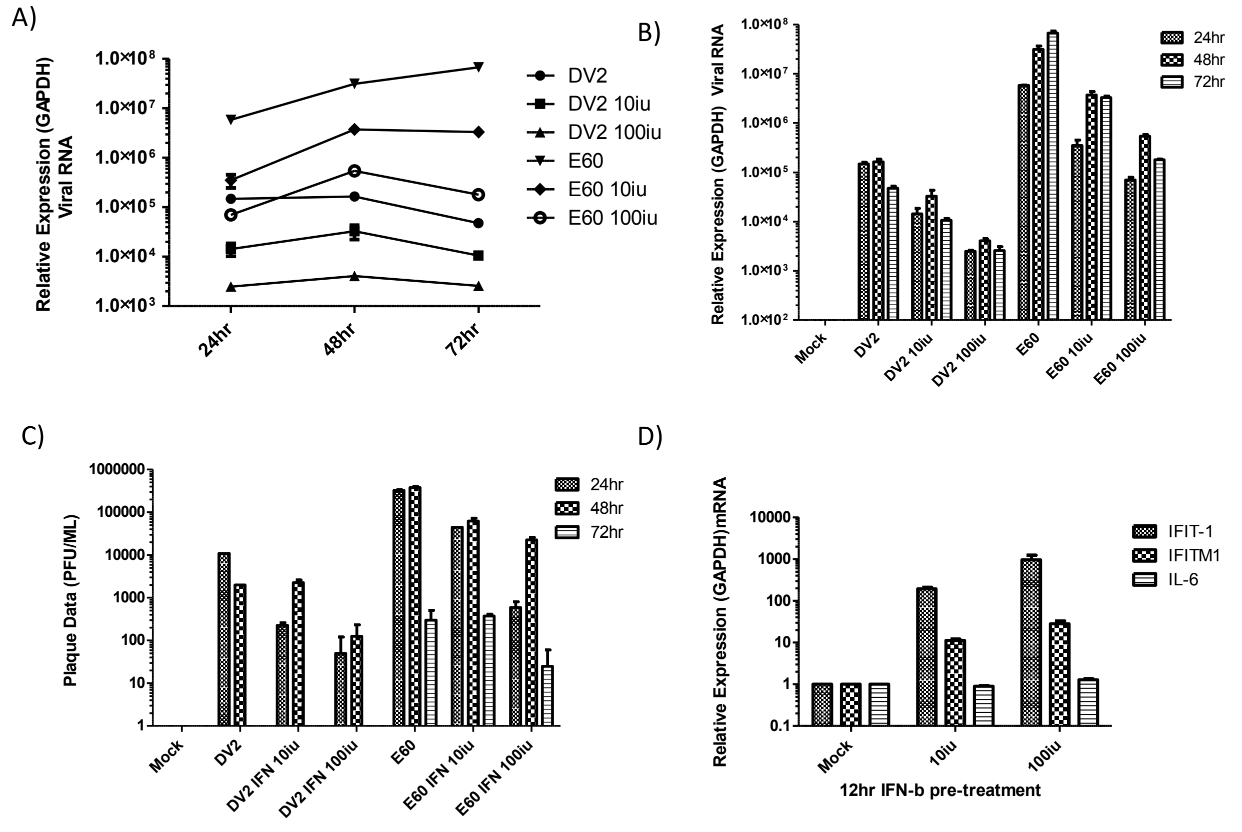


Figure 2.5 ADE overcomes the IFN induced antiviral state. U937 cells were pre-treated for 12 hrs. with 10 or 100IU IFN-beta and subsequently infected with DENV2 NGC at an MOI of 1 in the presence or absence of 0.001 ug/ml E60 antibodies. Cells and supernatant were harvested. A) qRT-PCR of cellular levels of viral RNA over time. B) qRT-PCR of cellular levels of viral RNA in histogram depiction. C) Plaque data of infectious virus output in PFU/mL D) qRT-PCR control data of cells pre-treated with IFN-beta for 12hr (time 0) to verify upregulation of JAK/STAT dependent ISGs: IFIT-1 and IFITM1. IL-6 was measured as a negative specificity control. Results are representative of 3 independent experiments.

To assess the relative proportions of infected cells between IFN pre-treatment conditions, infected cells were examined by flow cytometry. Following IFN treatment, U937 cells continued replication as expected and cell viability across time-points was consistent with no significant differences between comparison conditions (Figure 2.6B,C). The percentages of cells expressing endogenous viral envelope protein under non-ADE conditions were dramatically reduced following a 12hr pre-treatment with 10IU of IFN-beta and cells expressing Env were almost undetectable with 100IU pre-treatment (Figure 2.6A). Additionally, across all non-ADE conditions, the proportions of E positive cells decreased from 24 to

48hrs post-infection. Conversely, under enhancement with E60, the percentage of infected cells increased from 24 to 48hrs across all conditions and >1% of cells pre-treated with 100IU IFN-beta expressed Env. Similar results were obtained using an MOI of 5 for non-enhancing conditions (data not shown).

Under normal ADE conditions DenV is rapidly infecting naive cells at a high rate resulting in a low median fluorescence intensity (MFI) among infected cells (left-skewed distribution). Conversely, under primary conditions the rates of colonization among monocytes is low and the MFI increases as a small population of infected cells become viral replication factories with high levels of viral protein (normal distribution). For the 10IU treatment under ADE conditions the MFI decreases, consistent with high rates of colonization, whereas at the 100IU IFN-b treatment condition the MFI rises abruptly at 48hrs post-infection (Figure 2.6D). These data suggest that at this time point infection of new cells is low.

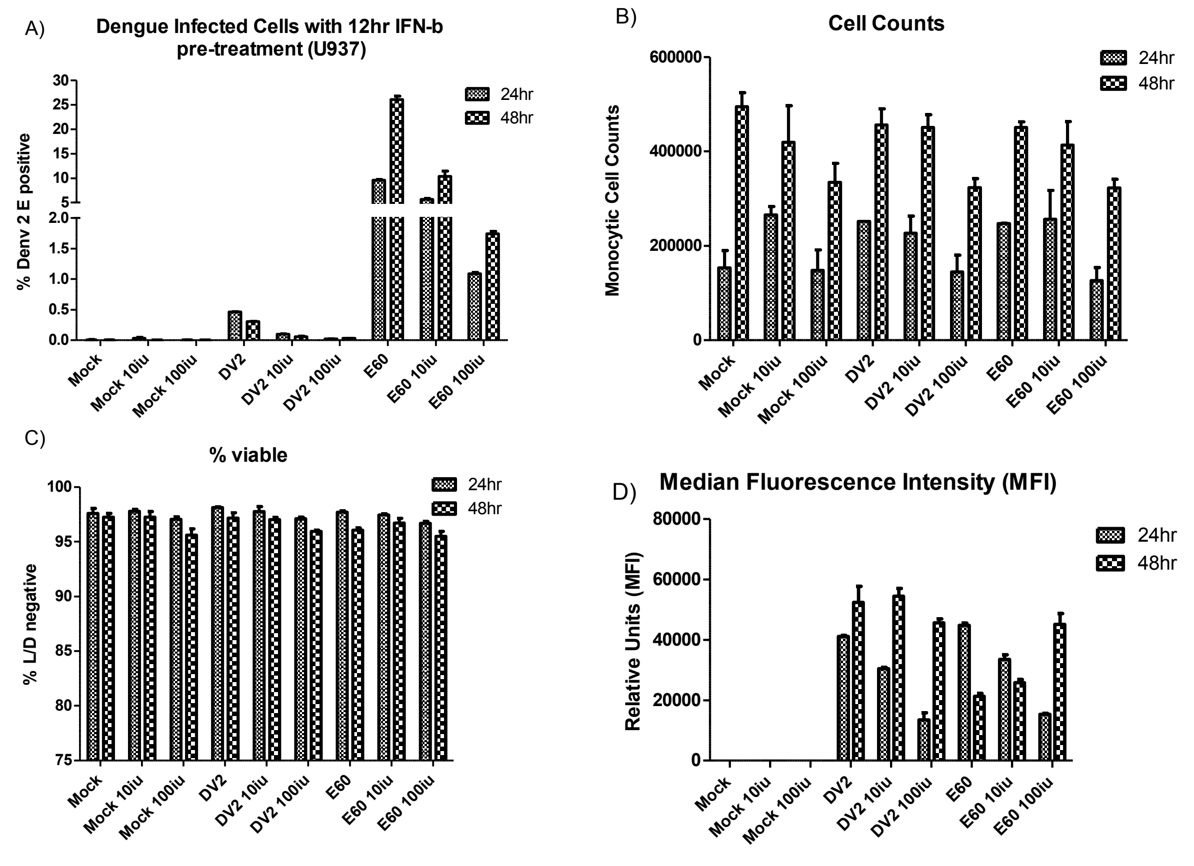


Figure 2.6 ADE increases infection during a pre-existing IFN induced anti-viral response. Cells were pre-treated for 12hrs with 10 or 100IU of IFN-beta and infected with DenV 2 NGC at an MOI of 1. Cells were

harvested and stained for cell viability and intracellular levels of viral Env protein. A) Percentage of DenV Env positive cells at indicated time points post infection. B) Absolute cell counts for each condition. C) Percentage of viable cells among absolute cells sorted. D) Median fluorescence intensity of DenV 2 Envelope protein positive cells. IU values under conditions indicate the concentration of IFN-beta pre-treatment 12 hours prior to infection. Results are representative of 2 independent experiments.

Therefore, these results strongly suggest that *in vitro,* ADE allows DenV entry, replication, virus production, and subsequent rounds of infection even in the presence of an established antiviral response. Taken together-the findings that the levels of infectious viral progeny decrease by 72hr post infection and the finding that MFI among infected cells increases-these data suggest that the antiviral state eventually restricts virus replication. Therefore, ADE mediated infection may allow for a moderately sustained or a transient level of viral replication.

Development of an ADE cell sorting assay to assess cell intrinsic innate immune activation

The data so far indicate that viral replication is highest among macrophage-like cells and monocytic cells support lower levels of viral replication but become highly susceptible to infection under enhancing conditions. Additionally, DenV is still able to productively infect monocytic cells with an activated antiviral response under ADE conditions. Therefore, these results indicate that during ADE fundamental differences exist between primary infection conditions whereby viral replication is enhanced and innate immune defenses are circumvented or evaded. However, all of data thus far has been obtained from experimental conditions in which only a minority of cells were infected. These low rates of infection prohibit any conclusions about the cell intrinsic consequences of ADE. Furthermore, the fact that ADE vs. non-ADE results in vastly different proportions of infected cells prohibits robust conclusions about the differential consequences of infection under each condition. Flow cytometry is a powerful tool for investigation of cell specific conditions. However, the paucity of innate immune antibodies (no type I IFN, limited ISG abs) limits the range of questions that can be asked.

To investigate the cell intrinsic consequences of ADE, I sought to develop tools that would allow for effective identification of cells that were infected with virus or experienced viral entry, enabling cell sorting and enrichment of the infected cell population. To achieve this a GFP-tagged DenV2 reporter virus was obtained with the full length GFP reporter gene inserted at the 5' end of the viral genome within the viral capsid structural protein gene region (Schoggins et al. 2012). This virus was an IC30P-A infectious clone of Dengue serotype 2 strain 16681. To assess rates of infection and the kinetics of GFP expression, U937 cells were infected in the presence and absence of E60 enhancing antibodies at an MOI of 10. Cells were harvested and fixed at 24 and 48hrs post-infection and GFP expression was analyzed by flow cytometry. Results indicated that the rate of infection and virus replication for the GFP tagged virus was prohibitively low for cell sorting even in the presence of enhancing antibodies (Figure 2.7).

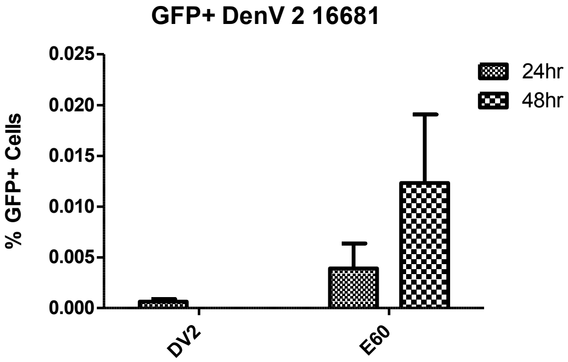


Figure 2.7 DenV-GFP IC30P-A reporter virus results in prohibitively low levels of infection. U937 cells infected with the GFP-DV2 16681 strain at an MOI of 10 in the presence or absence of E60 enhancing antibodies. Results indicate the percentage of GFP positive cells at the given time points.

Another method that has been used to study Dengue infection under enhancing and non-enhancing conditions is the DiD labeling of viral particles (Ayala-Nunez et al. 2011). The viral particles are labeled with a DiD fluorescent dye that is quenched at high concentrations. Upon viral entry and fusion with the endosome, the dye disperses and fluoresces. I reasoned that using this dye to label virus and then sort fusion positive cells following infection would allow me to concentrate infected cells for molecular analysis. One caveat to this approach is that viral fusion does not necessarily guarantee productive infection. However, from a pathogen recognition and innate immunity perspective, cells that experience a viral fusion event have multiple opportunities for pathogen recognition. This approach was only piloted and not optimized for DenV sorting (data not shown).

2.4 Discussion

Using an *in vitro* system for anti-body dependent enhancement of Dengue virus infection, I found that Dengue is able to overcome a pre-existing interferon induced antiviral response to maintain virus replication over a limited number of reproductive cycles in the presence of enhancing antibodies. I also found that monocytes are inefficiently infected by dengue virus under primary infection conditions, but are readily infected in the presence of enhancing antibodies. Macrophages, in contrast are infected at low frequencies and are only capable of undergoing limited infection enhancement, yet virus production per cell in macrophages was over 100 times greater than monocytes. Interestingly, across infection conditions the innate immune signature was only detectable at later time-points, correlated with the levels of infectious virus in the supernatant, and was of greater magnitude in macrophages. Collectively, these findings suggest a model whereby antibody dependent enhancement increases infection rates among monocytes resulting in reduced overall replication kinetics but greater infected cell frequency. During enhancement successive rounds of virus replication are able to persist even after pathogen recognition and initiation of the interferon-induced antiviral response. These findings are in agreement with PBMC profiling studies of acute Dengue patient's that have consistently demonstrated high proportions of monocytes infected (Durbin et al. 2008, Kou et al. 2008, Sun et al. 2011, Halstead 1977). Additionally, these findings are congruent with in vitro studies that have demonstrated virus output per infected cell increases under ADE conditions (Boonnak et al. 2008, 2011, Kou et al. 2011, Sun et al. 2011).

Previous reports using patient convalescent sera and THP1 or U937 cells, indicated that primary Dengue infection drove a profound pro-inflammatory and interferon induced antiviral response that was completely shut down through an IL-10 mediated mechanism during ADE (Ubol et al. 2010, Modhiran et al. 2011, Boonnak et al. 2011). In contrast, I found no evidence of a differential innate immune response between ADE and non-ADE conditions. Potential explanations for this discrepancy are that these authors used the DenV2 16681 hemorrhagic isolate laboratory strain that has been shown to be more inflammatory than other Dengue strains. Additionally, for all of the experiments performed by this group they used the same single patient's convalescent sera and it is possible that residual IL-10 in this sera could independently explain the IFN signaling collapse observed. In agreement with my findings another group using the DenV 2 NGC strain, and PBMC isolated monocytes, found limited innate immune signature that correlated with the levels of virus infection (Kou et al. 2011). However, an important limitation for the present analysis and all of these studies is that differential rates of infection existed between ADE and non-ADE conditions and only a minority of cells were infected at any given time. Other studies have reported a differential phenotype by titrating equivalent levels of virus between ADE and primary infection conditions by qRT-PCR or measuring rates of viral fusion (Boonnak et al. 2011, Chan et al. 2013). However, this approach results in differential biases in the level of DenV PAMP input during infection that can independently explain the reported differences in innate immune signature between ADE and non-ADE conditions.

The finding that Dengue replication and infection is able to transiently persist in the context of a pre-existing IFN-induced anti-viral state is intriguing and may help to explain how ADE drives the “cytokine storm” that can ultimately lead to “severe dengue” and shock. While this phenomenon was only examined in the context of monocytic infection, other FCyR bearing cells may also support infection. These data were consistent across IFN-beta treatment conditions, experiments, and monocytic cells types. The IFN feedback response results in the expression of a wide range of anti-viral factors that work to restrict viruses by inhibiting viral entry, trafficking, and egress (IFITM1, 2, 3), sequestering viral RNA (IFIT1,2,3, etc.), disrupting replication of the viral genome and enhancement of pathogen recognition (PKR, MX1, viperin), and destruction of viral RNA (OAS/RNase L), etc. (Schoggins et al. 2011 , Wong and Chen 2014, Brass et al. 2009, Jiang et al. 2010). Additionally, the IFN response also upregulates PRR abundance and augments cellular processes that shuffle PAMPs to PRRs for increased immune surveillance, expression of transcription factors with positive feedback for the IFN response (IRF1, IRF7, IRF5), as well as increasing antigen presentation and chemokine expression promoting a productive adaptive immune response (Schoggins et al. 2011 , Wong and Chen 2014). Therefore, continued virus replication in the presence of the ISG response may result in greatly enhanced levels of PRR mediated signaling that can ultimately drive a systemic inflammatory response among monocytes and other sentinel innate immune cells such as macrophages and dendritic cells.

Previous studies indicated that dengue virus was able to continue replication if IFN treatment was administered to cells following infection for both ADE and non-ADE infection conditions. Conversely, they concluded that virus replication was completely shut down if cells were pre-treated with IFN in both ADE and non-ADE conditions (Diamond et al. 2000, 2001). Examination of their data reveals fundamental differences in their methodology that may have influenced their results. The authors analyzed cellular rates of infection and infectious particle release 96hrs post-infection, yet they present no data on U937 viability. Within my culture system U937 viability wanes by 72hrs post-infection due to overcrowding and nutrient limitations. Therefore reduced cell viability could explain the lower virus output and rates of infection. Indeed my data suggested that the ADE effect of overcoming the IFN response was transient and tapered off by 72hrs post-infection. By 96hrs remaining virus would likely be in a steady state of decay. Reports have indicated that the half-life of DenV at 37C is roughly 12 hrs. (Pierson et al. 2007, Mukherjee et al. 2014). Finally, careful examination of the author's data reveals that at 96hrs post-infection their rates of viral infection and infectious virus output in the supernatant during ADE in the presence of IFN-beta were roughly equivalent to those found among cells that were infected under non-enhancing conditions. Therefore, their data are in agreement with my finding that IFN pre-treatment reduces virus infection and replication kinetics but is insufficient for complete infection restriction during ADE.

The mechanism whereby ADE transiently overcomes the IFN-induced antiviral state is unclear. One possible explanation is that co-receptor ligation or FCyR ligation may initiate downstream signaling pathways that promote degradation and inhibition of IFN induced antiviral effectors. A recent study reported that among a unique THP1 sub-clone population, Dengue infection in the presence of enhancing antibodies resulted in a blockade of the JAK/STAT mediated ISG response (Chan et al. 2013). They attributed this finding to immune complex co-ligation of the leukocyte immunoglobulin-like receptor B1 (LILRB1) inhibitory receptor during FC-receptor mediated entry, resulting in an SHP-1 mediated blockade of the JAK/STAT response. However, LILRB1 mediated shutdown of JAK/STAT expression would not explain Dengue viruses ability to overcome pre-existing protein-level expression of antiviral ISGs during ADE. It is also possible that the endosomes the virus utilizes through FCyR mediated entry do not contain restriction factors such as the IFITM3 family and may allow for differential subcellular localization for replication in compartments that have reduced antiviral effectors (Brass et al. 2008, Jiang et al. 2010). Similarly, Dengue virus infection has been shown to initiate and depend on the unfolded protein response (UPR) for efficient virion assembly and egress (Peña and Harris 2012, Hetz and Oakes 2015, Fischl and Bartenschlager 2011). Therefore, the virus induced UPR may restore cellular processes that are favorable for virus assembly as well as result in the degradation of viral restriction factors that were expressed as part of the ISG response (Hetz and Oakes 2015). However, no studies to date have investigated the UPR under ADE conditions. Alternatively, ADE may result in greater levels of virus fusion and entry per infected cell that is sufficient to overcome the ISG mediated antiviral state.

While the results from the present studies are provocative, there are a number of limitations. Most importantly, all of the infection data were obtained under conditions where differential rates of infection occurred and only a minority of cells were infected at any given time. Therefore, no robust conclusions can be made about the cell intrinsic effects of ADE on the innate immune response or the role that bystander cells play during infection. An additional limitation was that all infections were performed using lab adapted strains of DenV and immortalized cell types. A recent study reported differential rates of enhancement of lab adapted strains of virus compared to clinical isolates (Chaichana et al. 2013). Therefore, the finding that ADE overcomes the IFN induced antiviral state may be an artifact of the lab adapted virus strain and not a true representation of dengue related disease. Similarly, it is possible that the data on ADE overcoming the IFN induced anti-viral state could be attributable to a sub-clone population in the U937 cells that is unresponsive to IFN stimulation. Indeed, previous reports have indicated high rates of polyclonality among monocytic cell types such as THP1s (Chan et al. 2013, Tsuchiya et al. 1980). However, polyclonality exists to some degree within individuals and these findings were replicated using both THP1 cells and DenV4 H241. Therefore, it is unclear how divergent these results are from primary human cells and an IFN insensitive clonal population would have also needed to be present among the THP1 cells to explain this phenomenon.

Finally, all of these observations were made *in vitro* therefore, the dynamic interplay between the virus, physiological processes and diverse cell populations in the body, and the cross-talk between the innate and adaptive immune systems is absent from these findings. Thus, observations made using isolated *in vitro* systems can inherently over-simplify or misrepresent complex biological processes. This is especially true when trying to study the etiology of complex disease pathologies such as “severe dengue.”

Dengue virus infection under ADE conditions results in only limited detectable innate immune induction. The finding that the virus can continue replication and subsequent rounds of infection even following initiation of a robust type I interferon induced antiviral response, suggests that ADE is imparting a mechanistic advantage. However, it remains to be elucidated whether this is attributable to changes in cellular processes or overwhelming susceptible cells with virus. To study the cell-intrinsic innate immune response to dengue virus using relevant cell types in the context of ADE will necessitate the development of tools to selectively sort infected or exposed cell populations. The previously reported GFP-DV2 strain proved exceeding difficult to grow and was unsuitable for this purpose. However, preliminary data using DiD dye to assess viral fusion events may be a promising alternative for studying the cell intrinsic innate immune response. Therefore, to gain valid mechanistic insights into the processes involved in ADE, future research should utilize cell intrinsic tools that will allow for direct elucidation of the molecular processes involved.

Chapter 3. PLASMACYTOID DENDRITIC CELL INNATE IMMUNE RECOGNITION DURING FLAVIVIRUS INFECTIONS IS MEDIATED BY THE RIG-I LIKE RECEPTORS IN AN INFECTION DEPENDENT MANNER.

3.1 Background:

Plasmacytoid dendritic cells (pDCs) are specialized innate immune cells that play a pivotal role in the recognition of viruses. Functionally, pDCs detect and respond to RNA viruses via toll like receptor 7 (TLR7). pDCs are called interferon producing cells (IPC) because TLR7 ligation results in the production of large amounts of type I interferon and other pro-inflammatory cytokines that limit viral replication, influence pDC cell fate, and help orchestrate the resultant adaptive immune response (Siegal, Kadowaki et al. 1999; Kadowaki and Liu 2002; Kato, Takeuchi et al. 2006; Lee, Lund et al. 2007; Rodriguez-Madoz, Bernal-Rubio et al. 2010).

A defining feature of pDCs is the constitutive expression of interferon regulatory factor 7 (IRF7). In most cell types IRF7 is expressed following the interferon signaling cascade initiated by IFNAR and resulting in activation of the JAK/STAT pathway and the induction of interferon stimulated gene response (ISGs). Therefore, pDCs circumvent the need for the JAK/STAT feedback signaling and are primed to respond to viruses. TLR7 signaling in pDCs results in IRF7 activation which promotes the release of profound levels of interferon, principally IFN-alpha and beta, as well as nuclear factor kappa-b (NF-kB) resulting in pro-inflammatory cytokine release such as IL-6 and TNF-alpha (Siegal, Kadowaki et al. 1999).

An additional role for pDCs is to undergo programming to become Antigen Presenting Cells (APC). Upon TLR7 activation, activated NF-κB triggers the maturation process in pDCs. Upon maturation, pDCs migrate into draining lymph nodes and cross-present antigens to T-cells via the major histocompatibility complex II (MHC II). This important secondary pDC function results in a terminal cellular fate.

pDC mediated recognition of viruses has traditionally been thought to occur in the context of micropinocytosis or receptor mediated endocytosis of viral particles into TLR7 bearing endosomes. While many innate immune cell types use the RIG-I Like Receptor (RLR) family to sense and respond to replicative viral infections in the cytosol, mechanistic studies in pDCs have suggested that the RLRs do not participate in pDC pathogen recognition (Siegal, Kadowaki et al. 1999; Kadowaki and Liu 2002). Instead pDCs have been shown to utilize autophagic mechanisms to shuffle pathogen associated materials into TLR7 bearing endosomes for pathogen recognition (Lee et al. 2007, Severa et al. 2013). In contrast, a recent study that utilized synthetic transfected immunostimulatory viral RNA, demonstrated that pDCs can in fact utilize the RLRs to recognize foreign RNA motifs (Stone et al. 2013). However, we did not identify any studies demonstrating RLR involvement of pDCs in the context of an actual viral infection. Therefore, we hypothesized that in the context of productive viral infections in pDCs the RLRs may drive the interferon response.

An RNA virus family of immense public health importance are the Flaviviruses including West Nile Virus (WNV) and Dengue Virus (DenV). The type I IFN response has been shown to be of keystone importance for the immune control of Flavivirus infections (Kato et al. 2006, Tsai et al. 2009, Errett et al. 2013). Specifically, both the TLR7 and RLR-mediated interferon responses are critical for this IFN response (Nasirudeen, 2011). Since, pDCs are the most potent type I IFN producing cells, their contributions during Flavivirus infections may be vital. However, data highlighting the role of pDCs in the context of Flavivirus infection remains poorly characterized. Evidence indicates that mice lacking the RLR signaling adaptor protein, MAVS, still exhibit robust type I IFN responses when challenged with WNV (Errett et al. 2013). These findings suggest that pDCs may be the source of the persistent type I IFN response by WNV engagement of TLR7. Additionally, CNS infiltrating pDCs have been identified during encephalitic infection in mice (Brehin, et. al. 2008). Similarly, dengue virus disease severity correlates with low levels of pDCs in the blood as well as low type I IFN levels (Rodriguez-Madoz et al. 2010, Chau et al. 2009, Pichyangul et al. 2003, Simmons et al. 2007, Libraty et al. 2002). However, the exact contributions of pDCs in the context of DenV infection remains to be elucidated. Therefore, pDCs are likely important contributors to the IFN induced antiviral response, yet there are key mechanistic gaps in our understanding of how pDCs contribute to Flavivirus disease severity. We hypothesize that pDCs are important cells for orchestrating the innate immune response during Flavivirus infection by recognizing virus through both the TLR and RLR pathways, ultimately influencing pDC cellular fate and maturation.

3.2 Methods

Cell lines , viruses, and reagents

Vero African green monkey kidney cells and the *Aedes albopictus* C6/36 mosquito cells were grown in minimum essential medium supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT). The human pDC cell line CAL-1 was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 ug/ml streptomycin, and 100 U/ml penicillin. DENV2 New Guinea C (NGC) strain (Dr. Jay Nelson, Portland, OR) was propagated from viral reference stocks by single passage in C6/36 cells grown at 28C. Upon visual identification of cytopathic effect viral supernatants were harvested and subjected to centrifugation at 1500g for 20 minutes, and clarified through a 0.2uM filter. WNV isolate TX 2002-HC (WNV-TX) characterization and culture was described previously (Keller et al., 2006). Viral titers were quantified using the vero plaque assay. Sendai virus, Cantell strain, was purchased from Charles River. Cells were infected with 100 HA units per ml of Sendai virus and harvested at the indicated time-points post infection. R848 was purchased from Invivogen. Dual iODN was purchased from Enzo Lifesciences.

**The CAL-1 and Gen2.2 were extant in the Gale laboratory prior to Jonathan Florentin PhD and Russell Barlow joining the lab in June of 2013. Jonathan Florentin and Russell Barlow attempted to find a standing material transfer agreement (MTA) for both of these cell lines and were unable to do so.*

***In vivo* murine infections and splenocyte isolation**

C57BL6 mice were purchased from Jackson Laboratory and were maintained according to the University of Washington Guidelines for animal care established by the Institutional Animal Care and Use Committee. Age-matched 8 week old mice were inoculated subcutaneously (s.c.) in the hind footpad with 100 PFU of WNV-TX diluted in phosphate buffered saline (PBS). Mice were monitored daily for morbidity and mortality. To determine the in vivo levels of pDC infection, mice were euthanized 48hours post-infection. Spleens were removed and splenocytes were isolated by disruption of splenic connective tissue on frosted glass slides. Isolated cells were counted and re-suspended in complete RPMI 1640 prior to surface staining and flow cytometry analysis.

Vero cell plaque assay

The Vero cell plaque assay was performed as described previously (Pinto et al. 2013). Six log dilutions of virus stock were made from each supernatant sample and inoculated into six-well tissue culture plates containing confluent Vero cell monolayers. Virus adsorption was allowed to occur for 2 hr. Following adsorption the Vero monolayer was overlaid with complete minimal essential medium, containing 1% low-melting-point agarose (Invitrogen). Infection plates were incubated for 6 days at 37°C for and overlaid with the vital stain neutral red according to the manufacturers protocol (Sigma). Plaques were counted by visual inspection at 12-16 hrs following neutral red staining for determination of the number of infectious viral progeny as approximated by the number of plaque forming units (PFU) of Dengue virus or WNV per milliliter of supernatant.

Flow cytometry

FACSCalibur Canto2 or LSRII instruments (BD Biosciences, San Jose, CA) were used to monitor cell surface staining of anti-HLA-D4-PE-Cy7, anti-CD123-PE, anti-BDCA-2-APC, anti-CD11C-PE-Cy5, anti-CD83-PE and anti-CD40-APC (BD Biosciences). For monitoring of murine pDCs the anit-PDCA-1-APC (BD Biosciences, San Jose, CA) was used. For detection of cell viability a cellular permeability Live/Dead pacific blue stain was utilized (Invitrogen, Carlsbad CA). For detection of intracellular viral protein, cells were fixed and permeabilized with Cytofix/Cytoperm according to the manufacturers protocol (BD, Biosciences), and stained with E206 (anti-DV Env MAb) or E16 (anti-WNV Env MAb) conjugated to AlexaFluor-488 (Invitrogen, Carlsbad, CA) at 24 and 48hr post infection.

Western blotting

Western blot analysis was carried out as previously described (Loo et al. 2008). Briefly, cells were either mock infected or infected with Dengue virus 2 (NGC) or West Nile Virus TX in the presence or absence of enhancing antibodies at an MOI of 1 or 5 as indicated. Cells were harvested post-infection at defined time intervals and cellular lysates were extracted for total protein content. Harvested cells were lysed and protein was extracting using in extraction buffer (150 mM NaCl 50, mM Tris HCl [pH 7.5], 0.5% sodium deoxycholate, 1% NP-40, 1 mM EDTA, and 0.1% sodium dodecyl sulfate, supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotonin, leupeptin, and pepstatin). Following lysis extracts were centrifuged at 15,000g for 10 min at 4°C.Protein extracts were quantified using the Bradford assay (Bio-Rad) and 20ug of protein lysate were loaded per well. Cellular proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed via Western blot using targeted protein-specific antibodies. Mock-infected samples collected at each time point were also analyzed with each of the antibodies tested. The membranes were probed with mono-clonal and polyclonal antibodies specific for total IRF-3, phospho-IRF3 (p-IRF3, Ser396), MAVS (cardif AT107), ISG56

(IFIT-1), total IRF7, total STAT1, phospho-STAT1 (Tyr701), MDA5, RIG-I, and GAPDH (Santa Cruz Biotechnology), or WNV. All secondary antibodies were purchased from Jackson Immunoresearch. Immunoreactive bands were detected with Amersham ECL Plus reagent (GE Healthcare).

RNA extraction and analysis

Cells were harvested and lysed using the QiaShredder (Qiagen) kit per the manufacturer's recommendations. Total cellular RNA from experimental cultures was extracted for quantitative reverse transcription-PCR (qRT-PCR) using the RNAeasy kit (Qiagen) according to the manufacturers protocol. The extracted cellular RNA was then reverse transcribed using the iScript select cDNA synthesis kit using both oligo (dT) and random primers according to the manufacturers protocol (Bio-Rad). RNA amplification was conducted from 10 ng cDNA at a 10 µl reaction mixture in 384-well opitical plates. Amplification was performed under the following conditions: 30 min at 48 C, 1 cycle at 95 C for 10 min, 30 cycles at 95 C for 15 seconds and 1 min at 60 C and dissociation curve (95 C 1min, 65 C 2 min and 65C–95C at 2/sec). Cellular mRNA and viral RNA expression levels were determined by SYBR green qRT-PCR using gene- or virus-specific primers on a ViiA7 thermocycler (Applied Biosystems). Data was analyzed by the ΔΔCT method. Specific primer sets are as follows:

hIFITM1, forward: TACTCCGTGAAGTCTAGGGACAG; hIFITM1, Reverse: AACAGGATGAATCCAATGGTCA; hIFIT-1, forward: TTGATGACGATGAAATGCCTGA; hIFIT-1, reverse: CAGGTCACCAGACTCCTCAC; hIFIT-2, forward: AAGCACCTCAAAGGGCAAAAC; hIFIT-2, reverse: TCGGCCCATGTGATAGTAGAC; DENV, forward: TTAGAGGAGACCCCTCCC; DENV, reverse: TCTCCTCTAACCTCTAGTCC; WNV TX, forward: CGCCTGTGTGAGCTGACAAAC; WNV TX, reverse: WNV TX reverse, CATAGCCCTCTTCAGTCC; hIFN-a, purchased from SA biosciences; hIL-6, purchased from SA biosciences, hMAVS, purchased from SA biosciences.

ELISA

Enzyme linked immunosorbent assays (ELISA) was performed for absolute quantitation of IFNα (PBL Interferon Source) and IL-6 (eBiosciences) per the manufacturer's instructions.

Whole blood isolation of pDCs

Fresh leukophoresis packs were purchased from (SSS) and pDCs were immediately isolated using the Milteny Diamond Plasmacytoid Dendritic Cell Isolation Kit II (Miltenyi Biotech, 130-097-240) in accordance with the manufacturer's instructions. Isolated cells were confirmed by flow cytometry and used for infection within 8 hours post-isolation. For all pDC infection experiments fresh pDCs were supplemented with 10ng/mL recombinant IL-3.

CRISPR/CAS9 KO

The following MAVS and hAIM2 CRISPR targeting lentiviral plasmids were obtained from Dan Stetson, Seattle WA:

hAIM2: pRRL-gRNA10-Cas9-T2A-puro MAVS: RRL-gMAVS4-Cas9-T2A-puro

Sub-confluent 293T cells were transfected with 10ug of either hAIM2 or MAVS4 vectors containing the RNA guide sequence and the pRRL lentivirus constructs using Lipofectamine 2000 (Life Technologies). 48hrs post-transfection, lentivirus containing supernatants were harvest and filtered through a 0.2 um syringe filter and stored at 4C. CAL-1 cells were centrifuged at 500g for 5 minutes and the resulting supernatant was discarded. The pellet was re-suspended in 6mL of the filtered CRISPR lentivirus containing supernatant and incubated at 37C 5% CO2 for 48hrs. After 48hrs the media was replaced with complete RPMI containing 1 ug/mL of puromycin and cells underwent positive selection for 5-7 days. Following positive puromycin selection, the surviving cell population was frozen in aliquots and gene knockout was verified by qRT-PCR and Western Blot. The following RNA guide sequence targeting exon 4 of MAVS was used: GTCCTGCTCCTGATGCCCGC.

Statistical analysis

All data were analyzed using Prism software using the unpaired student's *t-test* (GraphPad Prism). P-values significant at the 0.05 alpha level were considered significant.

3.3 Results

pDCs support productive Flavivirus infection

In order for RLRs to play a role in the pDC recognition and response to Flaviviruses, we reasoned that pDCs must support productive infection of Flaviviruses. To test this we infected an immortalized pDC-like cell line known as CAL-1 with the Dengue virus serotype 2 strain New Guinea C (DenV 2 NGC) and the West Nile Virus Texas 02 strain (WNV TX) at multiplicity of infections (MOI) of 1 and 5 (Maeda et al. 2005, Karrich et al. 2012). Supernatants were harvested at various time-points following infection to assess the levels of infectious viral progeny. Our results showed clear increases in infectious viral particle output over time indicating a productive infection for both viruses (Figure 3.1A). To assess the relative proportions of cells infected over time, we performed flow cytometry analysis of CAL-1 post-infection and stained for cell viability and the presence of intracellular viral protein. Our results indicated rates of infection around 0.5-3% for DenV2 and WNV by 48 hrs. (Figure 3.1B). Analogous results were obtained using the Gen2.2 pDC-like immortalized cell line (data not shown).

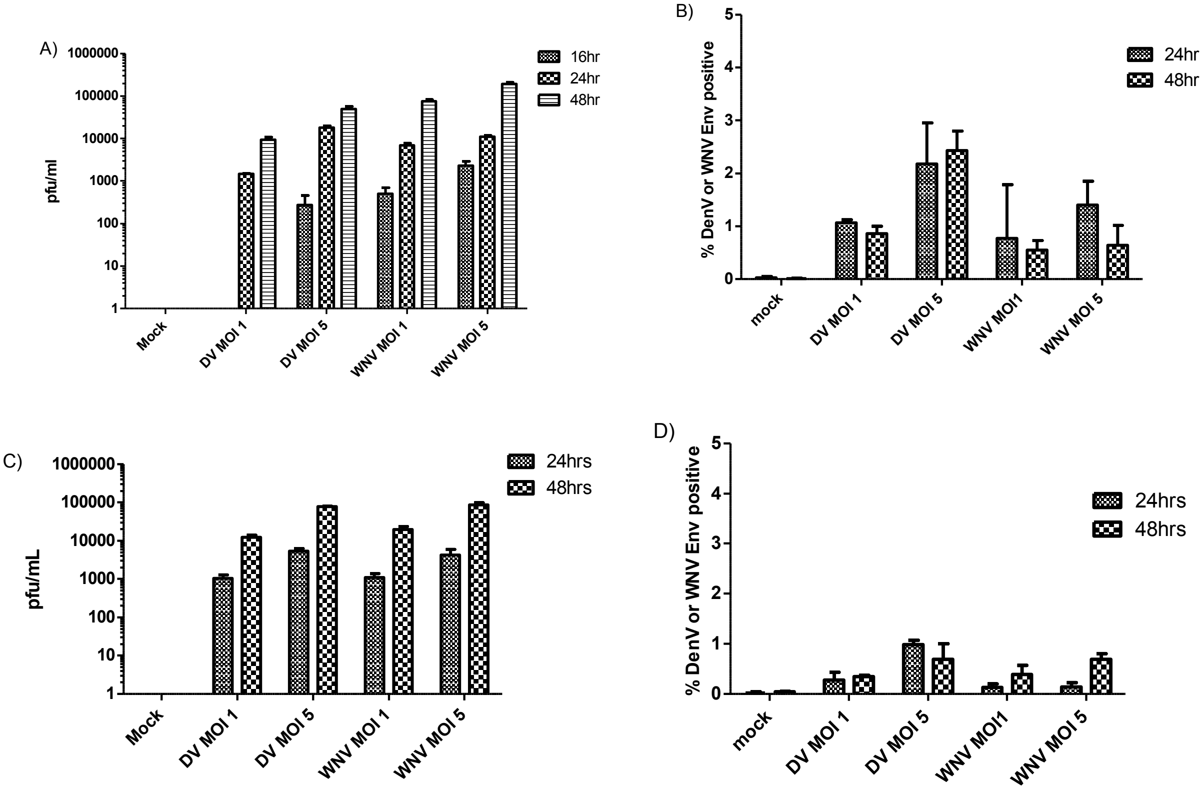


Figure 3.1 pDCs support flavivirus infection. Plaque assay and flow cytometry analysis. Cells were infected at MOIs 1 and 5 with DenV2 NGC or WNV TX. Supernatants were harvested at the indicated time-points for plaque assay analysis. Cells harvested, fixed, and intracellularly stained with an Alexa-Fluor 488 conjugated DenV 2 monoclonal ab (E206) or an Alexa-Fluor488 conjugated WNV monoclonal ab (E16) for flow cytometry analysis. A) plaque assay results for CAL-1 (pfu/mL) B) Percentage of CAL-1 cells infected at the indicated time-points post-infection. C) plaque assay results for primary CD123+ HLA-DR+ BDCA2+ CD11C- pDCs (pfu/mL) D) Percentage of CD123+ HLA-DR+ BDCA2+ CD11C- pDCs infected at the indicated time-points post-infection. Results are representative of at least 3 independent experiments. Error bars represent the standard deviation. Performed by RB (CAL-1) and JF (CD123+ HLA-DR+ BDCA2+ CD11C-)

To validate these findings we obtained freshly drawn peripheral blood mononuclear cells and isolated pDCs using the Miltenyi Diamond pDC isolation kit (Miltenyi-Biotech). Purification was verified by flow cytometry and our data indicated that 97% of the isolated cells were CD123+ HLA-DR+ BDCA2+ and CD11C- consistent with known markers of pDCs (A4). Isolated pDCs were then infected with DenV 2 NGC or WNV TX at MOIs of 1 and 5. Supernatants were harvested at 24 and 48hrs post-infection and infectious viral particle output was measured by plaque assay. Consistent with the CAL-1 results, we saw marked increases in infectious viral particle counts for both viruses (Figure 3.1C). Flow cytometry analysis and intracellular staining for WNV and DenV Env revealed detectable rates of cellular infection that were reduced relative to CAL-1 at 24hrs post-infection (Figure 3.1 D). In contrast to the CAL-1 infection, the rates of DenV infection among primary pDCs were decreased at 48hrs post-infection. This finding could be explained by the fact that primary pDCs produce greater levels of IFN-alpha and are short-lived replicating only slowly in cell culture. Indeed by 48hrs post-infection cell viability across conditions had dropped to roughly 70% (Data not shown). Therefore, the infected cell and the susceptible cell populations likely contracted between these time points. Collectively these data demonstrate that pDCs are susceptible to *in vitro* infection.

To confirm that pDCs are actually infected *in vivo*, we infected C57BL/6 mice with 100 pfu WNV TX and harvested spleens 48hrs post-infection. We found that pDCs represented ~0.4% of all splenocytes and ~6% of all CD3+Ly6C+PDCA1+ pDCs had detectable WNV Env protein consistent with infection (A5). These findings are intriguing because the rates of infection are higher than those obtained in our *in vitro* system and were higher than rates of infection among lymphocytes (~3%, Data not shown).

Flavivirus infection induces a type I IFN response in pDCs

Given that both DenV and WNV productively infect pDCs we sought to define the innate immune response following infection. CAL-1 and primary pDCs were infected with DenV 2 NGC or WNV TX at MOIs of 1 and 5. Cells and supernatants were harvested at 16, 24, and 48hrs post-infection and qRT-PCR analysis of innate immune gene induction and ELISA were performed to assess the magnitude and kinetics of innate immune activation. As a positive control for innate immune activation Sendai virus (SeV) or the TLR7 agonist R848 were used. To approximate the pathways involved, a representative panel of innate immune genes were analyzed by qRt-PCR. Specifically, IL-6 was chosen as this indicates NF-kB mediated pro-inflammatory activity, IFN-alpha is the primary interferon produced by pDCs and is indicative of immune detection and the antiviral response, IFIT-1 is indicative of IRF3/7 activation and/or JAK/STAT mediated transcription of interferon stimulated genes (ISGs). Finally, IFITM1 was chosen as this ISG has been shown to be exclusively dependent on JAK/STAT mediated signaling.

We found that CAL-1 cells infected with Dengue or WNV had detectable expression of IFN-alpha within 24 hours post-infection with maximal message at 48hrs (Figure 3.2C). The level of IFIT-1 was detectable within 24hrs post-infection peaking at 48hrs post-infection (Figure 3.2A). As expected IFITM1 expression was low to undetectable at 24hrs but peaked by 48hrs since this gene exhibits delayed expression due to the necessity for interferon positive feedback through IFNAR and subsequent JAK/STAT mediated transcription (Figure 3.2B). Additionally, only low levels of IL-6 message were detected across all time points (Figure 3.2D). Therefore these data suggest that immune detection of the Flaviviruses occurs in the context of viral replication.

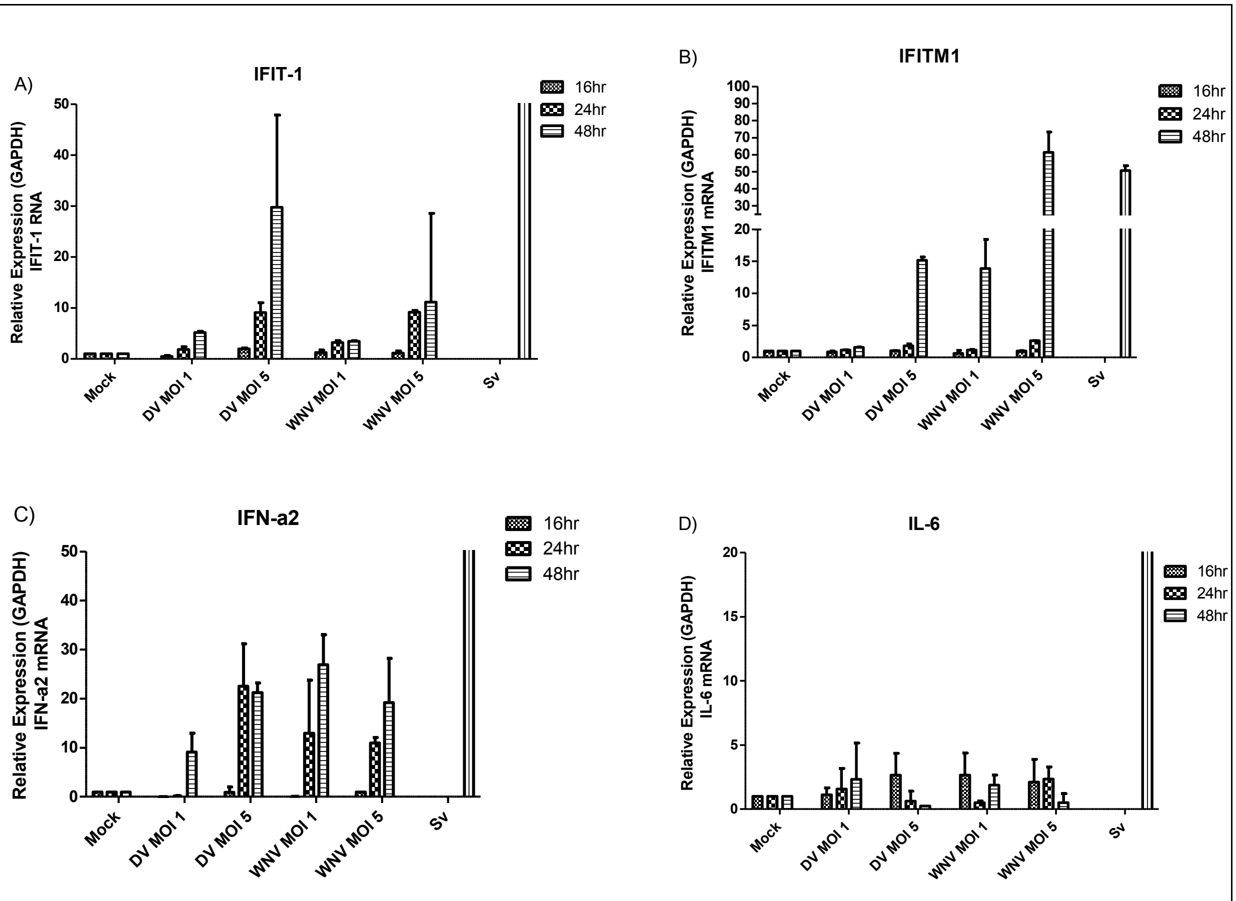


Figure 3.2 Flavivirus infection induces delayed innate immune message in pDCs. Innate immune gene induction was measured by qRT-PCR analysis for A) IFIT-1 B) IFITM1 C)IFN-a2 and D)IL-6 mRNA, relative fold induction after normalization to GAPDH was compared to that of mock infected cells. Results are representative of at 3 independent experiments with at least duplicate samples. Error bars represent the standard deviation. Performed by RB

Western blot analysis provided similar results with detectable increases in expression of the interferon inducible genes IFIT-1, RIG-I, and MDA5 expression at 24hrs and dramatically increased expression by 48hrs post-infection. Similarly, STAT-1 and IRF3 phosphorylation were detectable at 24hrs and peaked 48hrs post-infection for both viruses (Figure 3.3). IFN-alpha ELISA revealed that IFN-alpha production peaked at around 800 pg/ml at 24hrs post-infection for both viruses (Figure 3.4A). Importantly, infection with UV inactivated DenV or WNV resulted in no detectable innate immune signature by qRT-PCR or western blot analysis (Data not shown).

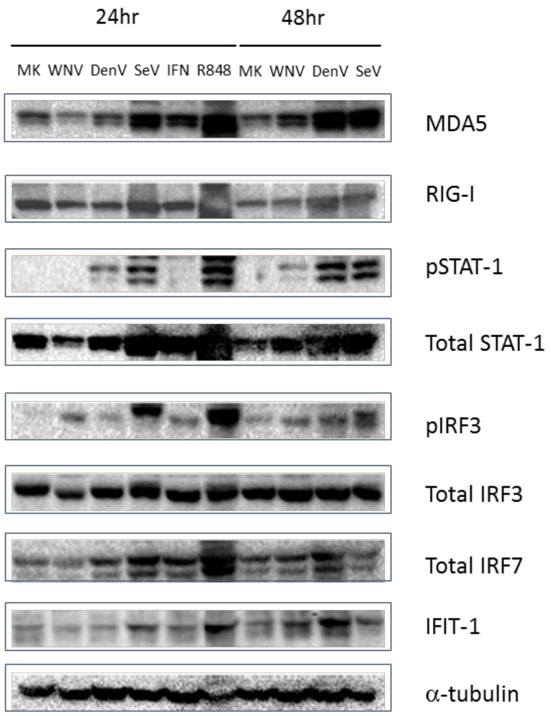


Figure 3.3 pDCs undergo innate immune activation following flavivirus infection. Western blot analysis of innate immune protein levels in CAL-1 cells infected with DenV2 NGC or WNV TX and an MOI of 1 and harvested at 24 and 48hrs post-infection. Sendai virus (SeV) and IFN-beta (IFN) were used at 100 HAU and 100 IU respectively as positive controls. Representative blot of at least 3 independent experiments performed by RB and JF.

CAL-1 cells are known to produce less type I IFN compared to freshly isolated whole blood pDCs. To confirm our CAL-1 in vitro findings, we again isolated CD123+ HLA-DR+ BDCA2+ CD11C- pDCs from PBMC. Isolated pDCs were infected with DenV 2 NGC or WNV TX at MOIs of 1 and 5 to assess the innate immune signature via qRT-PCR and ELISA. Fresh pDCs displayed a similar innate immune profile by qRT-PCR at 24 and 48hrs post-infection (Data not shown). IFN-alpha ELISA demonstrated elevated levels of IFN production relative to CAL-1 with similar kinetics (Figure 3.4B). TNF-alpha ELISA demonstrated both a time and MOI dependent increase in TNF-alpha production following infection. Therefore, these results indicate that pDCs are capable of recognizing and responding to both DenV and WNV in a manner that promotes type I IFN production and a pro-inflammatory cytokine response.

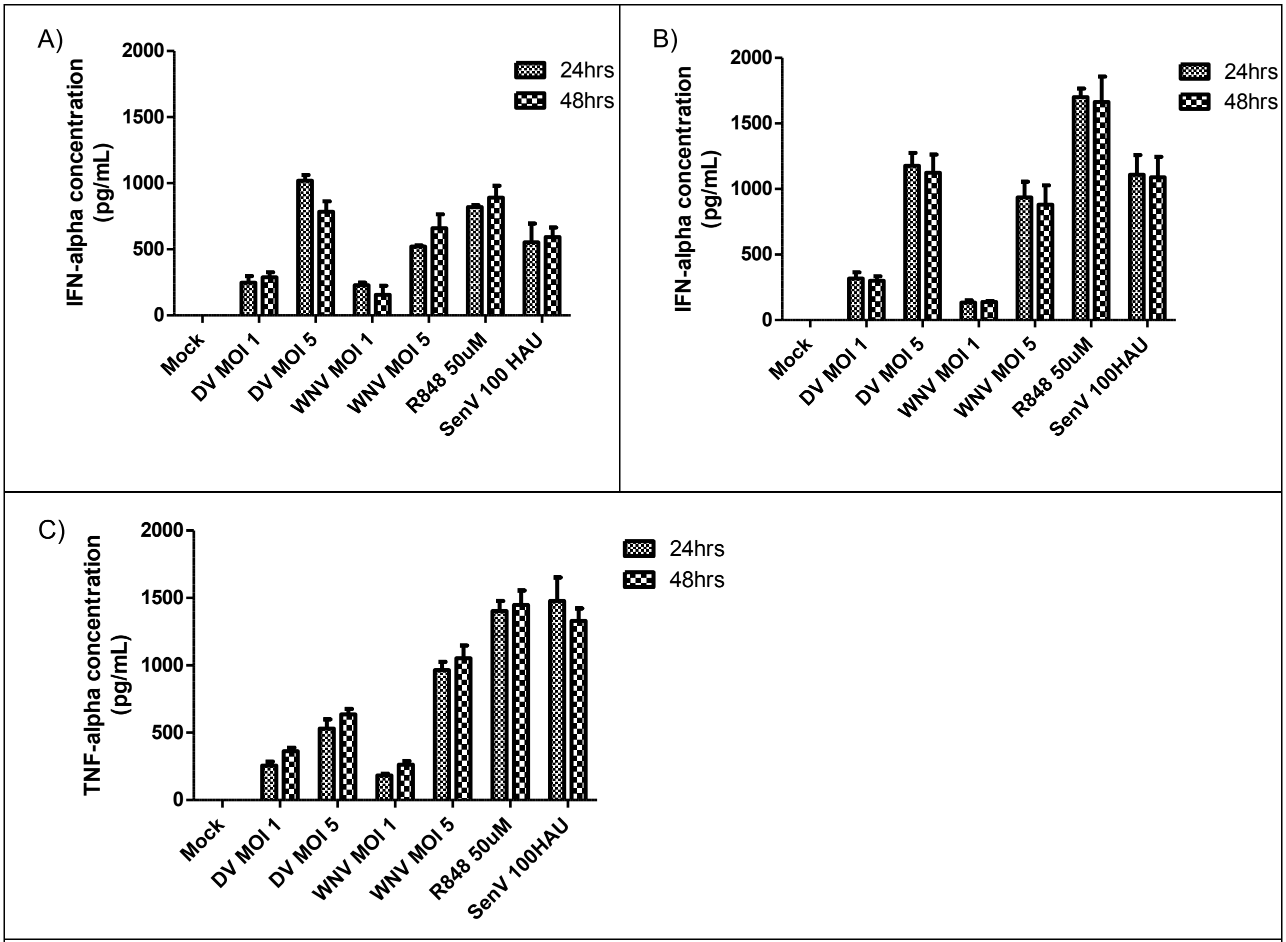
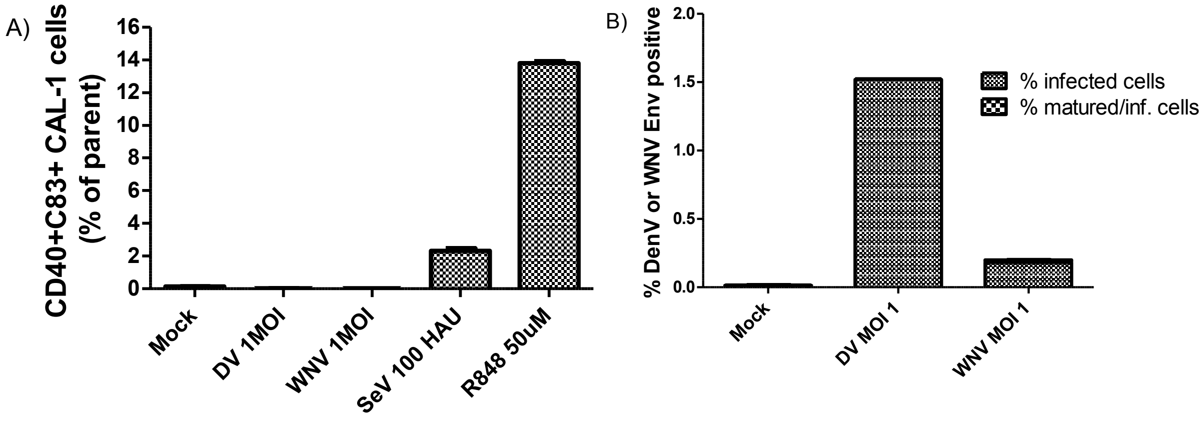


Figure 3.4 Flavivirus infection induces IFN and pro-inflammatory cytokine production in pDCs. CAL-1 or isolated CD123+ HLA-DR+ BDCA2+ CD11C- primary pDCs were infected with DenV 2 NGC or WNV TX and an MOI of 1 and 5 and subsequently harvested at 24 and 48hrs post-infection. A) CAL-1 IFN-alpha ELISA results. B) CD123+ HLA-DR+ BDCA2+ CD11C- primary pDC IFN-alpha ELISA results. C) CD123+ HLA-DR+ BDCA2+ CD11C- primary pDC TNF-alpha ELISA results. Sendai virus (SeV) and IFN-beta (IFN) were used at 100 HAU and 100 IU respectively as positive controls. Result are the pooled analysis of 3 independent experiments performed by RB (CAL-1) and or representative duplicate experiment JF(CD123+ HLA-DR+ BDCA2+ CD11C-)

Flavivirus infection does not drive pDC maturation

Both Dengue and West Nile viruses are known to infect mature dendritic cells with high efficiency (Pinto et. al. 2013, Boonnak et. al. 2008, 2010). Given that pDCs mature into an APC phenotype following TLR7 ligation, we sought to assess whether our infection results were influenced by maturation status and to determine whether Flaviviruses contribute to pDC maturation. Upon maturation, pDCs undergo extensive re-programming and transition from IFN-alpha production to increased antigen presentation. Cell surface markers for maturation include upregulation of CD40 and CD83 (Lee et al. 2007, Vu Manh et al. 2013). We reasoned that our infection and innate immune signature results could be biased if high levels of matured pDCs were present at the time of infection. Prior to infection we assessed maturation of CAL-1 cells via flow cytometry. The cytofix/cytoperm procedure for intracellular staining did not affect CD40 and CD83 surface marker expression. Our results indicated that >99% of cells were CD40-CD83- indicating that these cells had not matured (data not shown).

To assess the influence of Flavivirus infection on pDC maturation, CAL-1 cells were infected at MOIs of 1 and 5 with DenV 2 NGC or WNV TX and harvested 24hrs post-infection. Positive controls for maturation included the TLR7 agonist R848 at 50uM and Sendai Virus (SeV) at 100 HAU. Our results indicated no significant differences in maturation status following Flavivirus infection compared to mock ($p<0.01$, Figure 3.5A). Conversely, both R848 and SeV induced pDC maturation ($p<0.01$). Furthermore, analysis of the infected cell population revealed that less than 0.1% of infected cells upregulated CD40 and CD83 (Figure 3.5B). These data indicate that Flavivirus infection does not drive pDC maturation. Infection of primary pDCs revealed no detectable rates of virus induced maturation (Figure 3.5C). As anticipated the proportion of primary pDCs that underwent maturation was elevated following SeV and R848 treatment in primary cells compared to CAL-1 (Maeda et al. 2005).



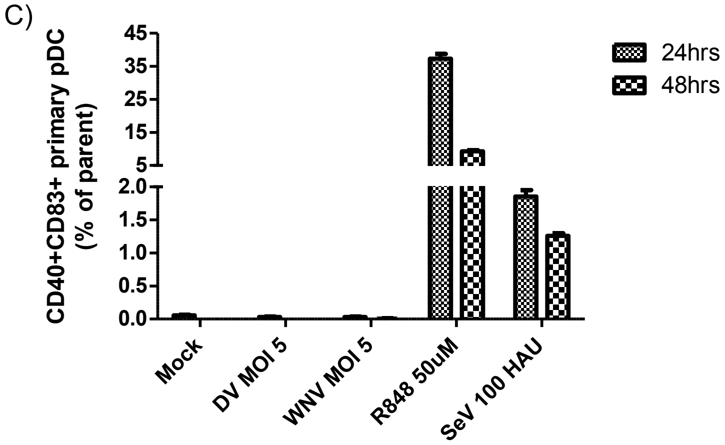


Figure 3.5 Flavivirus infection dose not drive pDC maturation. CAL-1 or CD123+ HLA-DR+ BDCA2+ CD11C- pDCs were infected an MOI of 1 with DV or WNV. 24hrs or 48hrs post-infection cells were fixed, surface stained, intracellular stained, and analyzed for CD40+CD83+ expression and DV or WNV Env expression. A) Percentage of CAL-1 cells double positive for CD40 and CD83. B) Histogram showing the proportion of CD40+CD83+ CAL-1 cells among DV or WNV Env positive cells. C) Percentage of CD123+ HLA-DR+ BDCA2+ CD11C- pDCs cells double positive for CD40 and CD83. Results are representative of at least 3 independent experiments performed by RB (CAL-1)and JF (of CD123+ HLA-DR+ BDCA2+ CD11C-)

We next sought to determine whether the Flaviviruses were evading TLR7 detection or were actively preventing maturation. Therefore, we titrated the R848 concentration on CAL-1 to determine the dynamic range for R848 mediated IFN-alpha production as a proxy for pDC maturation. We found that 2uM R848 was the minimum concentration where maturation could be detected with maximal maturation reached with 100uM (A7). Therefore we used 20uM R848 to promote maturation. CAL-1 cells were pre-infected for 24 hours with DenV and WNV and then 50uM R848 was added to promote maturation. Maturation status was analyzed by flow cytometry at 24hrs post-R848 treatment. Interestingly, we found that R848 treatment plus Flavivirus infection led to slightly increased pDC maturation over R848 alone although these findings were not significant (Data not shown). These data suggest that Flaviviruses do not antagonize pDC maturation and may induce weak TLR7 excitation that is insufficient for pDC maturation but synergizes with R848 to promote maturation.

CRISPR/CAS9 mediated knockdown of MAVS abrogates pDC IFN production

Collectively our data indicate that both DenV and WNV efficiently infect pDCs and that pDC infection results in a productive IFN response that results in only moderate pro-inflammatory cytokine production and no detectable maturation of pDCs into APCs. These data suggest that the Flavivirus induced immune response is not driven by canonical TLR7 ligation. To definitively elucidate the contributions of the RLR vs. TLR7 pathogen recognition pathways, we sought to knock down the RLR adapter MAVS (formerly IPS-1). MAVS specific SI-RNA knockdown was only about 40% effective (Data not shown). Therefore we utilized the CRISPR/CAS9 system to knock out MAVS. CAL-1 cells were transduced with MAVS targeting CRISPR lentiviral vectors or the expendable gene target hAIM2. Following puromycin selection we found that MAVS levels were over 80% reduced compared to mock transduced cells at the RNA level and no MAVS protein was detectable via Western Blot (Figure 3.6A,B). As expected there were no detectable differences in MAVS expression between the AIM2 transduced cells compared to wild type (WT) non-transduced CAL-1 cells.

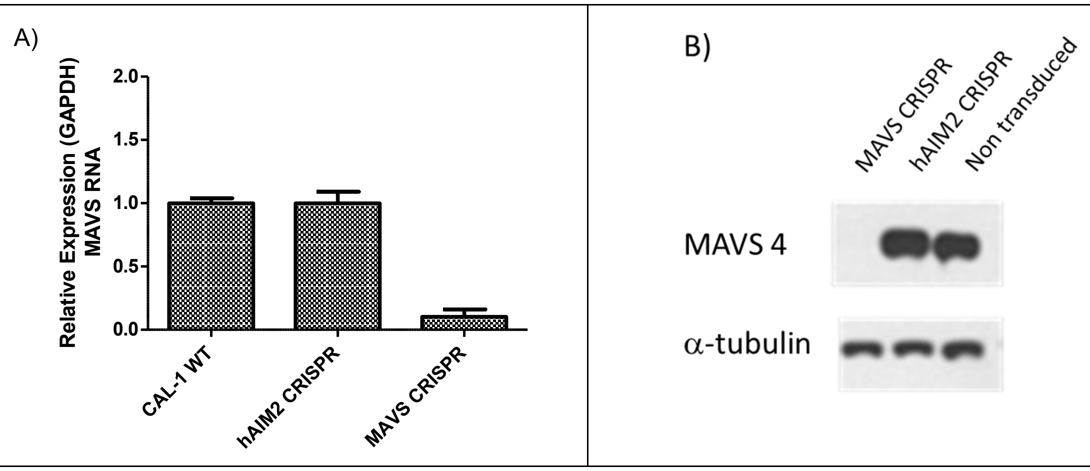
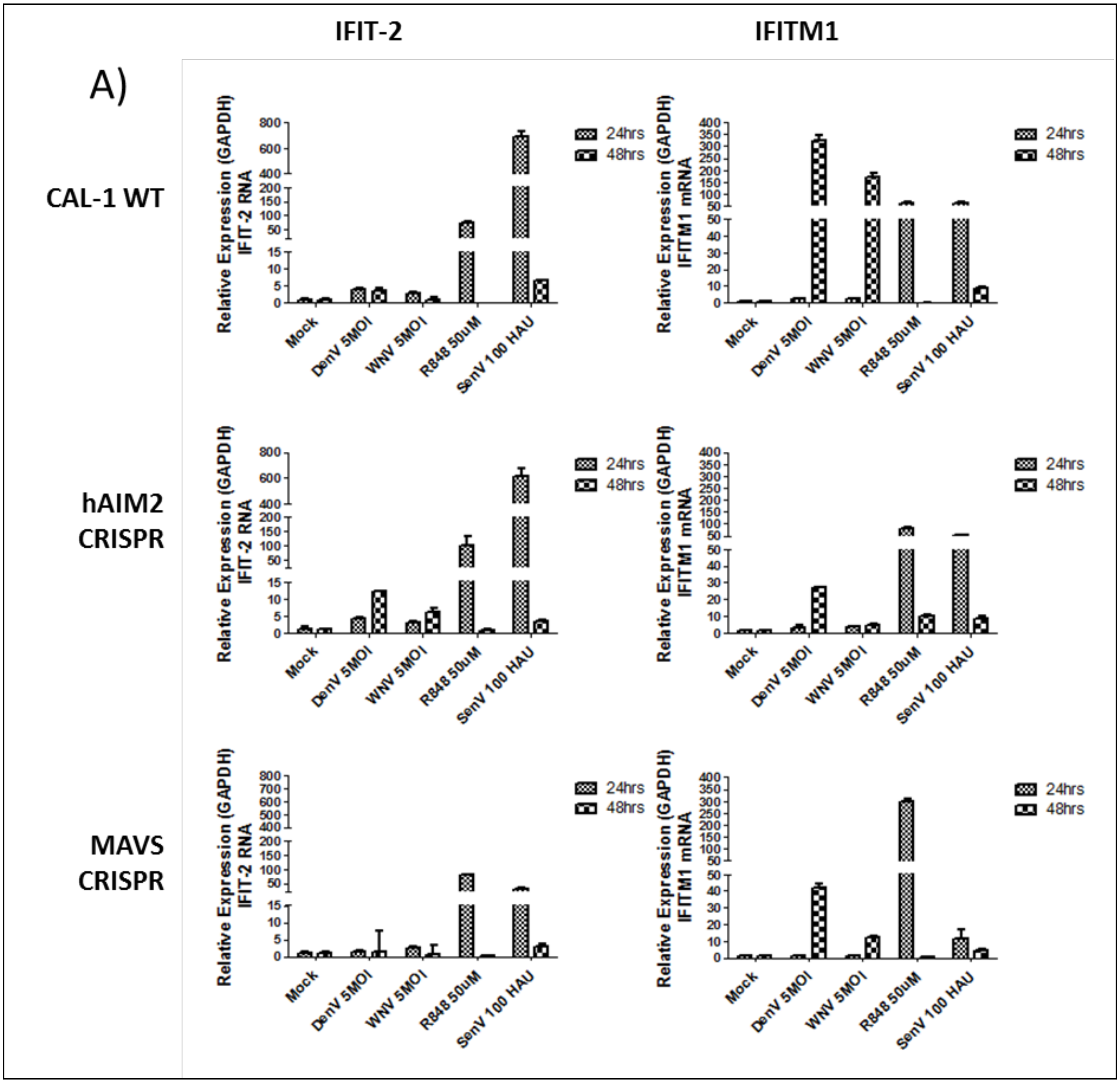


Figure 3.6 CRISPR mediated knockout of MAVS. CAL-1 cells were transduced with MAVS specific or hAIM2 specific lentiviruses and underwent puromycin selection for 7 days. A) qRT-PCR analysis of MAVS expression between transduced and non-transduced cell types following puromycin selection. B) Western blot analysis of MAVS expression between transduced and non-transduced cell types following puromycin selection. Performed by JF

WT, MAVS CRISPR, and AIM2 CRISPR CAL-1 cells were infected with DenV 2 NGC and WNV TX at an MOI of 5 and innate immune gene induction was measured by qRT-PCR and IFN-α protein was measured by ELISA. Strikingly, IFN-α message was almost completely lost among the MAVS CRISPR cells as well as the IRF/IFN-responsive gene IFIT-2, while IFITM1 expression was diminished for both hAIM2 and MAVS CRISPR cells (Figure 3.7A). In contrast IFN-α and IFIT-2 message were detectable and exhibited similar magnitudes of gene induction between the WT CAL-1 and the hAIM2 CRISPR transduced CAL-1 cells (Figure 3.7A). Interestingly IL-6 message was elevated for the MAVS CRISPR cells 24hrs post-infection for DenVpotentially indicated PRR compensation by the TLRs following loss of the RLR pathway. Consistent with the qRT-PCR data, ELISA results demonstrated ~80% reduction in the levels if IFN- α produced following infection of the MAVS CRISPR CAL-1 cells. In contrast, IFN-alpha production was roughly equivalent between the WT CAL-1 and the hAIM2 CRISPR CAL-1 cells (p<0.01 and p=0.23 respectively, Figure 3.7B). Importantly, the loss of IFN- α production in the MAVS CRISPR cells was apparent even during SeV infection, suggesting that SeV recognition by pDCs is also RLR specific.



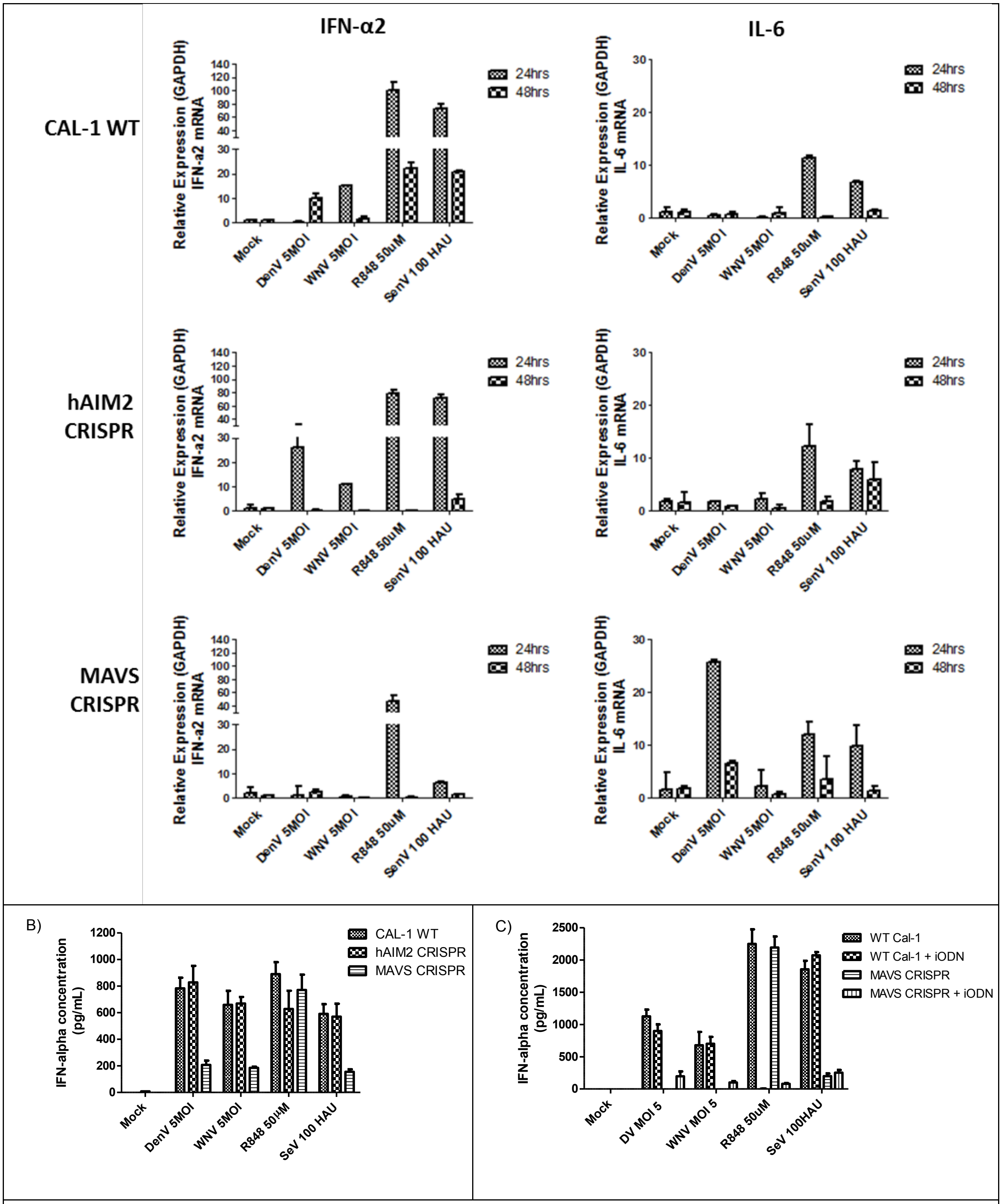


Figure 3.7 CRISPR mediated MAVS knockout results in diminished pDC IFN production. WT CAL-1, hAIM2 CAL-1, or MAVS CRISPR CAL-1 cells were infected at an MOI of 5 with either DV2 or WVN and cells were harvested at 24 and 48hrs post infection. A) qRT-PCR analysis for IFIT-2, IFITM1, IFN-a2 ,and IL-6 mRNA,

results indicated relative fold induction after normalization to GAPDH was compared to that of mock infected cells. B) IFN-alpha ELISA results 48hrs post-infection C) IFN alpha ELISA results 48hrs post-infection where cells were pre-treated for 1hr with the TLR7 inhibitor iODN prior to infection. Results are representative data from at least two independent experiments. Performed by JF

To assess the contributions of TLR7 for the residual IFN- α response that we observed in the MAVS CRISPR cells, we pre-treated WT CAL-1 and MAVS CRISPR CAL-1 for 1 hour with the TLR7 inhibitor Dual-iODN (Enzo Lifesciences) at 10ug/mL concentration and infected with DenV 2 NGC or WNV TX at an MOI of 5 and harvested 48hrs post-infection (Pawar et. al. 2007). iODN treatment did not adversely affect the CAL-1 cells as viability was over 90% at 48hrs post-infection. Interestingly, IFN- α production increased slightly across all conditions following iODN treatment with the exception of R848 where the IFN-alpha production was almost completely abolished (Figure 3.7C). However, for WNV infection and R848 treatment the IFN- α levels detected were below the limit of detection for this assay (~150pg/ml). Therefore, we found no evidence that TLR7 was contributing to residual l IFN- α production.

To confirm that the CRISPR knockdown did not alter the maturation status of CAL-1 cells and to ascertain the role that the MAVS pathway plays in pDC maturation, we assessed maturation status of the WT CAL-1, MAVS CRISPR CAL-1, and hAIM2 CRISPR CAL-1 before and after infection at 24 and 48hrs. Our data indicated that neither the MAVS nor the hAIM2 knockdowns altered maturation status of CAL-1 cells prior to infection (data not shown). Additionally, there were no significant differences in the proportions of cells that underwent maturation following Flavivirus infection, treatment with R848, or SeV (p>0.2, Figure 3.8A). Therefore, the loss of the interferon response among the MAVS CRISPR cells could not be explained by differential rates of CAL-1 maturation.

Next we determined the infected cell frequency between the WT CAL-1, MAVS CRISPR CAL-1, and hAIM2 CRISPR CAL-1 cells in the presence and absence of iODN. As expected, the loss of the RLR mediated interferon response led to significant increases in the proportion of cells expressing Flavivirus protein in the MAVS CRISPR cells compared to the WT CAL-1 for DenV infection but not for WNV although there was an increasing trend for WNV (p=0.03 and p=0.15 respectively, Figure 3.8B). Addition of iODN led to slight increases in infection rates for the MAVS CRISPR cells only although this was not significant (p>0.1). These data indicate that the RLR mediated interferon response is able to partially restrict virus replication, but the TLR7 pathway does not play major role in type I IFN production or restriction of flavivirus replication in pDCs. Finally, quantification of viral genomes in the supernatant revealed that MAVS CRISPR cells infected with DenV or WNV released roughly 1 log more virus per mL compared to WT CAL-1 (Figure 3.8C). This finding indicates that the RLRs in pDCs not only recognize Flaviviruses and initiate the IFN response, but also contribute to restriction of viral replication.

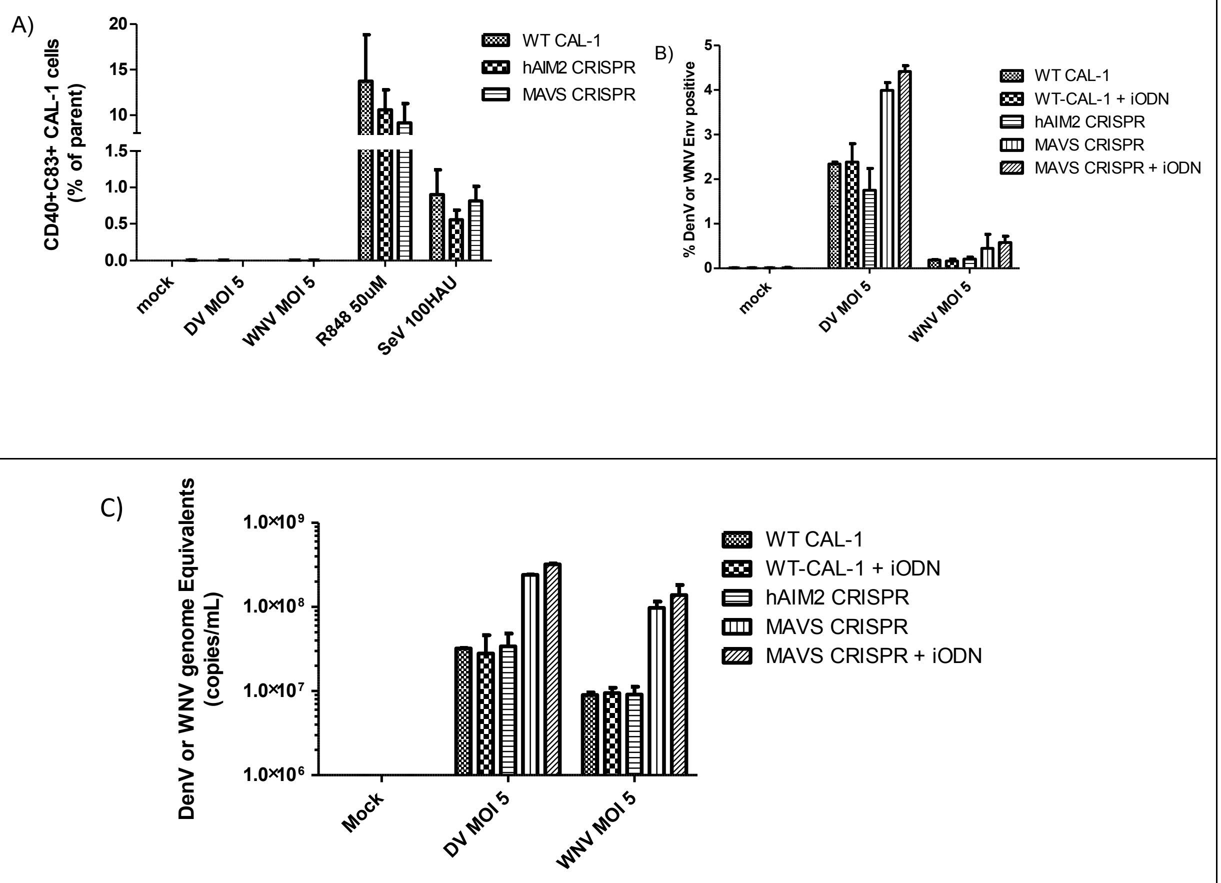


Figure 3.8 MAVS CRISPR results in increased flavivirus infection virus output. WT CAL-1,hAIM2 CRISPR CAL-1, and MAVS CRISPR CAL-1 cells were infected an MOI of 5 with DV or WNV in the presence or absence of a 1hr pre-treatment with the TLR7 inhibitor iODN. 24hrs or 48hrs post-infection cells were fixed, surface stained, intracellular stained, and analyzed for CD40+CD83+ expression and DV or WNV Env expression. At 48hrs cellular supernatant was harvested and analyzed for viral genome equivalents A) Percentage of CAL-1 cells double positive for CD40 and CD83 24hrs post-infection. B) Percentage of CAL-1 cells infected at 24hrs post-infection. C) Copies of viral genomes per mL of supernatant at 48hrs post-infection. Results are the representative findings of at least 2 independent experiments. Performed by JF

In conclusion, our findings of reduced IFN-alpha production were not attributable to differences in pDC maturation status or infection rates and there is no apparent role for the RLR pathway in pDC maturation. These data indicate that pDC mediated IFN- α production during Flavivirus infections is largely driven by the RLR pathway. The residual levels of IFN- α could be attributable to incomplete CRISPR knockdown or low level TLR7 mediated IFN- α production.

3.4 Discussion

Using the pDC-like cell line CAL-1, ex-vivo human PBMC isolated pDCs, and *in vivo* infections of mice, we consistently demonstrated that pDCs are productively infected by Dengue and West Nile Virus. We show that virus infection drives an RLR-mediated IFN- α response consistent with the pDC IPC phenotype. Furthermore, our data suggest that flavivirus infection does not appreciably initiate the canonical TLR7 mediated IFN- α response or drive pDC maturation into APCs. CRISPR/CAS9 knockout of the RLR adaptor MAVS resulted in a dramatic reduction in the level of IFN- α secreted following infection and demonstrated marked increases in rates cellular infection and virus output. Our data suggest that pDCs utilize the RLR pathway to recognize replicative RNA viruses and the resulting response contributes to the restriction flavivirus replication. Therefore, these data highlight a novel role for the RLRs in the recognition of replicative RNA viruses in pDCs. Our findings were obtained using multiple parallel cellular systems and molecular tools and highlight a new cellular target for flavivirus replication as well as build upon a previous report that RLRs are capable of recognizing immunostimulatory RNA in pDCs (Stone et al. 2013).

Multiple DenV studies have demonstrated reduced levels of type I IFN and reduced circulating levels of pDCs among DenV patients that ulitmately progress to severe dengue disease (Pichyangul et al 2003, Simmons et al. 2007, Libraty et al. 2002). Our finding that pDCs are infected and initiate IFN production during infection suggests that pDCs may play in important role in mounting the anti-viral response and restricting infection *in vivo*. However, we found no significant impact on pDC maturation status or cell viability following flavivirus infection that would explain the reduced cell counts or lack of interferon production observed in these previous studies. Therefore, the *in vivo* processes that lead to reduced pDC frequency and reduced type I IFN are unclear. Unexpectedly, the previous report that MAVS-/- mice have high levels of type I IFN in the blood during WNV infection is not explained by TLR7 mediated pathogen recognition in pDCs (Errett et al. 2013). It is possible that other cells types are able to recognize WNV through TLR7 or other pathogen recognition receptors such as TLR3 (not expressed in pDCs) or STING that may be responsible for the residual type I IFN levels (Aguirre et al. 2012, Yu et. al 2012). Indeed TLR3 has been implicated in DenV recognition and infection (Tsai et al. 2009, Nasirudeen et al. 2011). The residual IFN produced by the MAVS CRISPR cells during Dengue infection could be attributable to low levels of STING mediated detection and IRF3 expression. However, qRT-PCR and flow cytometry analysis of STING revealed exceedingly low to undetectable levels of STING expression in CAL-1 cells (Data not shown). Importantly, even if the residual IFN-alpha response was attributable to STING we can conclude that STING mediated detection of Dengue virus is not a significant contributor to DenV pathogen recognition. Alternatively, DenV may be disrupting the STING mediated antiviral pathway via the NS2/B3 protease (Aguirre et al. 2012, Yu et al. 2012).

Our finding that neither DenV nor WNV induced appreciable levels of pDC maturation nor did they actively antagonize the maturation process, suggests that DenV and WNV infection evades TLR7 mediated immune recognition. Thus, this finding is parsimonious with our conclusions that the RLRs are responsible for the pDC innate immune response. It is possible that Flavivirus replication within membranes limits the availability of nucleic acids that can shuffled to TLR7 bearing endosomes. Additionally, virus mediated initiation of authophagic processes and the UPR may also serve to disrupt TLR7 mediated pathogen recognition (Peña and Harris 2012, Hetz and Oakes 2015, Fischl and Bartenschlager 2011). The finding that virus entry does not initiate TLR7 mediated recognition may indicate that the virus capsid efficiently shields viral RNA from TLR7 during entry.

In congruence with our findings, multiple studies have demonstrated that Flavivirus and SeV mediated recognition by pDCs requires live replicative virus and viral entry (Lee et. al 2007, Sun et al. 2011, Wang et. al 2006). Therefore these data suggest that viral entry, fusion, uncoating, and replication are necessary for immune recognition. However, the strength of our approach was that we utilized relevant MOI's, demonstrated infection with both DenV and WNV across immortalized cell types and primary pDCs, and we were able to isolate infected splenic pDCs from C57BL6 mice that were inoculated with physiological levels of WNV at 48hrs post-infection. A recent study transfected cells with HCV PAMP and observed RLR mediated IFN production that was sufficient to restrict HCV infection in hepatocytes (Stone et al. 2013). However, multiple studies have demonstrated that HCV virion interacts with C-type lectin receptors to inhibit pDC mediated IFN- α production (Florentin et al. 2012, Stone et al. 2014). Furthermore, HCV does not productively infect pDCs and formal infections with HCV are immunologically silent. Therefore, we are unaware of any study that has investigated the role of the RLRs in the context of a relevant virus that is capable of infecting pDCs under natural infection conditions.

Previous studies using primary pDCs were unable to demonstrate infection for either of the flaviviruses DenV or WNV (Sun et al. 2011, Silva et al. 2007). One studies examined primary pDCs and mature dendritic cells (mDCs) during DenV infection and found as high as 1.5% of cells were DenV Env+ by 48hrs post-infection (Sun et al. 2011). However, the mock samples in their experiment had high positivity for DenV Env, therefore the authors concluded frequencies of infection were "not significant." Interestingly, consistent with viral replication, copy number of the viral negative sense RNA strand in this same study increased between 12 and 24hrs post infection between both mDCs and pDCs with parallel slopes between time-points. Another possible explanation for the author's discrepancy in infection results was the fact that they used an MOI of 10 that drove almost 10 times the IFN-alpha production per well than we obtained from primary pDCs using an MOI of 1. Similarly, a study using WNV virus suggested that WNV infection does not occur in pDCs (Silva et al. 2007). Their study design differed in the respect that they used MOIs of 100 and 1000, driving extreme levels of IFN-alpha production that were far above physiological levels and likely impacted viral replication and cell viability. Neither of these studies presented cell viability data. Therefore, the artificiality of these systems and the concentration of virus with IFN producing cells could have negatively biased their results.

Our finding that pDCs use the RLR pathway to detect replicative RNA virus infections contradicts previous reports that productive infections with pDCs are only recognized by autophagy mediated shuffling of virus replication intermediates into acidified TLR7 or TLR9 bearing endosomes for pathogen recognition or during virus entry (Lee et al. 2007, Severa et al. 2013, Wang et al. 2006). Lee et. al. investigated the role of autophagy in pDC mediated recognition during VSV infection. To demonstrate a role for autophagic processes in innate immune recognition during infection, these authors used chemical inhibitors of autophagy and mice chimeric for the ATG5-/- autophagy protein to show reduced IFN-alpha production. Disruption of autophagy may have altered other cellular processes that are vital for pathogen recognition, virus replication, or IFN-alpha secretion, thereby independently explaining the loss of IFN-alpha production observed. Another study investigated the role of Influenza A and Dengue Virus recognition via TLR7 (Wang et al. 2006). Using a TLR7 inhibitor they demonstrated reduced IFN-alpha production. Subsequently, they inactivated virus or used viral entry inhibitors to prevent viral replication and fusion and saw a similar reduction in DenV mediated IFN- α production. It is unclear why their finding of TLR7 mediated signaling differed from ours. However, their finding that blockade of viral fusion impairs pathogen recognition and IFN production is congruent with our RLR results, since virus entry is essential for RLR mediated recognition. Importantly, neither of these studies formally investigated the contributions of the RLR pathway during infection.

This study had limitations. Most importantly, pDCs represent less than 1% of all cells in the bloodstream despite being potent producers of type I IFN (Tversky et al. 2008). Therefore, the overall significance of pDCs during flavivirus infection is unclear. The use of the immortalized cell line CAL-1 may have influenced our results if these cell types are more permissive for infection or utilize altered means of pathogen recognition compared to primary pDCs. However, we validated all our in vitro infection, maturation, and innate immune phenotype results in primary cells and found strikingly similar results. Therefore, the CAL-1 cells displayed a comparable phenotype to primary pDCs highlighting the strength of this cellular mode. Our finding that CRISPR mediated knockdown of the RLR adaptor MAVS abrogated the flavivirus induced IFN response could have been biased if MAVS mediated knockdown altered other innate immune signaling processes unrelated to the RLRs. Indeed, MAVS has been shown to play diverse and perhaps unidentified roles in apoptosis and the NLRP3 mediated inflammasome response (Subramanian et al. 2013, Guan et al. 2013). However, the finding that iODN treatment in WT CAL-1 cells did not significantly reduce IFN-alpha production provides strong evidence that the RLRs are solely responsible for flavivirus recognition. Primary pDCs are short-lived and therefore MAVS could not be knocked out. Therefore, it is possible that loss of MAVS in primary pDCs would not abrogate the innate immune response. However, the lack of pDC maturation (a marker for TLR7 ligation) following DenV and WNV infection strongly suggests that the RLRs were indeed responsible for innate immune recognition in pDCs.

In conclusion, we report that plasmacytoid dendritic cells are productively infected by the flaviviruses resulting in RIG-I like receptor mediated pathogen recognition and a robust type I interferon driven anti-viral response. TLR7 does not play a significant role in flavivirus recognition thereby preventing changes in pDC programming toward an APC phenotype. The lack of pDC maturation may impact the kinetics and breadth of the adaptive immune response during infection. Furthermore, our data suggest that pDCs are more permissive to Dengue virus compared to WNV *in vitro*. Given that DenV is reliant on the human host for transmission, pDC infection may impart advantages to the virus *in vivo* such as dissemination or bias of the innate immune micro-environment that were not elucidated in this present analysis. Interferon signaling competent mice are not susceptible to Dengue virus infection. Therefore, evaluation of the role of pDCs *in vivo* during DenV infection is not currently possible. However, WNV infection in WT C57BL6 mice recapitulates many of the hallmark symptoms of WNV mediated disease in humans. A C57BL6 model with the diphtheria toxin receptor (DTR) under control of the CLEC4c promoter (expressed almost exclusively on pDCs) has been used to selectively deplete pDCs and assess their *in vivo* contributions to disease following the administration of diphtheria toxin (Swiecki et al. 2013). Thus, future research should evaluate the role of pDCs during WNV infection among wild type B6 mice and CLEC4c DTR mice in the presence and absence of diphtheria toxin to evaluate the *in vivo* relevance of pDCs during Flavivirus infection. A priori outcomes that can be assessed are: survival, weight loss, histology, and clinical scoring.

3.5 Author Contributions

Russell Barlow (RB) and Jonathan Florentin (JF) conceived all of the experiments and scientific basis for the pDC project. RB performed CAL-1 infection experiments, qRT-PCR, plaque assay, and flow cytometry for maturation and infection. Primary pDC infection, CRISPR/CAS9 analysis, iODN inhibitor analysis and *in vivo* WNV infections were performed by JF.

Chapter 4: CONCLUSIONS

Dengue virus is an emerging infection responsible for over 100 million apparent infections and 250,000-500,000 cases of severe disease each year (Murray et al. 2013, Blatt et al. 2013). Since the first cases of severe hemorrhagic fever and shock were identified in the 1950s and 1960s, considerable research has been performed to understand the virus and to explain the disease pathology (Halstead 1970). Pre-existing heterotypic humoral immunity, decreased interferon production, virus determinants, and reduced pDC frequency have all been implicated in severe disease (Halstead 1977, Guzman 2002, 1990, OhAinle 2011, Dejnirattisai 2010, Simmons et al. 2007, Pichyangkul 2003, Vaughn et al. 2000). Unfortunately, unraveling the molecular mechanisms that ultimately drive the DenV induced immune pathology has remained far more elusive. In the present studies we sought to focus on the direct interactions of DenV with the innate immune system using *in vitro* culture systems to better understand two aspects of the dengue immune pathology: antibody dependent enhancement of infection and the pDC response to flaviviruses.

Collectively the data presented herein provide some understanding of-and appreciation for-dengue virus's cellular promiscuity and ability to overcome the innate immune response. Throughout the ADE and pDC experiments, we demonstrated continued virus replication and naive cell infection even in the presence of high levels of Type I IFN. Specifically, during ADE we demonstrated that virus is still able to productively infect cells that have been primed for antiviral control of virus replication. Understanding this phenomenon may provide insights into the molecular mechanisms that DenV exploits during ADE as well as explaining; 1) how the virus replicates to high enough levels to allow transmission and 2) the role of ADE in the initiation of an over-exuberant immune response. Measurement of the DenV induced innate immune signature across both studies demonstrated delayed pathogen recognition and induction of the innate immune response. These data collectively suggest that DenV does not significantly engage the TLRs during initial infection. Instead delayed virus recognition by the RLRs appears to drive the primary innate immune response.

Delayed pathogen recognition may provide sufficient time for the virus shutdown the JAK/STAT driven ISG response via NS5a and NS4b mediated STAT2/1 degradation respectively (Ashour et al. 2005, 2009, Jones et al. 2010, Morrison et al. 2013, Perry et al. 2011, Munoz-Jordan et al. 2003, 2005, Ho et al. 2007). NS5a and NS4b mediated shutdown of the ISG response may explain how DenV can persist and continue replication within already infected cells after IRF3/7 mediated type I IFNs are produced. However, these virulence factors do not explain how virus is able to colonize and infect IFN primed cells.

Therefore, unidentified molecular mechanisms or virulence determinants must exist to allow continued cycles of infection.

Dengue virus's slow growth may underlie strategies for immune evasion. Studies have indicated that the virus translates viral protein and assembles virions within the ER during early stages of infection and only at later stages do significant levels of viral RNA accumulate (reviewed in Fischl and Bartenschlager 2011, Green et al. 2014, Paranjape and Harris 2010). During early stages of infection, both circulating levels of viral RNA for new virions and dsRNA from replication complexes would be of low abundance providing less DenV PAMP for pathogen recognition. The tradeoff being that DenV is left with less template for new genome copies and for translating viral protein, resulting in delayed initial replication kinetics. One would expect that the consequence of such a strategy would be delayed and reduced virus output per cell early during infection with low levels of innate immune detection. Indeed this is the viral phenotype that we observed during DenV infections compared to WNV, JEV and other distantly related viruses. Similar strategies may be employed by other successful human pathogenic viruses such as HCV, HPV, and HIV that are exceedingly difficult to grow *in vitro,* suggesting that these viruses utilize strategies to evade immune detection or require specific environmental conditions favorable for replication.

A recent WNV study found that a more efficiently replicating mutant virus produced greater amounts of viral RNA early during its replicative lifecycle. The early nucleic acid amplification promoted rapid innate immune recognition by the RLRs resulting in greater IFN-beta production and lower peak viral titers compared to wild-type virus (Scherbik et al. 2013). However among IFN insensitive cells such as BHK, the mutant virus displayed improved replication kinetics and yielded significantly greater peak viral titers. Similarly, a serotype 2 dengue virus has been shown to preferentially replicate subgenomic viral RNA (sfRNA) early during infection, resulting in prevention of TRIM25 de-ubiquitylation and delayed RLR mediated recognition of the virus (Manokaran et al. 2015).

The studies presented here and the vast majority of the innate immune literature in general, do not accurately capture the cell intrinsic consequences of virus infection. Instead many studies have used highly permissive cell types and elevated MOIs potentially introducing important biases. However, these approaches are often not feasible or relevant during circumstances where differentially biased conditions will exist between comparison groups, such as ADE. Therefore these limitations constrain the breadth of questions that can be asked about virus-host interactions. However, emerging technologies for infected cell sorting such as virus tagging or DiD labeling may represent powerful new tools to investigate innate immune mechanisms during virus infection using relevant cells types, physiological MOIs, allowing direct 1-1 comparisons between infected cell populations, ex-vivo analyses, and providing robust data even under circumstances where differential rates of infection exist (Schoggins et al. 2012, Alaya-nunez et al. 2011). Furthermore, the advent of single cell transcriptomic tools may also prove useful for identifying cell-intrinsic processes during virus replication as well as providing insight into sub-populations of susceptible cell types (Shalek et al. 2014, Saliba et al. 2014). Finally, the availability of new mouse models with selective knock-out of pDCs (CLEC4c DTR) or IFNAR in dendritic cells (CD11c+ CRE) and monocytes and macrophages (LysM+ CRE), may provide more relevant models to study DenV induced immune pathology related to antibody dependent enhancement and the reduced type I IFN/reduced pDC phenomena identified during epidemiological studies (Zust et al. 2014, Pinto et al. 2014,Swiecki et al. 2013).

In conclusion, dengue virus is a well-adapted human pathogen that is surprisingly adept at circumventing and overcoming the host innate antiviral response allowing for elevated viraemia sufficient for transmission. We found that during ADE transient viral entry and replication persisted in the presence of an established anti-viral response. This finding may help to illuminate underlying mechanisms of immune evasion during ADE that explain the increased levels of viraemia and the cytokine storm found in patients with severe dengue. We found that dengue virus infects pDCs resulting in RLR mediated recognition of the virus and a robust type I IFN response, despite reports that type I IFN levels and pDC frequency inversely correlate with severe disease progression. Furthermore, dengue infection of pDCs had no apparent effect on pDC maturation status or viability. Therefore, the mechanisms whereby type I IFN and pDC frequency are reduced are likely independent of direct pDC infection or occur through a more dynamic interplay of the innate and adaptive immune systems and the virus *in vivo*.

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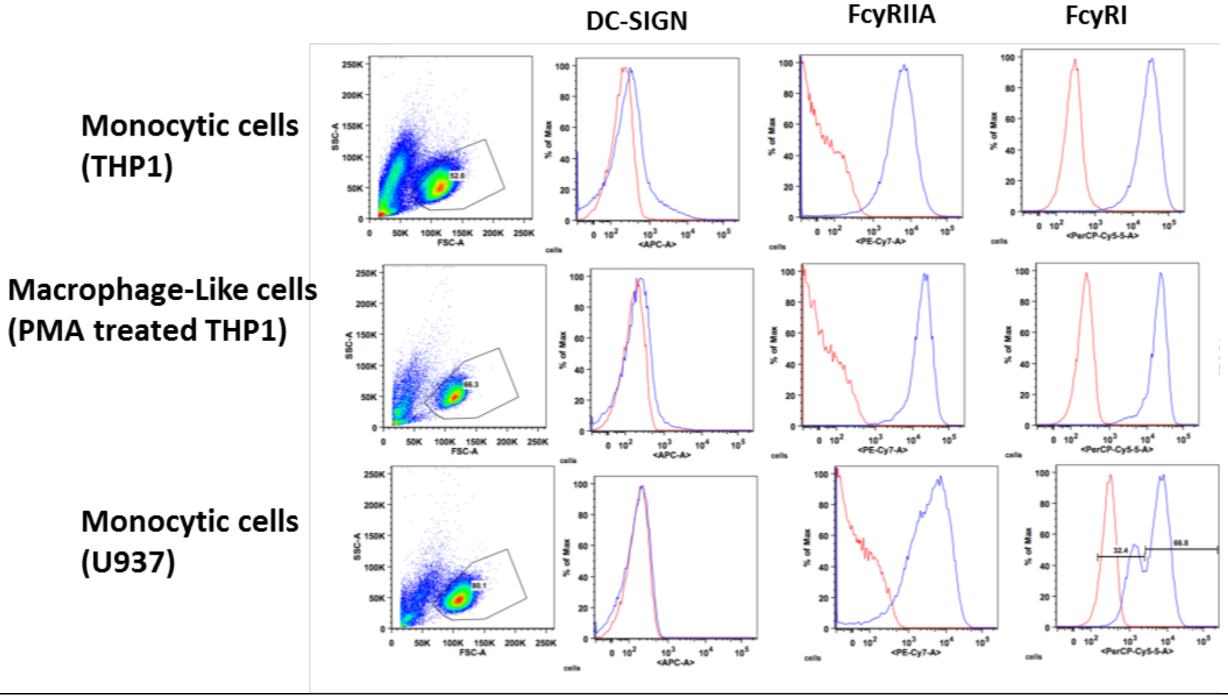
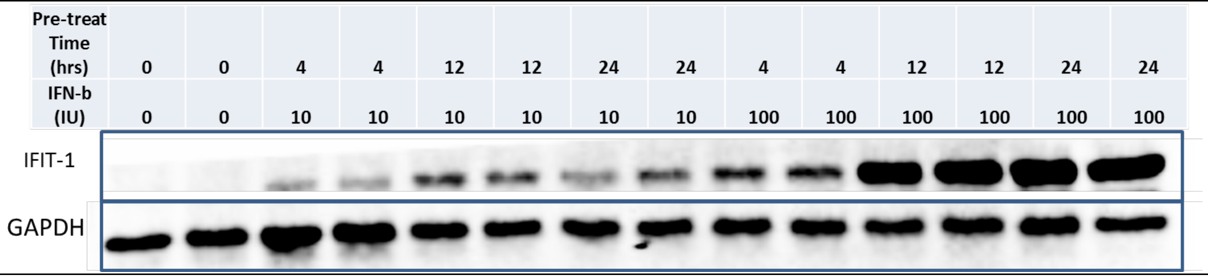
Appendix A1

Flow cytometry analysis of surface receptors involved in Dengue infection: DC-SIGN, FCyRIIA and FCyRI. Red lines indicate unstained mock cells that were fixed and stained. Blue lines indicate levels of respective receptor expression among stained cells.



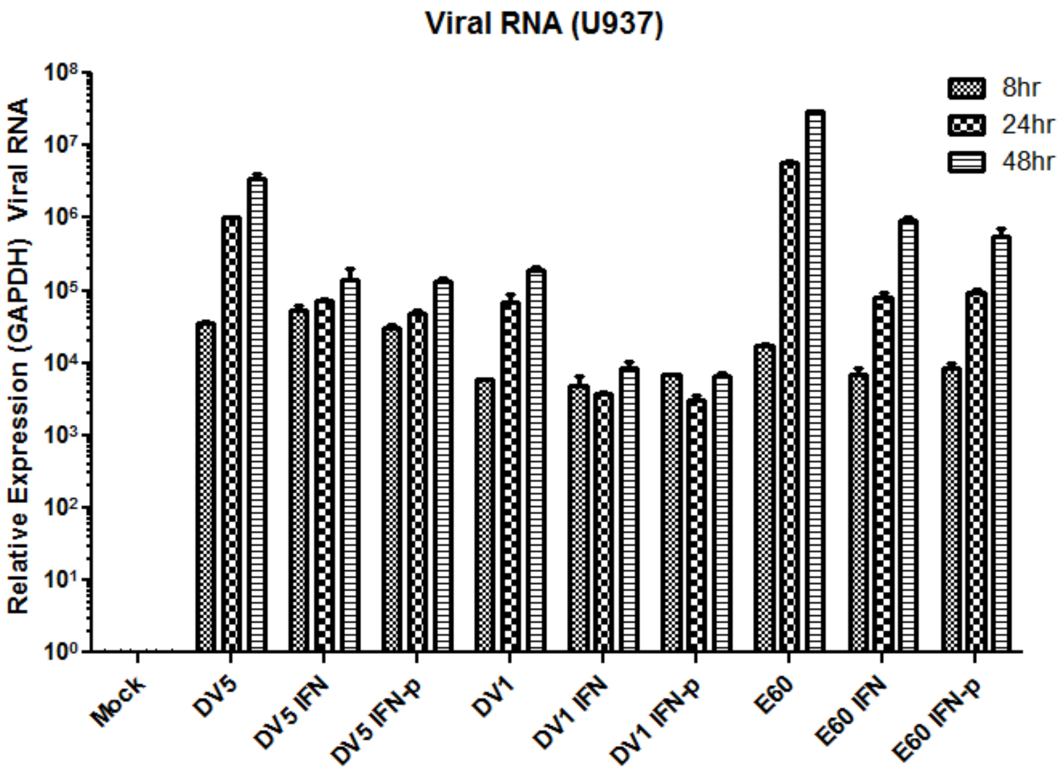
Appendix A2

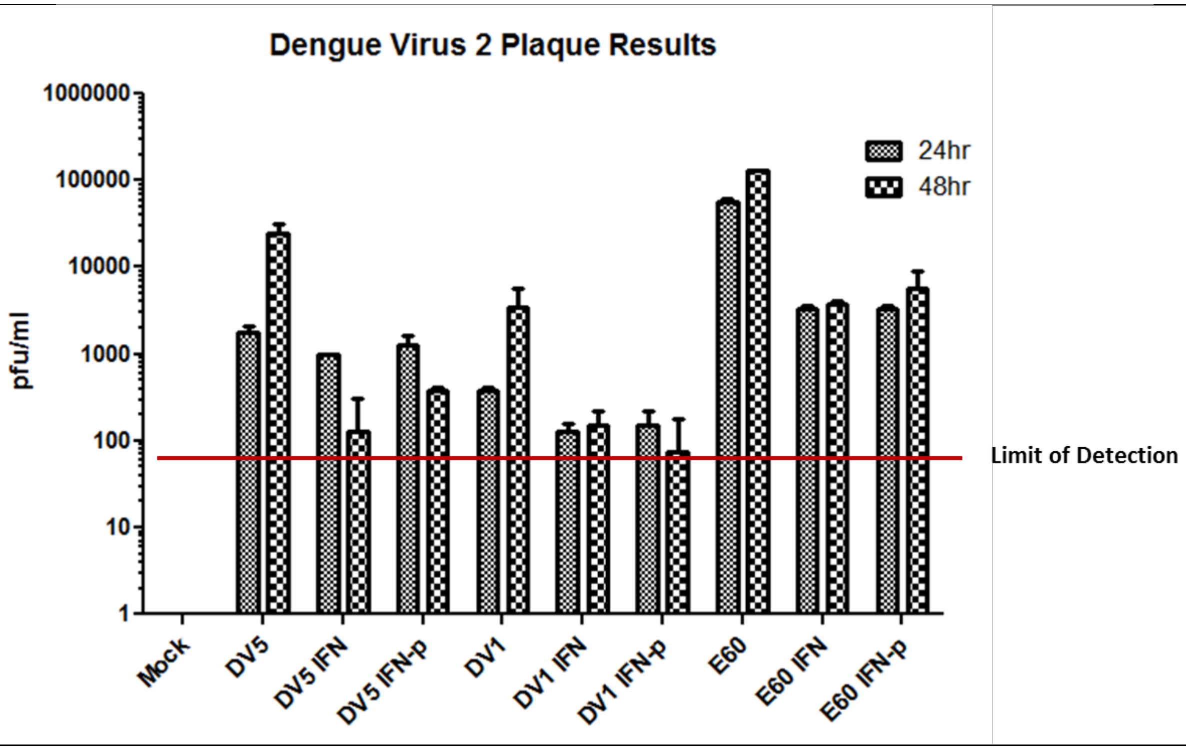
IFN-β pre-treatment experiment to determine the kinetics of ISG expression at the protein level. U937 cells were pretreated with the indicated IU of IFN-β. Cells were harvested at the indicated time-points post treatment and analyzed by western blot for the expression of IFIT-1.



Appendix A3

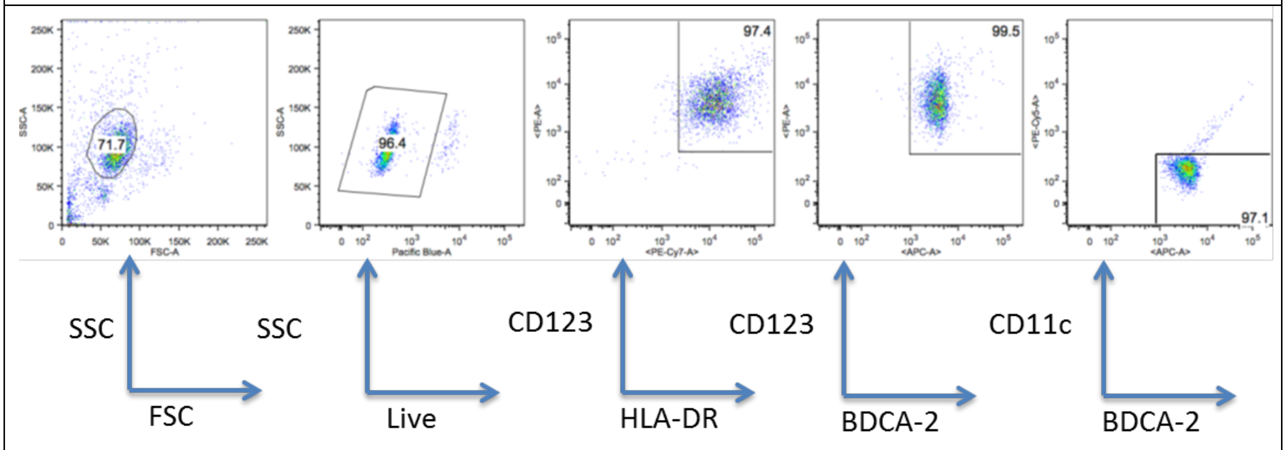
Effects of ADE infection on U937 cells following 12-hr pre-treatment with 50IU IFN-β. Conditions with IFN-p in the label indicate cells that were both pre-treated with 50IU IFN-β prior to infection as well as treated with another 50IU of IFN-beta following infection.





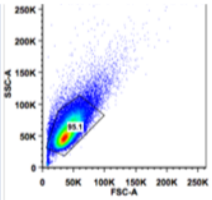
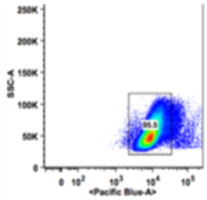
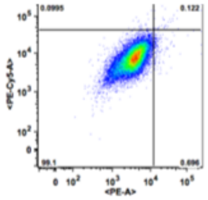
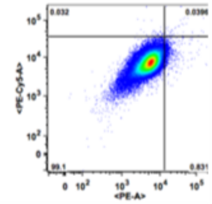
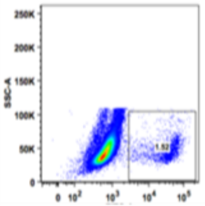
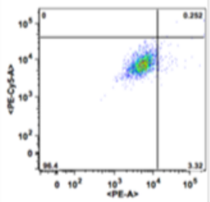
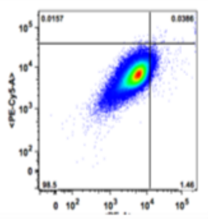
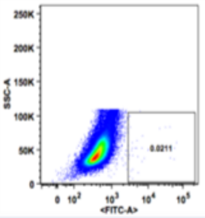
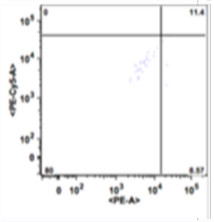
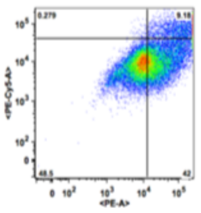
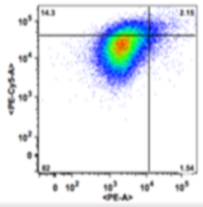
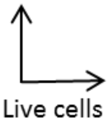
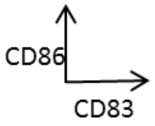
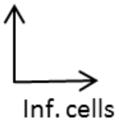
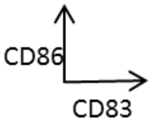
Appendix A4

Representative flow plots and gating strategy to confirm isolation of human pDCs from whole blood PBMCs



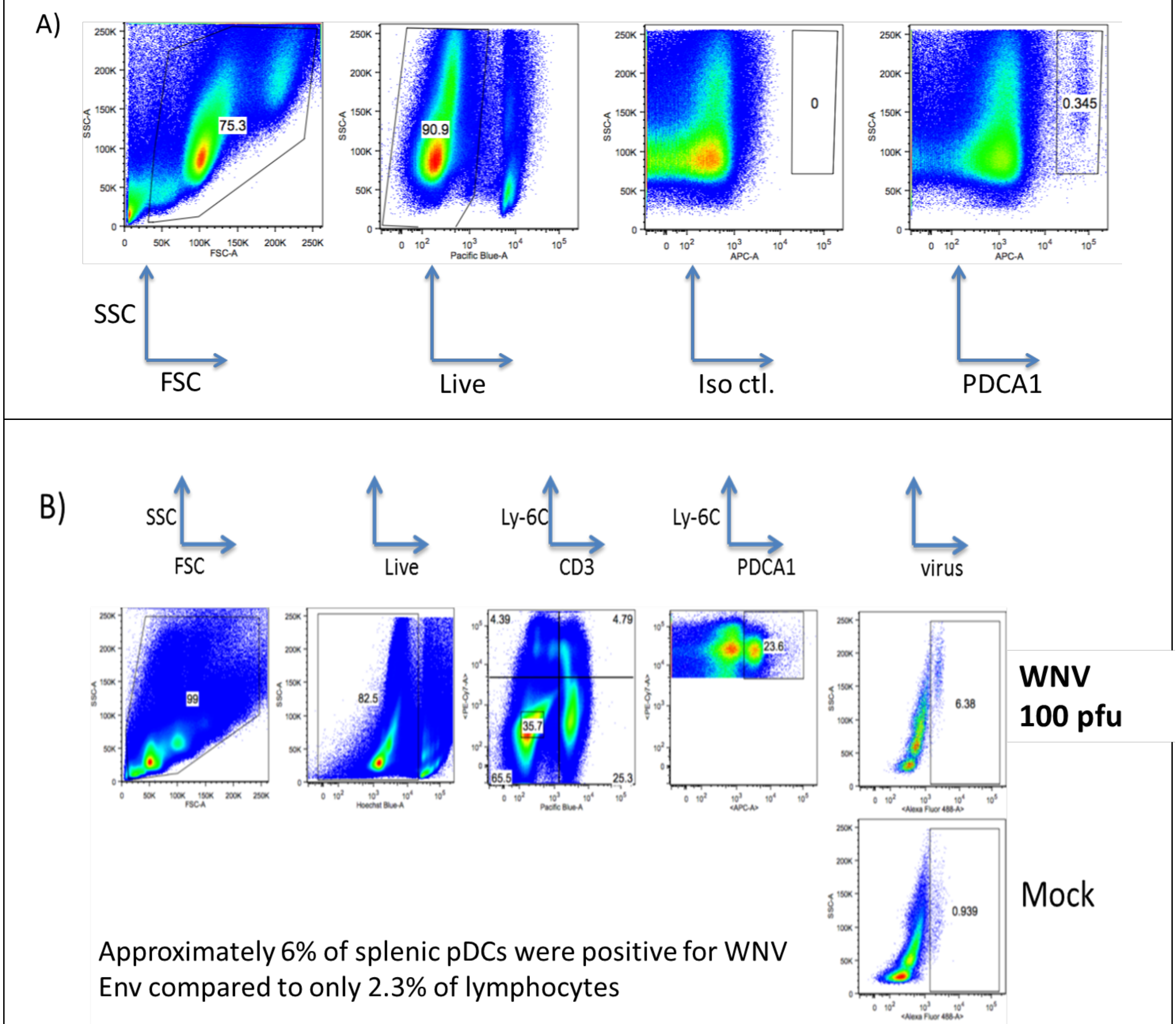
Appendix A5

Representative flow plots and gating strategy to quantify pDC maturation into CD40+CD83+ APC

	Cells	Live cells	Matured cells / live cells	Inf. Cells	Matured cells / inf. cells
Mock					
DenV, 1MOI					
WNV, 1MOI					
SenV, 50 HAU					
R848, 20 uM					
					

Appendix A6

Isolation and WNV infection of murine pDCs. C56BL6 mice were infected with 100pfu WNV TX spleens were harvested and analyzed by flow cytometry for expression of pDC markers and the presence of WNV envelope protein. A) representative gating strategy and % of total splenocyte population positive for the murine pDC marker PDCA1. B) Gating strategy and representative flow plot for CD3+Ly-6C+PDCA1+ pDCs expressing WNV Env+ (% of all total pDC population).



Approximately 6% of splenic pDCs were positive for WNV Env compared to only 2.3% of lymphocytes

Appendix A7

IFN-alpha ELISA results (pg/mL)for CAL-1 cells treated with a range of concentrations of the TLR7 agonist R848. Supernatants were tested by IFN-alpha ELISA 24hrs post-R848 treatment. Number values on X axis indicate uM concentrations of R848 that were administered.