TOWARDS UNDERSTANDING PLASMABLAST DEVELOPMENT IN DENGUE VIRUS INFECTION

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ABSTRACT

Dengue is a re-emerging global public health threat that over one-third of the world's current population stands at risk of developing. Persistent upticks in climate warming, population growth and global transportation have the potential to spread the four dengue virus (DENV) serotypes to new geographical areas, thereby increasing the number of at-risk individuals. Annually 50 to 100 million dengue cases develop worldwide; approximately five hundred thousand of these cases degenerate into severe disease. In some instances, regions lacking the means to provide supportive therapy have experienced 40 percent case-fatality rates. The economic and global impacts of dengue remains unchallenged due to inadequate control measures and the absence of licensed vaccines. A more thorough understanding of DENV pathogenesis would open the door to designing the effective vaccine and antiviral drug candidates essential to the global health community's efforts to control dengue. This thesis focuses on understanding a recently observed characteristic of DENV pathogenesis: four to seven days after disease onset, peripheral plasmablasts undergo a massive expansion. Relative to other heavily studied cell types, very little is known about the role plasmablasts play in the overall model of DENV pathogenesis. At the start of this project, it was unknown if this robust plasmablast response to DENV originated from the DENV specific memory B-cell subset or if it was driven by naïve B-cells. To facilitate investigation of the events relating to DENV-induced B-cell activation, peripheral blood mononuclear cells (PBMCs) were collected from healthy individuals with previous DENV exposures. Using an *in vitro* model, I showed that DENV was

able to drive differentiation of DENV specific memory B-cells into DENV specific IgG secreting plasmablasts. After observing that DENV could drive a plasmablast response, I also demonstrated that monocytes, the primary target cells, played an important role in this plasmablast development. This work leads to the important conclusion that DENV is able to drive the differentiation of DENV specific memory B-cells into IgG secreting plasmablasts with the help of monocytes. However, it is unlikely that the secondary response mounted by this memory subset will be sufficient to produce the high percentage of plasmablasts seen in acute patients. These research findings are important in the ongoing effort to identify the early events essential for driving the robust plasmablast response observed during DENV infections.

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ABBREVIATIONS

ADE	Antibody Dependent Enhancement
APRIL	A Proliferating-Inducing Ligand
ASCs	Antibody Secreting Cells
BAFF	B-cell Activating Factor
BCR	B-cell Receptor
C	Capsid Protein
CFSE	Carboxyfluorescein succinimidyl ester
CDC	Center for Disease Control and Prevention
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3) grabbing non-integrins
DENV	Dengue Virus
DF	Dengue Fever
DHF	Dengue Hemorrhagic Fever
DSS	Dengue Shock Syndrome
E	Envelope Protein
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-Linked ImmunoSpot
ER	Endoplasmic Reticulum

GC	Germinal Center
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-10	Interleukin-10
IFN-γ	Interferon-gamma
M	Membrane Protein
MIP-1β	Macrophage inflammatory protein-1beta
NS1	Non-structure protein 1
NS2A	Non-structure protein 2A
NS2B	Non-structure protein 2B
NS3	Non-structure protein 3
NS4A	Non-structure protein 4A
NS4B	Non-structure protein 3
NS5	Non-structure protein 5
prM	Pre-membrane protein
PWM	Pokeweed Mitogen
SAC	Staphylococcus Aureus Cowan
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor-alpha
VV	Vaccinia Virus
WHO	World Health Organization

1.0 INTRODUCTION

Dengue is the most important mosquito-borne viral infection occurring in tropical and sub-tropical regions. This disease has emerged as an alarming public health threat, especially in developing countries. An estimated 50 to 100 million dengue virus (DENV) infections occur annually [1], with over one hundred countries endemic with at least one DENV serotype. The number of individuals at risk for contracting DENV infections may increase in upcoming decades as the habitat range of its vector, the *Aedes* species mosquito, continues to spread geographically [2, 3].

There are currently four genetically distinct DENV serotypes (1-4) scattered around the world. During the past few decades, many countries that were once endemic with a single DENV serotype now have multiple circulating serotypes. Overall, the reported incidence of severe dengue has increased over 500-fold since the 1950s [4, 5]. It is suspected that this higher prevalence of severe infections is linked to the increase in heterotypic DENV infections in a given region, but this remains an outstanding question [6-9].

Other than supportive treatments, there are currently no licensed vaccines, anti-virals or therapeutics available to prevent or treat severe infections. Despite the decades devoted to unraveling the factors which drive the immunopathogenesis of DENV infections, the dynamic host and viral factors that contribute to the development of severe disease have yet to be fully elucidated [10]. Therefore, the pathogenesis of DENV infections requires further research.

1.1 GLOBAL IMPACT OF DENGUE

1.1.1 Disease Burden

Dengue threatens an estimated 2.5 billion people -or about one-third of the world's population- with significant medical, social and economic burden. In 2010, the World Health Organization (WHO) reported the overall severe disease burden to be approximately five hundred thousand cases annually with a 1-5 percent case-fatality rate. In the absence of supportive therapy, this case-fatality rate rose up to 40 percent in some regions [9, 11]. However, many experts believe that these disease burden estimates are highly conservative. A recent mapping exercise conducted by the Spatial Ecology and Epidemiology Group at Oxford suggests that the actual burden of dengue is about three times higher than current WHO estimates [12].

1.1.2 Emergence

Dengue has accompanied human society throughout history. The earliest records of dengue-like symptoms were documented during the Chin Dynasty, 265-420 A.D [13]. Throughout the 18^{th} and 19^{th} centuries, dengue was primarily observed in parts of Asia and the Americas with a major epidemic in Philadelphia in the 1780s [14]. However, these localized dengue outbreaks differed from the global disease present today because expansion of mosquito vectors and infected human hosts occurred at a much slower rate. During World War II, movement of viremic troops and refugees across the globe facilitated the spread of dengue to new areas [15]. Combined with post war population growth, rapid urbanization and poor vector

control, this heightened trans-continental travel led to the establishment of dengue hyper-endemic areas in Asia and the Pacific [16]. Dengue's reach continues to expand dramatically. Many countries in Asia, Africa and the Americas now show all four dengue serotypes in circulation when during World War II and up to twenty years ago they evidenced only one or two serotypes [17].

1.2 DENGUE VIRUS

1.2.1 Genome/Structure

DENV is part of the genus *flavivirus* from the family Flaviviridae. The Flaviviridae family includes other relevant, serologically related viruses such as West Nile virus, yellow fever virus, and Japanese encephalitis virus. The genome is an ~11 kb single stranded positive sense RNA genome that has one open reading frame which is translated into a single polyprotein. This genome encodes three structural proteins: the nucleocapsid (C), the membrane associated protein (M), and the E protein. It also encodes seven non-structure proteins that are essential for viral replication: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 [18-20]. Electron micrographs show the virion to measure 50 nm as an immature particle and 60 nm as a mature particle [21]. Inside the virion, viral C proteins encasing the viral genome form the nueclocapsid which is surrounded by a host-derived lipid bilayer with an icosahedral scaffold consisting of 180 copies of E protein monomers. The E protein plays an essential role in facilitating the virion's attachment and fusion with susceptible cells. All four serotypes share similar epitopes on the E protein that lead to extensive cross-reactivity [19, 22-24].

1.2.2 Transmission and Life Cycle

The *Aedes aegypti* mosquito is the principal DENV vector; *Aedes albopictus* is the secondary vector. The *Aedes* mosquito species are found in urban areas. They prefer to lay their eggs in standing water near or under houses. Once a mosquito feeds on an infected host and contracts DENV, it is infected for life [21]. DENV is transmitted to the human host when an infected mosquito inserts its proboscis into the upper dermis layers while searching for the capillaries to take a blood meal. The virus enters the human host through the mosquito saliva that is injected during feeding [21, 25, 26]. Disease onset occurs typically four to six days after the virus is introduced into the skin [27].

Once the transmitted virus makes contact with susceptible cells, it enters the cells through receptor-mediated endocytosis using receptors such as dendritic-specific intercellular adhesion molecule 3(ICAM-3)-grabbing nonintegrin (DC-SIGN) [19]. *In vitro,* it has been observed that the virus primarily targets monocytes/macrophages for DENV infection, as well as Langerhan cells and dermal dendritic cells. However, the precise *in vivo* targets are still not known [28]. After entering the cell, acidification of the endosome triggers a conformational change which causes the projection of fusion peptides located on the E protein to facilitate the fusion of the virus membrane with the host membrane [24]. This opens the viral particle, releasing the viral genome into the cytoplasm. In the cytoplasm, the viral RNA dissociates from the nucleocapsid and associates with endoplasmic reticulum (ER) derived-membranes. Replication of the viral genome and assembly of the virion is initiated in the ER. Translation of the genome involves the production of a single polyprotein that is cleaved at specific sites by both host proteases and the viral NS3/NS2B protease. The immature particles travel through the Golgi and are released from

the cell by exocytosis. Mature particles are produced when the endoprotease furin cleaves the pre-membrane protein (prM) to produce virus associated M, an infectious particle [19, 29, 30].

1.3 CLINICAL CLASSIFICATIONS AND MANIFESTATIONS

1.3.1 Changes in Clinical Classifications

Over the past decades, it became apparent that the previous system for classifying DENV infections was no longer suitable for standardizing treatment and clinical practice. This system, based on the original 1975 guidelines, proved too complicated for medical professionals to use in the proper treatment and classification of dengue patients. In an effort to ease the triage process, the WHO revised this classification system in 2009 to account for the variable disease manifestations recorded across multiple geographical regions [31-33].

In the past, dengue outcomes were categorized as dengue fever (DF), and dengue hemorrhagic fever (DHF) grades I-IV with IV representing dengue shock syndrome (DSS). The 2009 system removes this grading system and only considers three different categories of disease: dengue, dengue with warning signs and severe dengue [33]. This new system is hoped to ease management of clinical patients and allow for greater sensitivity to a severe dengue diagnoses [31, 32, 34, 35]. However, some researchers opine that categorizing dengue as a spectrum of disease outcomes will impede the study of DENV pathogenesis. They argue that understanding dengue as a distinct set of disease outcomes is essential to elucidating the factors driving severe disease development as different immune mechanisms may be involved in different clinical outcomes [32, 33]. Advocates of the new system cite the lack of progress in

understanding DENV pathogenesis under the old system as justification that the classification changes will not have any negative effects on research [32].

1.3.2 Clinical Manifestations

The disease outcomes of DENV infections range from asymptomatic infections to severe, life-threatening infections. Based on the WHO/SEARO 2011 Comprehensive Guidelines for Prevention and Control of Dengue and DHF, which use the older classification system, cases can be categorized as undifferentiated febrile illness (UF), DF, DHF, and DSS. UF is commonly seen in primary dengue. Confirmation of DENV infections in UF cases relies on serological testing, as healthcare professionals cannot diagnose these patients based on clinical observation alone. DF, an acute febrile illness also called break-bone fever, is associated with severe headaches, joint pain, and occasionally haemorrhaging. DHF manifests similar symptoms to DF during the febrile phrase. The development of significant plasma leakage and abnormal haemostasis distinguishes DHF from DF. Warning signs such as persistent vomiting, abdominal pain, lethargy or restlessness can precede severe disease outcomes. DSS occurs when the plasma leakage observed during DHF becomes so severe that it causes the patient to go into shock [27, 36, 37].

Implementation of the new classification system is hoped to allow for the distinct classification of patients. The category of dengue is assigned to patients who recover after defervesecence. Patients classified as dengue with warning signs suffer from symptoms such as persistent vomiting, abdominal pain, lethargy, etc. Severe dengue indicates patients who develop severe plasma leakage or severe bleeding. This new system has yet to be practically shown as a relevant alternative to the previous system. To optimize this dengue classification system,

researchers may need to implement further modifications as well as incorporate elements from the previous naming system [34].

1.4 PRINCIPAL RISK FACTOR: SECONDARY INFECTIONS

In the late 1970s Scott Halstead proposed the "sequential infection hypothesis". This hypothesis was the first to suggest that pre-existing immunity to any DENV serotype may be linked to the development of severe disease [6]. To investigate his theory, Halstead studied a group of patients admitted to the Children's Hospital in Bangkok, Thailand. Forty percent of the 523 children hospitalized with DHF had symptoms of shock. When analyzing the serum of these children, Halstead found a correlation between antibodies to a secondary DENV serotype and the development of shock and [38]. In the 1980s, Donald Burke and several others conducted a follow-up study in Bangkok, Thailand. They also found evidence indicating that an infection with a second DENV serotype was a principal risk factor for developing severe disease. Fifty percent of the children recruited into the study had already become infected with at least one serotype. When they actively surveyed these children for seven months, 69 percent of the children who developed secondary symptomatic infections were described as DHF. In this study, they observed a higher prevalence of DHF infections in children with pre-existing immunity compared to children without said immunity [6]. This study was the first comprehensive study to link the presence of pre-existing immunity to DENV with the severity of disease manifestation observed [6, 8, 38, 39].

Another important study occurred in Cuba during the 1970s and 1980s. For the first time, investigators were able to directly study the effects of a second DENV serotype entering into an

already exposed population [40, 41]. Between 1977 and 1979, 50 percent of the Cuban population was infected with DENV-1. Two years later, DENV-2 was introduced into the population, producing the first major DHF/DSS epidemic in Cuban history [41]. DHF/DSS was observed in the adult population for the first time, and 98 percent of the DHF/DSS adult cases were found to have pre-existing immunity to the previously endemic DENV serotype. Among the 124 child cases of DHF, 122 of these children with DHF/DSS had pre-existing immunity to DENV. There were no hospital cases or fatalities in children who were born after the DENV-1 epidemic. The observations made in this study were consistent with other studies of DHF in children with secondary exposures and with Halstead's hypothesis of sequential infection [42].

1.5 CAUSATION OF SEVERE DENV INFECTIONS

1.5.1 Antibody Dependent Enhancement

The current central dogma explaining the causation of severe disease development is antibody dependent enhancement (ADE) [38, 43, 44]. ADE is based on the hypothesis that in an environment of sub-neutralizing antibodies, enhanced infection of monocytes and other susceptible cells with Fcγ receptors is promoted through the uptake of sub-neutralized virus-antibody complexes. This mechanism of enhanced infection due to ADE has been previously observed *in vitro* with other viruses [45-47]. It is proposed that when an individual with pre-existing immunity to DENV is infected with a different serotype the antibody response produced is sub-neutralizing and specific for the original infecting serotype. These sub-neutralizing antibodies are presumed to bind to the heterotypic DENV due to cross-reactivity and enhance the

infection of monocytes and macrophages. These increased levels of infected monocytes leads to higher levels of viremia, which is linked to the development of severe disease outcomes [48].

However, despite multiple clinical studies, direct evidence in support of ADE and its role in human infections has yet to be confirmed [43, 46]. Mice models provide the main *in vivo* model to study ADE. These studies have shown that antibodies can either neutralize or enhance DENV infections depending on their concentrations [43]. In humans, evidence in support of ADE come from epidemiological studies that report a high incidence of severe infections among infants, aged six to nine months, with waning passive maternal immunity received from dengue immune mothers [38, 49]. In addition, a study conducted in Thailand observing the ADE qualities of serum collected pre-infection with a second dengue serotype yielded evidence that suggested *in vitro* ADE activity was a good predictor for severe disease outcome [50]. Despite *in vitro* and clinical observations, the evidence in support of ADE remains circumstantial. Severe dengue infections are also observed in individuals with primary infections, so researchers have concluded that other factors must play a role in driving severe disease development. [46].

1.5.2 Other cellular factors

The role of cellular immunity during severe dengue infections has been closely studied, especially since increases in soluble factors such as interleukin-2 (IL-2), interferon-γ (□□□–γ), tumor necrosis factor-alpha (TNF-α), macrophage inflammatory protein-1beta (MIP-1β), and soluble CD8 in patient serum is linked to severe disease outcomes [51]. When monocytes become infected, they secrete TNF-α, interleukin-6 (IL-6), and IFN-α that lead to the reactivation of DENV specific $CD4^+$ memory T-cells. This T-cell activation also leads to increased cytokine expression [52]. Some groups predict that the reactivation of memory T-cells

specific for the original infecting serotypes results in a skewed memory response consisting of low avidity cross-reactive T-cells, also known as original antigenic sin. This skewed response, based on *in vitro* studies, is proposed to alter the kinetics and the profile of cytokine secretion leading to an increase in IFN-γ, TNF-α and MIP-1β [51].

Until recently, most pathogenesis studies focused on factors related to humoral or cellular immunity such as the antibody profiles present during infection, or the skewed T-cells responses driving the cytokine storms, but few studies focused on the roles which other cell types might play. Outside of the dynamic cross-reactive antibody profiles produced by the B-cells, few studies have delved into the other possible roles of this cell type. Current observations suggest that researching B-cells may shed further light on the factors driving dengue disease progression.

1.6 HUMORAL IMMUNITY

1.6.1 B-cells

B-cells, historically called bone-marrow derived lymphocytes, are the lymphocyte population responsible for secreting antibodies against specific antigen epitopes leading to neutralization and clearance. They are primarily identified by the expression of CD19, as all B-cell lineage cells express this surface molecule [53]. CD20 is also a surface marker for identifying mature B-cells. However, this marker is down regulated once committed to developing into plasmablasts, a professional antibody secreting cell described in later sections [53, 54]. B-cells are essential for long-term humoral memory and protection as antibody titers after natural or vaccination-induced immunity is considered a biomarker for a ‘correlate of

protection' [55, 56]. Intimate knowledge of this cell type constitutes an important step towards unraveling the role they may play in immunopathogenesis as well as providing specific long-term protection against pathogens [57].

Ever since their discovery and characterization in the mid-1960s, B-cells have been divided into multiple subsets based on their unique combinations of surface markers and the different roles each distinct phenotype plays [53].

1.6.1.1 Development of Memory B-cells, Plasmablasts and Plasma Cells

The activation of naïve circulating B-cells after antigen challenge, which leads to their differentiation into acute plasmablasts or antibody secreting cells (ASCs), is relatively well-defined [57]. Upon recognizing the challenging antigen, activated naïve B-cells migrate to the secondary lymph node organs and home to the T-cell-B-cell border where they interact with follicular T-cells and their cognate T-helper cell [58]. This interaction pushes the naïve B-cell to differentiate into first responder low-affinity acute IgM ASCs or to become founder cells for germinal centers (GC) [56, 59]. Somatic hypermutation (SHM) and class switching occurs in the GC to select the B-cell clones with the highest affinity to the invading antigen [56]. After founder B-cells form a GC and undergo SHM, they begin the process of either differentiating into acute plasmablasts, memory B-cells or long-lived plasma cells.

Plasmablasts are an intermediate subset between an activated B-cell and a plasma cell. Plasmablasts and plasma cells are similar in that they both are professional antibody secreting cells that down-regulate CD20 and up-regulate CD38 and CD27. These two subsets differ in that plasmablasts still undergo cell cycling and proliferation and have not started to express CD138, while plasma cells express CD138, have extremely low CD19 expression and are terminally

differentiated [54, 60]. If the plasmablasts do not directly differentiate into plasma cells and survive in the lymph node, they will exit into circulation and survive only a short time during the infection to secrete high amounts of antigen specific antibodies [60]. It is still not clear if these acute plasmablasts are able to home to survival niches such as the bone marrow and differentiate into long-lived plasma cells [54, 60].

Memory B cells represent the immunological memory branch of the immune system because they are the main mediators of the secondary responses to infection. This subset is distinguishable from naive B-cell subsets based on their expression phenotype $CD19^{+}CD27^{+}CD38^{-}$, their longevity and their ability to produce high affinity antibodies [54, 61]. Memory B-cells have high affinity B-cell receptors (BCR) that are extremely sensitive to their cognate pathogen. After sensing their antigen, memory B-cells will rapidly differentiate into IgG secreting plasmablasts because their signal and cytokine requirement is much lower compared to a naïve B-cell [57, 62, 63]. However, the particular signals necessary to drive the secondary response facilitated by re-activated memory B-cells remain poorly understood. Some studies identify cell populations such as T-cells or monocytes as important while others suggest that activation through the BCR by the antigen alone is sufficient to drive the classic anamnestic response [62, 64]. After the antigen is cleared, the memory B-cells remain quiescent in the periphery; how this subset is maintained for decades in the absence of antigen remains unknown. Throughout an individual's lifetime, the memory B-cells generated by primary and secondary exposures persist for life-providing protective immunity against reinfection [56, 57]. One caveat is that any defect or alteration in the signals required for regulating this process can promote pathogenic outcomes, either through a lack or overabundance of antibodies due to an unregulated immune response.

1.6.2 Role of T-cells and Monocytes in Plasmablast Development

The particular microenvironment where the activated and developing B-cell resides regulates their phenotypic outcome. The cytokine profile is particularly important in modulating the transcription factors that influence the up or down regulation of certain surface molecules. In the 80s and 90s, a myriad of cytokines such as IL-6, interleukin-10 (IL-10), a proliferating-inducing ligand (APRIL) and B-cell activating factor (BAFF) were discovered and found to promote survival, proliferation, isotype switching and differentiation of B-cells [59, 60]. The particular combinations of these cytokines and others are responsible for regulating the development of the different subtypes; however, researchers still have not pinpointed the exact signals required to drive the differentiation of a memory B cell versus a plasmablast [59].

It is known through *in vitro* studies that T-cells are important for this process as IL-2 enhances proliferation upon CD40L interactions, which is provided by direct T-cell interactions [59]. However, the role of T-cell help in the formation of plasmablasts is still not clear especially in secondary verses primary infections [54]. IFN-α has also been identified as an important cytokine to push the differentiation of B-cells into plasmablasts in an IL-6 environment. IL-6 is predominantly secreted by monocytes [59]. Previous publications provide evidence that the microenvironment where plasmablasts mature are rich in IL-6 and APRIL, which are secreted by monocytes/macrophages and dendritic cells [58, 60].

As mentioned above, plasmablasts enter the peripheral blood after their formation in the GC, retaining their ability to both proliferate and secrete high amounts of antigen specific antibodies. Multiple studies have noted that the plasmablast's kinetics are fairly consistent. Regardless of vaccine or antigen based activation, plasmablasts peak in the periphery 6-7 days post primary contact with the antigen [54, 65, 66]. This response lasts as long as viral antigen is

present in the system, but afterwards the number of plasmablasts rapidly drop off as the infection clears [54].

1.6.3 Humoral Immunity and DENV Infections

In the context of DENV infections, very little is known about the role plasmablasts play because most studies merely focus on the cross-reactive or the neutralizing profile of the antibodies produced. Recent studies delving into the role these cells may play have found that individuals infected with DENV have a very robust plasmablast response that peaks four to seven days after the onset of symptoms [54, 67-69]. One study completed in Thailand using a cohort of adults and children hospitalized with dengue observed that six to seven days post onset of symptoms there was a massive plasmablast response. The kinetics of this response occurred within the expected timeframe, but the extremely high presence of plasmablasts observed was distinctive. On average, the peak plasmablast response was 47 percent of all peripheral $CD19^+$ B-cells with some individuals reaching 80 percent [68]. During other acute infections or vaccination boosters, the peak in plasmablasts usually occurs six to seven days post exposure, but only around 5 to 20 percent of the total CD19 population develop the plasmablast phenotype [66]. In subjects receiving the primary yellow fever virus vaccine or the influenza vaccine, only 2 to 3 percent of their $CD19^+$ cells were identified as plasmablasts [68]. In addition to this, at least 70 percent of the plasmablasts produced were dengue specific IgG secreting cells indicating this is a targeted rather than a massive non-specific response [68]. There was no observable difference between the plasmablast responses mounted in individuals infected with different dengue serotypes.

Our lab has recently published data observing a similar pattern using a cohort of individuals from Brazil. We found that among the individuals who were suffering from severe dengue infections, there was a massive plasmablast response peaking four to seven days after the onset of symptoms that averaged 47 percent and rose as high as 87 percent of the peripheral $CD19^{+}$ B-cell population. Unlike the previous study, there were enough individuals with primary infections to compare the plasmablast responses between severe secondary and primary DENV infections. We found that there was a significant difference in the number of plasmablasts between severe primary and secondary infections. In this study, we also found that over 70 percent of these plasmablasts were specific for DENV. The antibodies produced by these plasmablasts were three times greater in their reactivity against the current infecting DENV serotype, not the previous infecting serotype [67].

It is not clear how this very potent DENV specific plasmablast response fits into the current model of DENV pathogenesis, but it is believed that this response plays an important role. Some speculate that the reactivation of DENV specific memory B-cells early in infection facilitates the production of antibodies which lower virema. In turn, this lowers the overall viral load to decrease the chances of severe disease progression [69]. However, no studies have conclusively investigated the profiles of these antibodies produced during secondary infections. It is also possible that a large reactivation of memory B-cells specific to the previous infecting serotypes may drive ADE, causing a high viral load and severe infection. The founding population of the plasmablasts that drive this robust plasmablast response remains unknown. It is possible that a skewed memory B-cell response could drive this potent response and influence the overall humoral response to the heterotypic DENV infection.

Identifying the origins of this plasmablast response and its impact on the disease may provide new opportunities to design more targeted vaccine candidates. When developing an effective vaccine, especially for DENV, it is essential that the vaccine protectively targets and 'teaches' the immune system how to properly respond to the infection when re-challenged. Therefore, more research is required to elucidate whether or not this potent plasmablast response is the result of an immune response skewed towards protection or pathogenesis.

2.0 PROJECT AIMS

Before moving towards future studies to link this potent plasmablast response to disease outcomes, I first wanted to investigate the possible origins of this plasmablast response. To understand if these plasmablasts originated from a skewed memory B cell response or a robust activated naïve B-cell response, I first decided to focus on the reactivation potential of DENV specific memory B-cells. For this study, my primary goal was to examine the *in vitro* requirements for re-activating DENV specific memory B cells and driving them to differentiate into IgG secreting DENV specific plasmablasts.

AIM 1: Investigate the ability of DENV to stimulate the differentiation of DENV specific memory B-cells into plasmablasts. Hypothesis: DENV is able to directly stimulate DENV specific memory B-cells and drive them to differentiate into DENV specific plasmablasts. Approach: I will stimulate PBMCs from individuals who had previous DENV infections with DENV before culturing for seven days. Then I will assess the phenotype of the ASCs by flow cytometry and their specificity by ELISPOT to evaluate the ability of DENV to drive the differentiation of plasmablasts in culture.

AIM 2: Determine the role of monocytes in promoting the *in vitro* expansion of plasmablasts in response to DENV stimulation. Hypothesis: Other cell populations such as

monocytes may play a role in assisting the differentiation of DENV specific memory B-cells into plasmablasts. Approach: Using magnetic beads, $CD14^{+}$ monocytes will be depleted from the PBMC samples before stimulating with DENV and culturing for seven days. Flow cytometry analysis will be used to observe if this causes a decrease in the frequency of plasmablasts produced in culture. After forty-eight hours, I will look for the presence of infected monocytes by utilizing fluorescently labeled 2H2 antibodies to probe for the presence of intracellular virus.

3.0 MATERIALS AND METHODS

Isolation of PBMCs from blood donors

The University of Pittsburgh IRB approved blood collection from healthy donors with and without pre-immunity for dengue, and these donors gave their informed consent. Three of the six donors used in this study had been exposed to dengue >10 years prior to the blood draw. ELISA confirmed the presence of dengue specific antibodies in their serum. The three remaining donors self-reported that they had had no known exposure to the dengue virus or history of potential dengue-like disease. ELISA showed a lack of dengue specific antibodies and Confirmed their dengue naïve status. Venous blood was collected using heparin or acid citrate dextrose tubes once or multiple times during a 9 month period depending on the donor. PBMCs were isolated using gradient centrifugation over Ficoll-Hypaque. Any contaminating red blood cells were lysed by ACK treatment for 10 minutes RT. The PBMCs were then washed twice in MACs Buffer (PBS, 0.1 EDTA, 5% BSA, HEPES) and re-suspended in 10% dimethylsulfoxide (DMSO)/fetal bovine serum for cryopreservation at a concentration of $1x10^7$ cells/ mL per vial.

Virus-capture ELISA

Determination of the presence of DENV specific antibodies in the serum samples collected from the donors was performed by virus-capture ELISA. The ELISA plates were coated using 1ug/well of 4G2 antibody (a pan DENV envelop antibody, produced and kindly gifted by Eduardo N, Center for Vaccine Research, University of Pittsburgh PA USA) in sodium

bicarbonate carbon buffer pH 7.4. 50 uL/well of this mixture was then incubated overnight at 4ºC. The 4G2 was washed off with wash buffer (PBS, 0.1% Tween-20) then incubated at 37ºC for an hour in blocking buffer (5% milk in PBS). Afterwards, the plate was washed 3x with wash buffer before the addition of an equalized DV-2 and DV-3 virus supernatants in C6/36 growing media (DMEM, 2% FBS, 1% phosphate broth) (gifted by Eduardo N.). The plate was incubated for 2 hours at 37ºC and then washed 5 times with wash buffer. Serum samples from each donor were diluted at 1:100 in 1% milk PBS before being added to each well and incubated overnight at 4ºC. The plate was then washed 2x before the addition of 1:10000 anti-IgG antibody (Jackson) in 1% milk PBS and incubated at 37ºC for 1 hour. The plate was washed 3x in wash buffer and 1:10000 of anti-IgG HRP in 1% milk (Jackson) was added to the plate. After the plate was incubated for an hour at 37ºC, the plate was washed 3x with wash buffer, and 50 uL/well of TMB was added for 20 minutes. After 20 minutes the reaction was stopped by adding 25 uL/well of 2N HCl. The OD was read and then analyzed using Graphpad Prism 6.

Stimulation of cryopreserved PBMCs with virus

The cryopreserved PBMCs were quick thawed in a 37ºC warm water bath and washed in R-10 (RPMI-1640, 10% FBS, 1% Pen/Strep, 1% L-glut, 1% Non-essential amino acids, 1% sodium pyruvate, 1% HEPES) culture medium. They were rested in 10 mL of medium for 2 hours prior to stimulation with DENV in 5% CO_2 at 37ºC. The cells were stimulated in 15 mL conicals using virus re-suspension medium alone (20nm HEPES, 3% FBS, DMEM) or with an MOI 3 of DENV 2 or 3, influenza virus (H_7N_9 a gift from the Ted Ross, previously Center for Vaccine Research, University of Pittsburgh, PA USA) or 240 nm of SIV_{251} capsid protein (a gift from Jeff Listone). After re-suspending $5x10^5$ cells/275 uL in a mixture of virus and virus re-suspension media or virus re-suspension media alone, the cells were incubated in 5% CO_2 at

37^{o}C for two hours. Then the virus was washed off the cells by filling the 15 mL conicals with R-10 medium and spinning the cells down. The cells were re-suspended in R-10 medium at a concentration of 100, 000 cells/100 uL. As a positive control some of the cells not stimulated with virus were stimulated with a mixture of *Phytolacca Americana* pokeweed mitogen (1/100,000 dilution), Staphylococcus Aureus Cowan (SAC) (Sigma) (1/10,000 dilution) and 6ug/mL of CpG 2006 (Invitrogen). They were cultured ~171, 000 cells/well in a 96 round-well culture plate in 5% CO_2 for 7 days.

Detection of plasmablast by flow cytometry

After the PBMCs were either thawed or cultured for 7 days, they were incubated at various concentrations in the master mix of different combinations of the fluorochrome-labeled Ab from BD science. The main antibody panel used was CD19 (clone HIB19 PE-Cy7), CD20 (2H7 APC-H7), CD27 (M-T271 V450), CD3 (PE-Cy5), CD38 (AT-1; Stem Technologies). Some experiments used CD95 (DX-2 APC) or CD21 (B-ly4 PE-Cy5) (all from BD Science except CD38). Before the cells were stained for extracellular targets, they were incubated with LIVE/DEAD (Invitrogen), which is an amine-reactive fluorescent dye that stains dead and dying cells positive. All samples were run on an LSRII flow cytometer (BD Biosciences) and the data was analyzed using Flowjo software (Tree Star).

Detection of infected monocytes 48 hrs post stimulation by cytometry

The detection of infected monocytes occurred after the PBMCs were exposed as described above to DENV-2 or DENV-3 before culturing for 48 hours. The cells were collected and MACs Buffer was added to the wells. Each well was washed vigorously until all adherent cells were removed from the plate. Then the cells were incubated with LIVE/DEAD (Invitrogen) as described above; the Ab panel from BD Science used was CD19 (PE-Cy7), CD14 (PB), CD3

(PE-Cy5), HLA-DR (FITC) and CD11c (PE). Intracellular staining for virus followed the surface staining of the cells. The cells were fixed and permeabilized using Cytofix/Cytoperm kit (BD Biosciences), followed by staining with a Alex647 conjugated 2H2 antibody. The 2H2 is an IgG_{2a} isotype antibody produced in lab using a hybridoma from a mouse immunized against all 4 dengue serotypes. Therefore, this is a pan-dengue antibody that recognized the prM protein. An Alexa674 anti-mouse IgG_{2a} (Invitrogen) was used to label this antibody following the manufacturer's protocol. Following the intracellular staining, the samples were run on an LSRII flow cytometer and analyzed using Flowjo as previously described.

Growth/ultracentrifugation of DENV prototypes 16681 DENV-2 and H87 DENV-3

The DENV prototypes used in this study were DENV-2 (16681) and DENV-3 (H87). They were propagated on C6/36 cells. At day zero $1x10^6$ cells were infected with an MOI 0.01 of virus for 2 hours before the virus was washed off. Then the cells were cultured for 15 days in DMEM, 2% FBS, 1% Pen/Strep and 1% phosphotase Broth. Supernatants were collected and stored at -80^{o}C at days 5, 7, 9, and 15 dependent on the amount of CPE observed. Then the supernatants were pooled and concentrated by centrifugation at 100,000 x *g* for 4 hours over a 20% glycerol cushion.

DENV specific ELISPOT Assay

The ELISPOT assay used was previously published [67]. To give a brief summary, 96 well ELISPOT plates were coated with goat anti-human IgG (10 ug/mL; Bethyl Laboratories), UV-inactivated DENV-2 (16681) or DENV-3 (H87) that were grown on C6/36 cells and concentrated as described above to detect IgG secreting plasmablasts or DENV specific plasmablasts, respectively. The concentration of virus used per well was $1x10^5$ PFU/mL and the control used was a 2% BSA and IgG coated well with a media only culture. The plates were

coated overnight at 4^{o}C. Before adding the stimulated cells- either CpG cocktail or DENV which had been stimulated for 7 days- the plates were blocked with RPMI 1640 with 10% FBS for 2 hours. The desired concentration of cells was then added to each well. After an18-20 hour incubation at 37^{o}C, the cells were discarded and washed before a 1 hr incubation with biotinylated goat anti-human IgG-Fc (Bethyl Laboratories) at room temperature. Then Streptavidin-conjugate alkaline phosphatase (Bio-Rad) was added and the plates were developed using an alkaline phosphatase conjugate kit (Bio Rad). CTL ImmunoSpot reader and counting software was used to count the spots produced (Cellular Technologies).

Depletion of CD14^{+} monocytes

Depletion of CD14^{+} cells was achieved by using the MACs cell sorting kit (Miltenyi Biotech). This kit positively labels CD14^{+} cells; the depletion was carried out following the manufacturer's protocol and using a LS depletion column.

CFSE cell Proliferation Kit

In order to follow the proliferation of CD19^{+} in culture for seven days, the Cell Trace^{TM} CFSE Cell Proliferation Kit, for flow cytometry by Invitrogen was used. The protocol issued with the kit was followed. Briefly, 1 uL of the Cell Trace^{TM} stock solution was used per 1 mL of protein-free buffer. 5 uL of Cell Trace^{TM} was used per 5 mL of 0.1% BSA (BSA and PBS) and 5x10^{6} million PBMCs. The cells were re-suspended in the dye solution and left in the 37^{o}C hot water bath for 30 minutes. Then the cells were washed thoroughly and re-suspended in complete R-10 media and left in the hot water bath at 37^{o}C for another 10 minutes. After staining the PBMCs, they were stimulated with the CpG cocktail, as described above, or cultured in media only for 7 days. At day 7, the PBMCs were collected and stained as described above to look at CD19^{+} population.

4.0 RESULTS

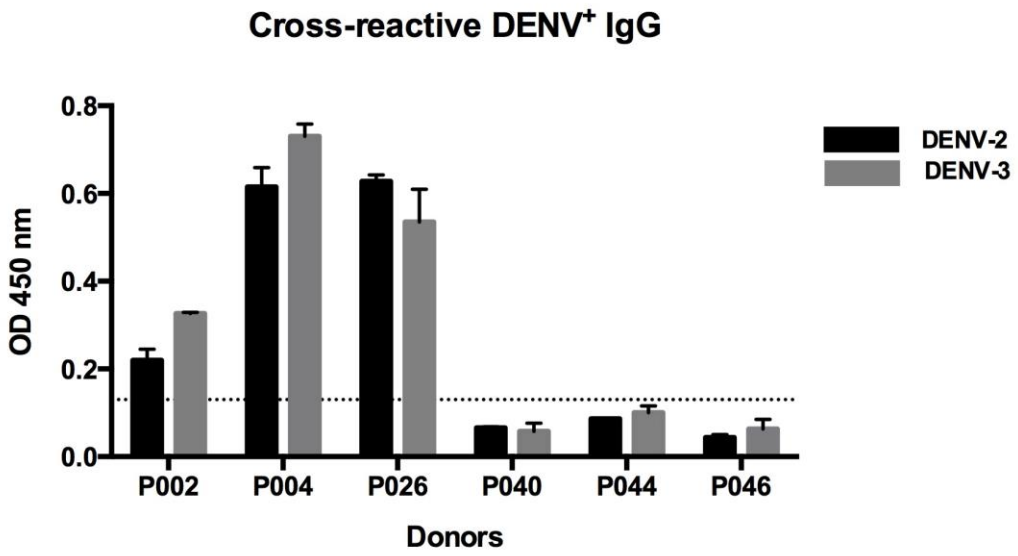
4.1 SPECIFIC AIM 1 RESULTS

AIM 1: Investigate the ability of DENV to stimulate the differentiation of DENV specific memory B-cells into plasmablasts.

4.1.1 Patient and sample characteristics

For this study, healthy donors were recruited from the local community. From this group of local donors, six reported previous infections with DENV and eight had no history of travel to dengue endemic areas. To confirm the immune status of these donors, their serum was tested by an in-house anti-DENV IgG ELISA. I confirmed that the serum for all six donors with previous exposure to DENV was positive for antibodies that could bind to DENV. Five of the presumed naïve donors did not have DENV binding antibodies in their serum. The remaining three donors were dropped from the study due to borderline negative results. Of these remaining donors I chose three of the confirmed naïve and three of the confirmed immune to include in the study. Figure 1 shows the anti-DENV IgG virus-capture ELISA results for these six donors. The demographics and clinical histories for each of the donors are described in Table 1. Additionally, plaque reduction neutralization assays were performed using the serum samples to confirm the DENV serotypes to which the donors reported being previously exposed. The neutralizing

antibodies detected were DENV-3, donors P002 and P004, and DENV-1 and DENV-2, donor P026 (Table 1).



The dotted line represents the cut-off OD where all readings below the line are negative and all the readings above are positive serum samples for DENV antibodies

Figure 1. Detection of cross-reactive anti-DENV IgG antibodies in donor serum samples.

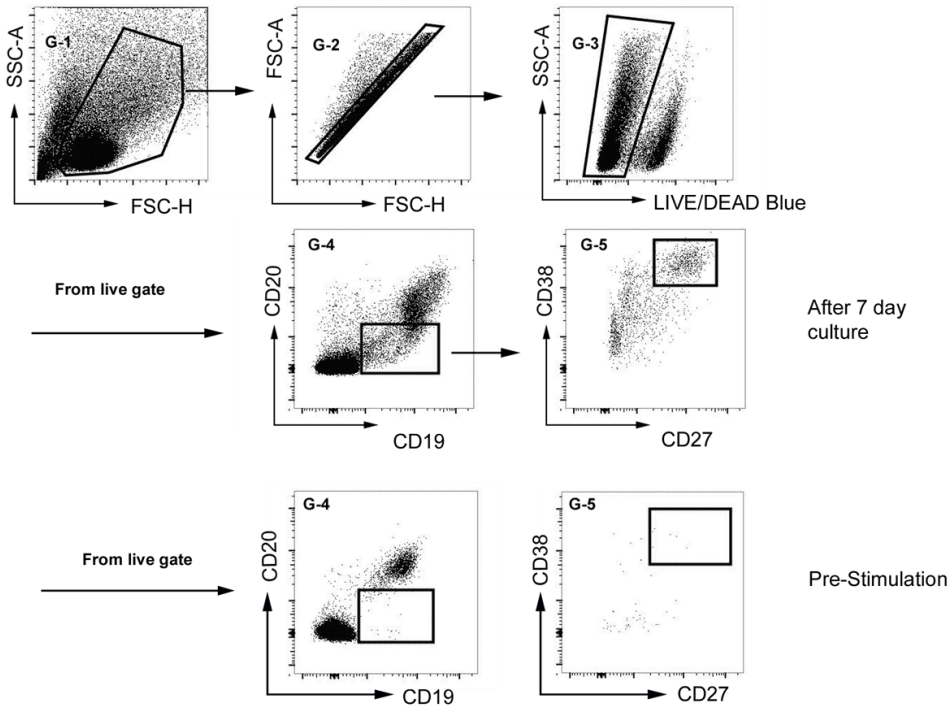
Table 1. Demographic and serological characteristics of the donors.

DONOR ID	P002	P004	P026	P040	P044	P046
Age	30s	30s	30s	30s	20s	20s
Gender	M	F	F	F	F	F
Type Infection	DF	Asymptomatic	DHF	Naïve	Naive	Naive
Neutralizing Antibodies	DENV-3	DENV-3	DENV-1 DENV-2	-	-	-
Year Infected	2003	2003	-	-	-	-

PRNT data kindly provided by Priscila Castanha, MS from Ernesto Marques lab.

4.1.2 Examining the phenotype and kinetics of the plasmablasts produced after DENV exposures

For this study, I defined plasmablasts as the $CD19^{+}CD20^{-}CD38^{++}CD27^{++}$ population based on previously published classifications [54]. The gating strategy depicted in Figure 2 was used throughout the entire study to classify this cell population.

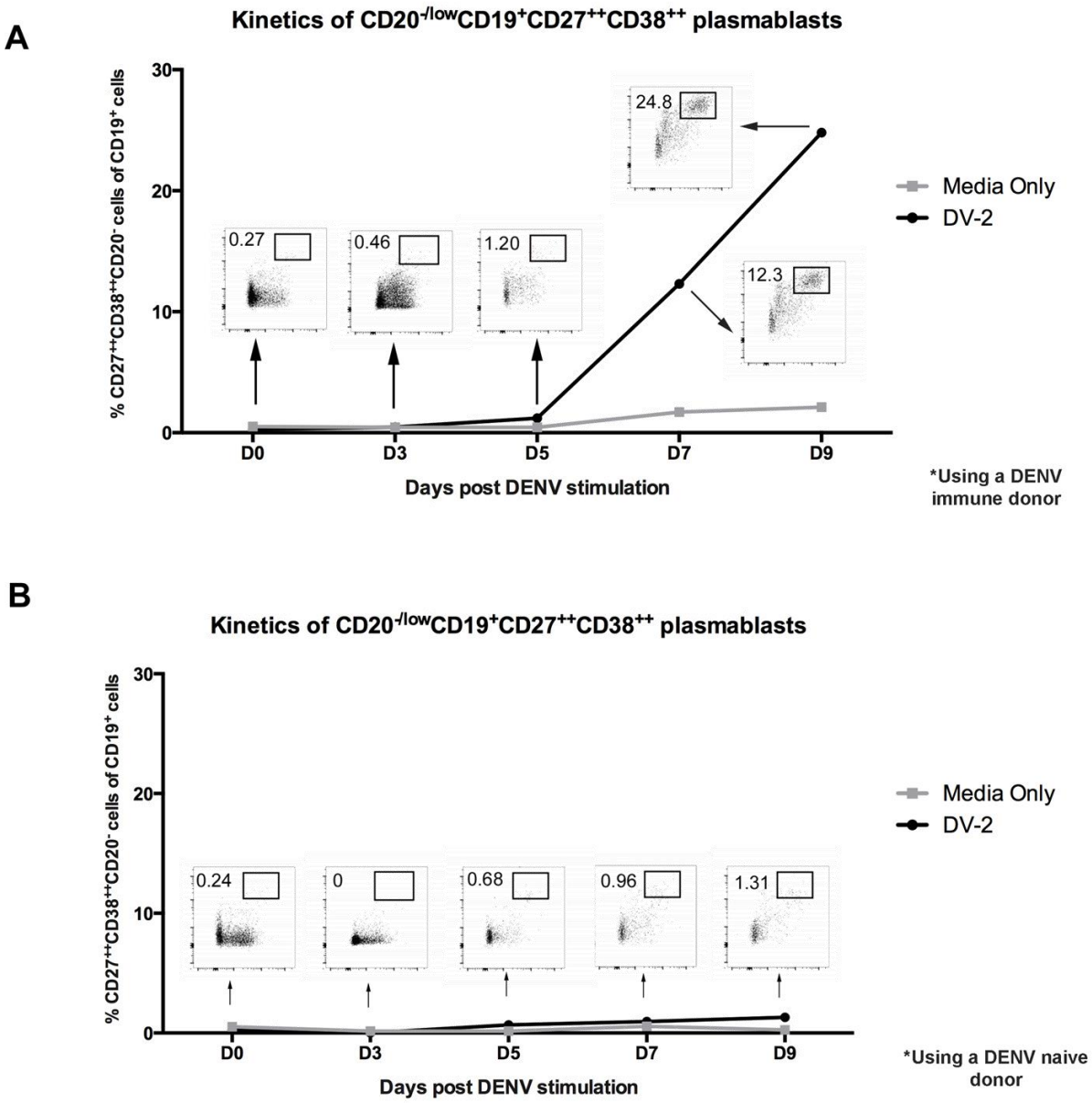


Representative gating strategy for how plasmablasts were defined from DENV immune individual post DENV stimulation and pre-stimulation. **G-1** gates on the total cells that have the appropriate Forward and Side Scatter to indicate they are neither dead cells or debris. **G-2** gates on single cells. **G-3** gates on live cells. **G-4** gates on the $CD19^{+}CD20^{-/low}$ B-cell population. **G-5** gates on the $CD38^{++}CD27^{++}$ plasmablast population.

Figure 2. Gating strategy to define plasmablasts.

A kinetics curve was performed to observe when B-cells start differentiating and up-regulating $CD27^{++}CD38^{++}$, as well as down regulating CD20, which is the classic phenotype of plasmablasts. Vaccine, clinical and *in vitro* studies observed that this response takes place

between five to ten days post exposure and peaks at six to seven days [65, 67, 70, 71]. Therefore I took samples from day three until day nine, two days before the presumed beginning stages of the plasmablast development and two days after the estimated peak, respectively. PBMCs from P026, a DENV immune donor, were used to define this curve, as it was expected that DENV would drive a defined plasmablast population (Figure 3A). P040 was used as the negative control for a response to DENV without any specific B-cells (Figure 3B). Since memory B-cells have high affinity BCR against their specific antigen, they are more sensitive and require less activation signals compared to a naïve B-cell; therefore, they rapidly differentiate into plasmablasts after antigen exposure *in vivo* [54]. To ensure that donors with DENV specific memory B-cells did not produce a peak plasmablast response before donors without DENV specific memory B-cells, I tested donors from both groups. Due to naïve B-cells requiring strong activation signals, I speculated that non-specifically activated cells may peak after the reactivated memory B-cells population. The amount of plasmablasts in both donors increased gradually each day during the nine day kinetics study. This suggests that after DENV stimulation *in vitro*, the plasmablast response uniformly developed in each donor regardless of immune status; however, as expected, the overall frequency of the plasmablasts was greater when the donor had DENV specific memory B-cells. Based on this curve, day seven was chosen as the time point to look for the plasmablasts in the subsequent studies (Figure 3A). Despite the higher percentage of plasmablasts at day nine, day seven was chosen because of the difficulty of culturing fragile primary B-cells for long periods.



(A) Kinetics curve for P026 a DENV immune donor. **(B)** Kinetics curve for P040 a DENV naïve donor

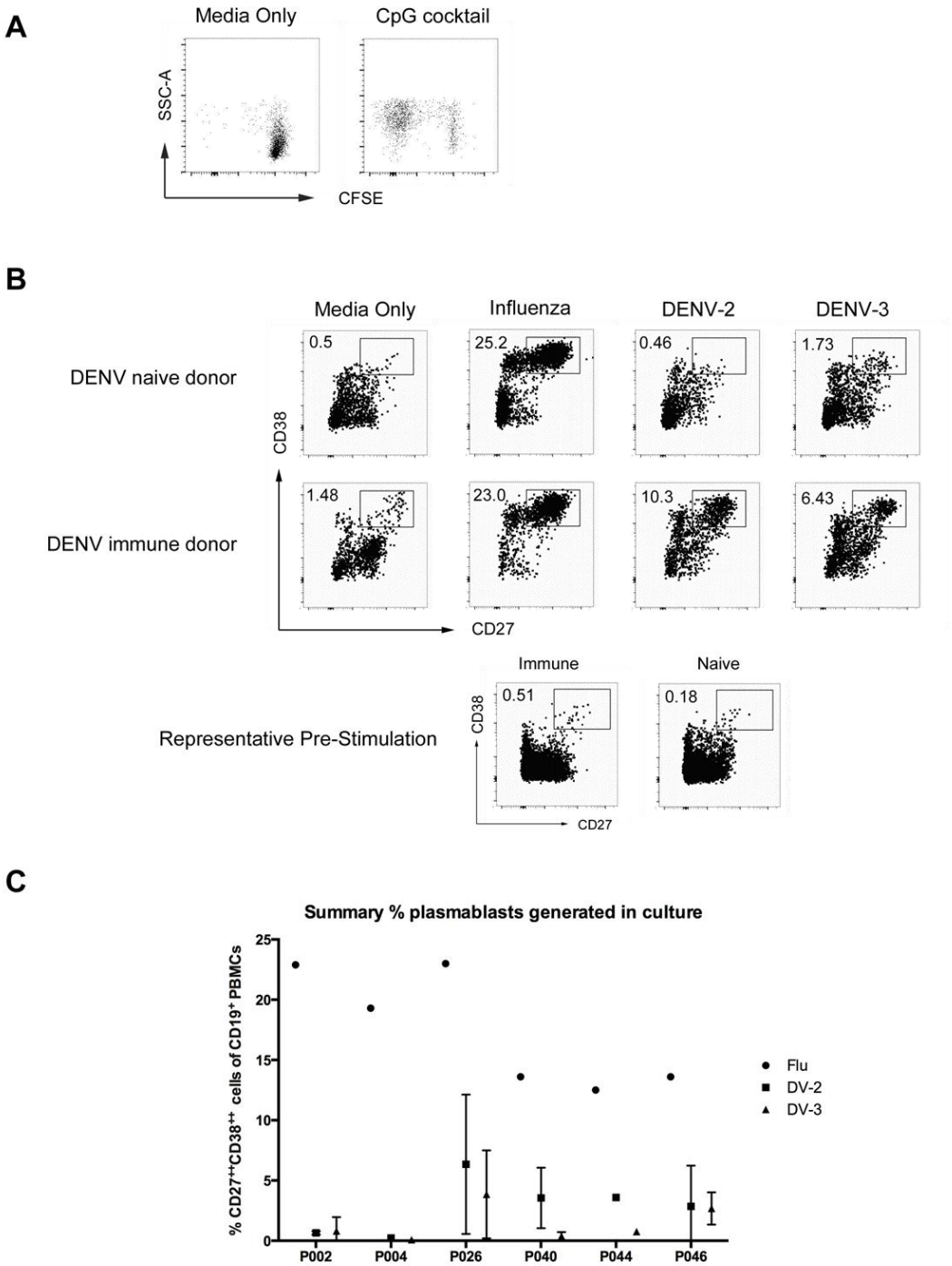
Figure 3. Kinetics curve for plasmablast differentiation in culture.

4.1.3 Generation of plasmablasts by DENV-induced stimulation

After establishing that day seven was the optimal day to look for plasmablasts, I compared how selected DENV immune and naïve donors responded in culture. Our hypothesis was that donors with DENV immunity would produce a strong plasmablast response in culture as seen in influenza vaccine studies or clinical dengue infections. As a positive control virus for antigen derived plasmablasts in culture, influenza virus was chosen. In the United States (US), the Centers for Disease Control of Prevention (CDC) estimates that 38.8 percent of adults received the influenza vaccine 2011-2012 [72], so many US adults have a well-maintained population of influenza virus specific memory B-cells. The six donors chosen for this set of experiments all mounted a high frequency plasmablast response after *in vitro* stimulation. A carboxyfluorescein succinimidyl ester (CFSE) study was also completed to show that B-cells could be driven to proliferate in culture. Figure 4A shows the $CD19^+$ cells seven days after being cultured in media only or a strong CpG mitogen cocktail. As shown by the large number of cells that had a distinct reduction in florescence, the majority of the $CD19^+$ were induced to proliferate. In contrast, the media only control population mainly stayed a uniformed high florescent population indicating they did not undergo proliferation during the seven days.

It was expected that both DENV naïve and DENV immune donors would have strong plasmablast responses in culture to influenza while only the DENV immune donors would produce a distinct plasmablast response to DENV (Figure 4B). However, based on multiple experiments, only one DENV immune donor produced a defined, but varied, plasmablast response in culture. The three naïve donors produced a low-level plasmablast response. The two remaining DENV immune donors produced a very low plasmablast response in culture. Figure 4C shows the range of plasmablast responses observed in each donor throughout the study. The

specificity of these plasmablasts produced as a result of DENV stimulation could not be detected by flow cytometry. Based on the low levels of plasmablasts observed in the naïve donor samples, DENV is likely able to cause some level of non-specific activation. Another experimental approach was taken to determine the specificity of these plasmablasts, as well as whether or not this suggests DENV can drive reactivation and proliferation in the DENV specific memory B-cell population.

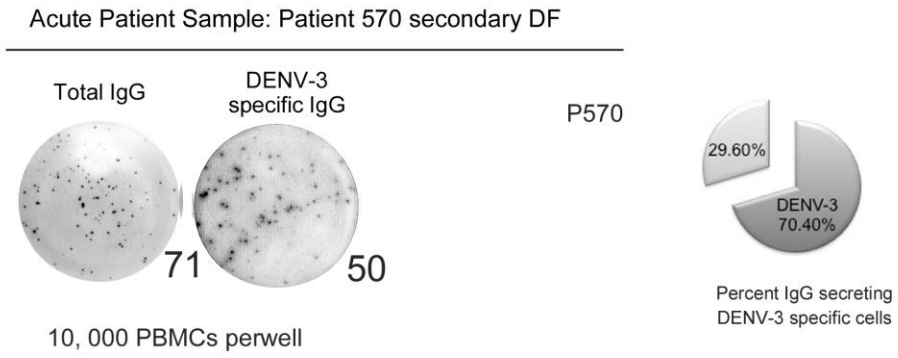


(A) CFSE study. Both dot-plots represent the $CD19^+$ population of the total PBMCs cultured. The left dot-plot represents the media only control showing very little proliferation while the right dot-plot shows high levels of proliferation in the CpG cocktail treated PBMCs. **(B)** Representative dot-plots of the plasmablast response hypothesized to be observe. **(C)** Range of plasmablast responses observed in each donor throughout the study

Figure 4. Generation of plasmablasts in culture.

4.1.4 Specificity of plasmablasts in DENV patients

The flow cytometry method used in the previous experiments could only measure the overall frequency of the plasmablasts produced by the different donors in culture. A system to detect the specificity of these plasmablasts by flow cytometry was not available. Therefore an in-house ELISPOT assay was used to determine the specificity of the DENV activated plasmablasts. A previous study showed that ~70 percent of IgG^+ secreting plasmablasts obtained from a dengue patient were specific for DENV-3 [67]. Since this patient was already characterized, I used them as a positive control for the assay. Figure 5 shows that the assay was working accurately since ~70 percent of the total IgG secreting plasmablasts were specific for DENV-3.



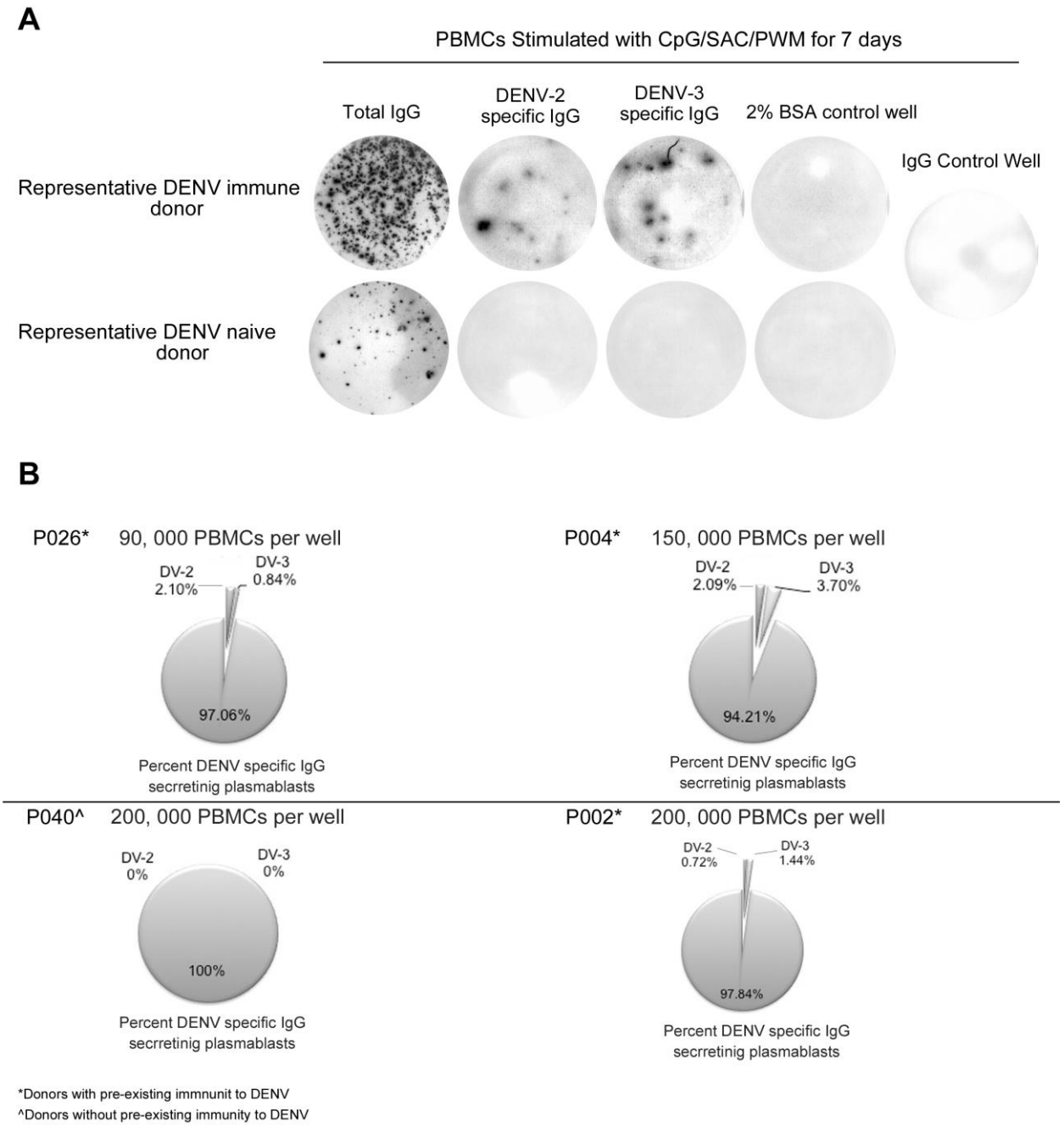
10,000 PBMCs were plated per well. Patient 570 was a previous characterized patient sample with >70% of their plasmablasts specific for DENV-3

Figure 5 Highly DENV specific plasmablast response in patient 570.

4.1.5 Frequency of long-lived DENV specific memory B-cells

Before looking for DENV specific IgG^+ plasmablasts after stimulating PBMCs from immune individuals with DENV, tests were designed using a strong mitogen cocktail to make sure there was a detectable population of DENV specific IgG^+ memory B cells in the peripheral blood. Most investigators searching for class switched memory B cells use a strong mitogen when measuring their frequency. The class switch population has been reported to be 250 to 300 cells/$1x10^6$ PBMCs with the percentage of antigen specific B-cells reported to be ~0.01 to 0.1 percent of this class switched population depending on the study and antigen [65, 66, 73]. In order to characterize the frequency of DENV specific memory B cells in each DENV immune donor, I stimulated PBMCs for seven days using a strong B-cell mitogen cocktail (see methods) to drive $CD27^+$ memory B-cells to differentiate into antibody secreting cells. I then transferred the cells in an ELISPOT plate pre-coated with DENV and incubated for twenty hours to capture any IgG or DENV specific antibodies (Figure 6A). All 3 DENV immune donors had quite a large DENV specific IgG^+ memory B-cell population relative to the observations referenced above. Over 0.7 percent of the circulating IgG^+ memory B-cells in the immune donors were DENV specific. Two of these donors had over 2 percent of their circulating memory B-cells specific for one or two dengue serotypes (Figure 6B). A donor with no pre-exiting immunity to DENV was also stimulated with the same strong mitogen cocktail for seven days and, as expected, no DENV IgG^+ secreting plasmablasts were detected (Figure 6A). These results suggest that the DENV immune donors have a large DENV specific IgG^+ long-term memory B-cell population in their peripheral blood that could be detected by ELISPOT. Since antigen stimulation is much weaker compared to a strong mitogen cocktail, if DENV specific antibodies

are undetectable by ELISPOT after seven days, this is not related to an absence of DENV specific memory B-cells.

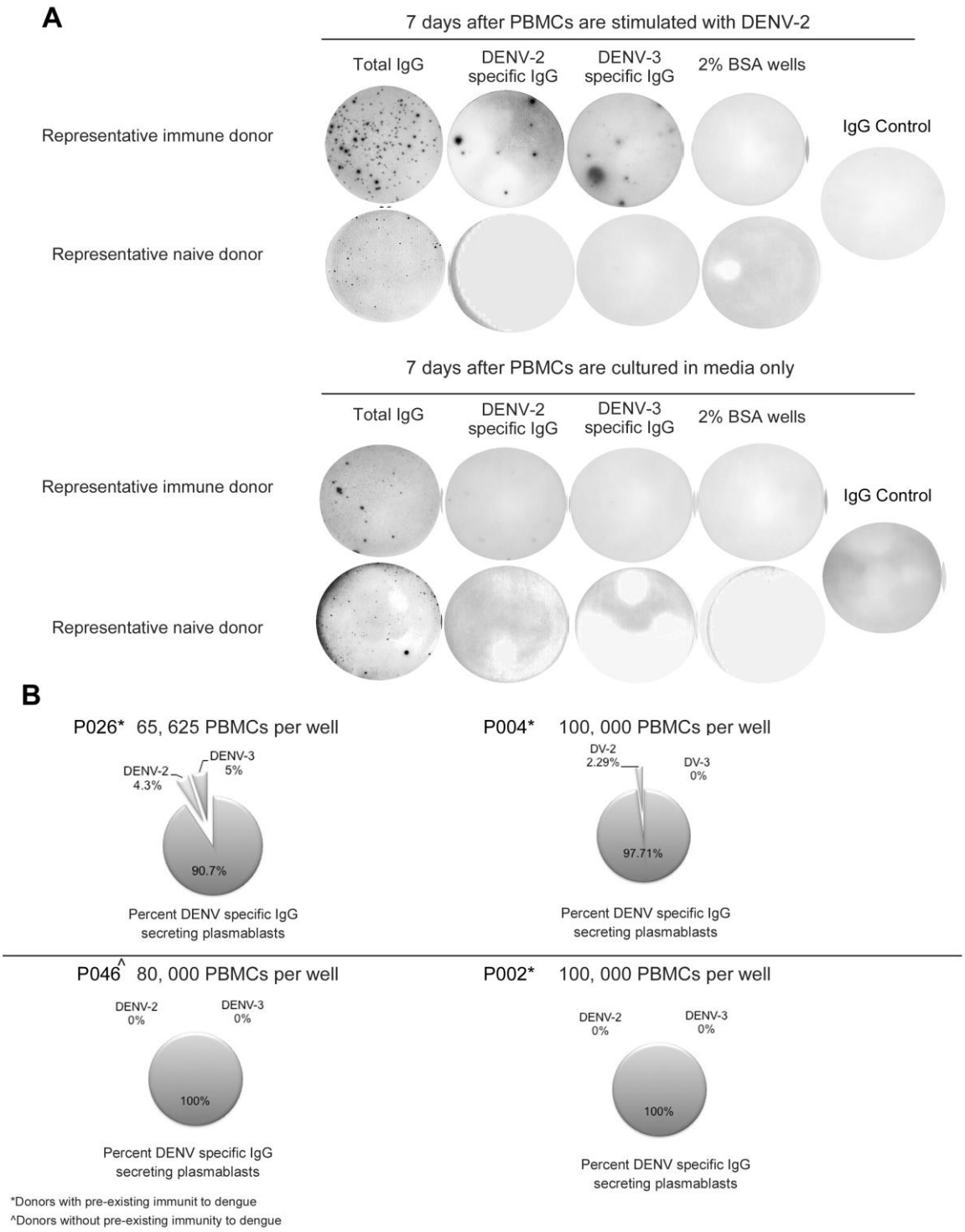


(A) Representative ELISPOT wells of DENV immune and naïve donors after stimulation with DENV for 7 days and remaining un-stimulated in culture. This assay detected the plasmablasts secreting Ab reactive against IgG, DENV-2, DENV-3 and BSA. **(B)** Pie charts representing the frequency of DENV specific IgG plasmablasts per total IgG plasmablasts.

Figure 6. Frequency of DENV specific memory B-cells.

4.1.6 Specificity of plasmablasts generation after stimulating with DENV-2

Testing was conducted to determine if DENV alone is a strong enough stimulus to specifically reactivate the circulating DENV specific IgG^+ memory B cells to proliferate and differentiate into DENV specific IgG secreting plasmablasts. After stimulating the PBMCs with DENV-2, I plated a range of cells, $1x10^5$ to 32,500 cells, in each ELISPOT well and incubated for twenty hours to capture any DENV specific antibodies secreted by the ASCs. The DENV naïve donor used as a negative control produced a low level IgG^+ response, supporting the initial observation that the plasmablasts produced in culture were non-specific. In the media only controls, there was also a low level of IgG^+ plasmablasts after seven days (Figure 7A). As shown in Figure 7B, in two of the donors with a known history of DENV infection, only 2 to 5 percent of their ASCs were specific for DENV. The third donor with DENV immunity had undetectable levels of DENV specific IgG secreting cells. It is possible their response was so low because I needed to plate a higher number of cells per well. These results show that the plasmablast population observed by flow cytometry after seven days in culture does not represent a dominant DENV specific population as was expected. Even though the percentages of DENV specific IgG secreting cells are lower than expected, they still show that DENV can drive DENV specific memory B cells to differentiate. These results suggest that the re-activation of DENV specific memory B-cells and the plasmablasts they differentiate into do not predominantly make up the potent plasmablast response observed in acute patients.



(A) Representative ELISPOT wells of DENV immune and naïve donors after stimulation with DENV for 7 days and remaining un-stimulated in culture. This assay detected the plasmablasts secreting Ab reactive against IgG, DENV-2, DENV-3 and BSA. **(B)** Pie charts presenting the frequency of DENV specific IgG secreting cells per total IgG secreting cells

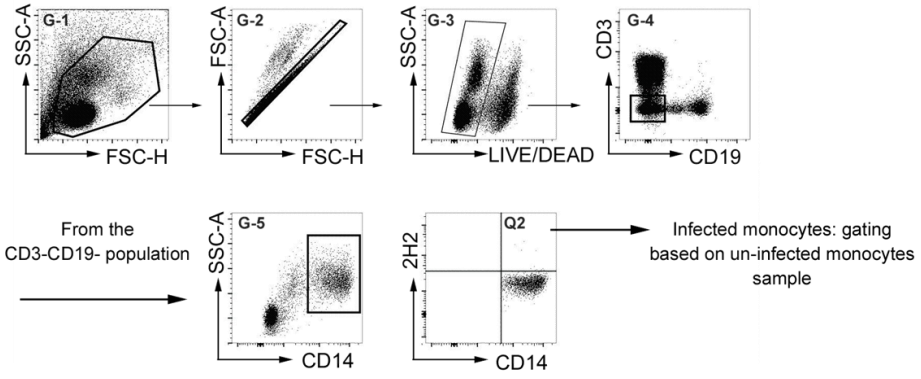
Figure 7. Specificity of the plasmablasts produced after stimulation with DENV-2.

4.2 SPECIFIC AIM 2 RESULTS

AIM 2: Determine the role of monocytes in promoting the *in vitro* expansion of plasmablasts in response to DENV stimulation.

4.2.1 Gating Strategy to define $CD14^+$ Monocytes

The target monocyte population is defined as $CD3^-CD19^-CD14^+$. The gating strategy shown in Figure 8 was used to look for DENV infected monocytes and to confirm that $CD14^+$ monocytes were depleted from the PBMC samples during the preceding experiments.



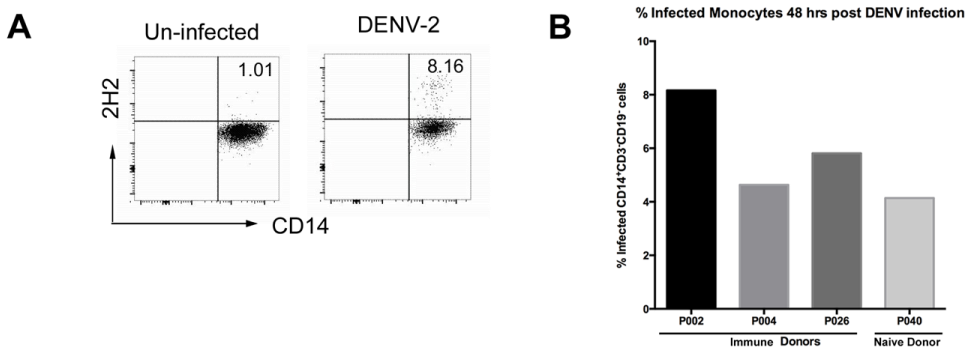
Representative gating for identifying monocytes using an un-stimulated PBMC sample. **G-1** gates on the total cells that have the appropriate Forward and Side Scatter which show they are neither dead cells nor debris. **G-2** gates on the single cells. **G-3** gates on the live cells. **G-4** $CD3^-CD19^-$ population. **G-5** gates on the $CD3^-CD19^-CD14^+$ monocyte population. **Q2** where the $2H2^+$ $CD14^+$ monocytes are found with the gating based on a control un-infected PBMC sample.

Figure 8. Gating strategy to define $CD14^+$ monocytes.

4.2.2 Detection of DENV infected monocytes using intracellular 2H2 antibodies

After exposing PBMCs from selected donors to DENV and culturing for forty-eight hours, a fluorescently labeled 2H2 pan-dengue antibody against for the prM protein was used to probe for the presence of intracellular virus. I detected a distinct population of monocytes, $CD3^{-}CD19^{-}CD14^{+}$, that were positive for 2H2 (Figure 9A). No other cell types, $CD3^{+}$ or $CD19^{+}$, were positive for 2H2 after forty-eight hours (data not shown) confirming previously published data which states that monocytes are the primary target cells for DENV infection [74, 75]. When PBMCs were stimulated with completely UV inactivated DENV, I did not detect a 2H2 positive monocyte population above background after 48 hours suggesting that this uptake of DENV is related to infection (data not shown). This is in-line with previous literature stating that monocytes and not T-cells or B-cells are the targets for infection [76].

After testing all three of our DENV immune donors and one DENV naïve donor for $2H2^{+}$ monocytes at forty-eight hours, we found that all donors had similar levels of $2H2^{+}CD3^{-}CD19^{-}CD14^{+}$ monocytes (Figure 9B). The frequency of this monocyte population is not a unique characteristic to DENV immune individuals which suggests that their monocytes are not uniquely susceptible to DENV infection. There was also no observable link between the frequency of the $2H2^{+}$ monocytes and the ability of the matched donors to produce a defined plasmablast response in culture.



(A) Representative dot plots of the un-infected PBMC control sample and the matched donor PBMC sample 48 hours post exposure to DENV-2. **(B)** Bar graph showing the percent of monocytes infected after 48 hours using PBMCs from 3 DENV immune donors and 1 DENV naïve donor

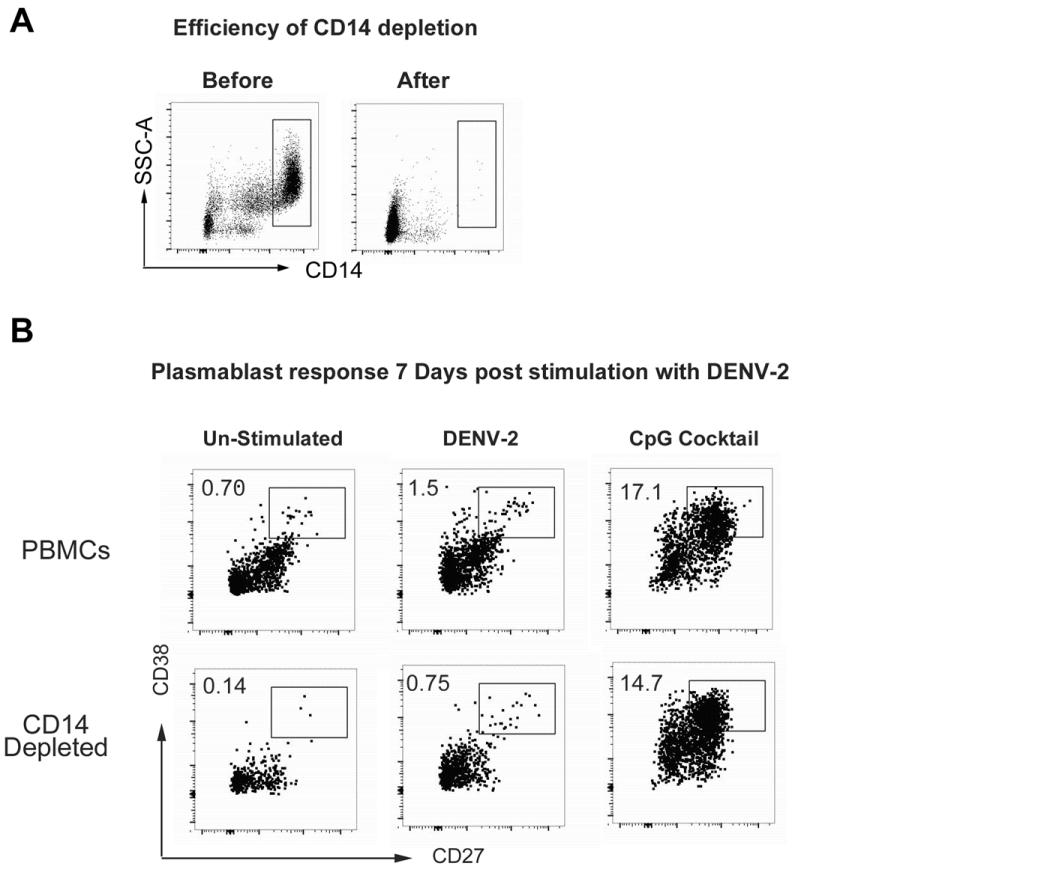
Figure 9. Detection of DENV infected monocytes by intracellular 2H2 antibodies

4.2.3 Plasmablast generation after $CD14^+$ depletion of DENV stimulated PBMCs

To directly measure the role monocytes play in the *in vitro* differentiation of plasmablasts, the $CD14^+$ cell population was depleted using magnetic beads before DENV stimulation. Figure 10A shows that the depletion of the $CD3^-CD19^-CD14^+$ monocyte population from PBMCs was >95 percent efficient. After seven days, there was a 50 percent reduction in the $CD19^+CD20^-CD38^{++}CD27^{++}$ plasmablast population in the $CD14^-$ PBMC sample compared to the PBMC sample (Figure 10B). This suggests that monocytes are not the only cell population important for the expansion of plasmablasts in culture, as the production of plasmablast in culture was not completely abolished. It is possible other cell types such as T-cells play an important role. However, this is difficult to confirm without further supporting evidence as the general plasmablast response in culture was very low.

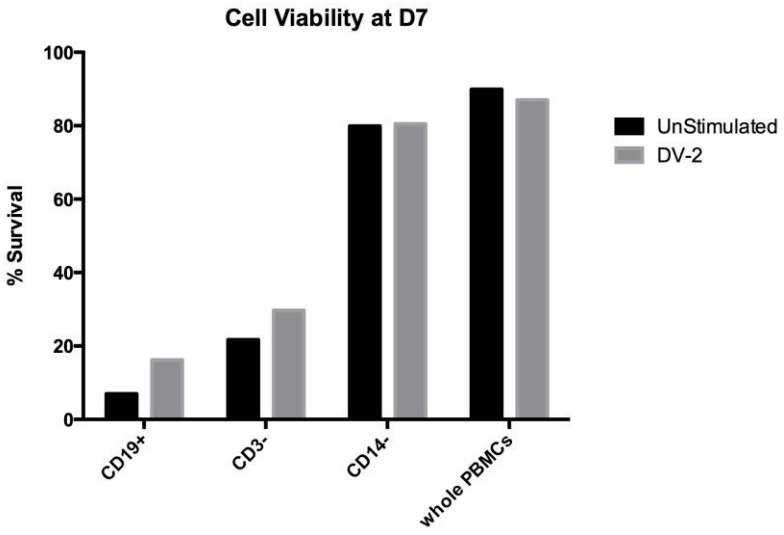
Previous experimental data using a $CD19^+$ purified cell population exposed to DENV did not produce a robust plasmablast response in culture (data not shown). An attempt to look at the

role of T-cells by depleting the $CD3^+$ population before stimulating with DENV was inconclusive; however, it was observed that the removal of $CD3^+$ cells dramatically affected the overall cell viability at day seven (Figure 11). It is known that isolated B-cells have poor viability in culture and the viability data suggests that $CD3^+$ cells may play an important role in the long-term survival of $CD19^+$ cells, as well as other cell types. Even though the frequency of infected monocytes does not seem to have a significant role in driving plasmablast production, the presence of $CD14^+$ cells does play a role in the distinctness of the population. Most likely, the monocyte population plays an important role in the survival, proliferation and differentiation of the B-cell population due to the secretion of cytokines such as IL-6 and IL-10 [77].



(A) Efficiency of depleting the $CD14^+$ cells from PBMCs using CD14 magnetic beads. **(B)** Top row shows the plasmablast response after stimulating PBMCs with DENV. The bottom row shows the plasmablast response after simulating PBMCs depleted of $CD14^+$ cells after DENV stimulation. **(C)** The percent reduction of plasmablasts between the whole PBMC and $CD14^+$ depleted PBMC samples.

Figure 10. Generation of plasmablasts in the absence of $CD14^+$ cells.



As expected, the $CD19^{+}$ population has poor viability while the whole PBMCs have over 80% viability after 7 days in culture. When CD3 is depleted from the PBMCs with viability although better than purified B-cells is still poor compared to CD14 depleted and whole PBMCs.

Figure 11. Viability of PBMCs/B-cells after 7 days in culture.

5.0 DISCUSSION

As outlined in the introduction, despite decades of research devoted to unraveling the pathogenesis of dengue, the immunological events driving DENV infections are still poorly understood. Relative to other intensely studied cell types, very little is known about the role plasmablasts play in the overall model of DENV pathogenesis. At the start of this project, it was unknown if this robust plasmablast response to DENV originated from the DENV specific memory B-cell subset or if it originated from a robust response driven by naïve B-cells. The overall goal was to tackle a part of this question by investigating the ability of DENV to drive memory B-cells from a previous DENV infection into IgG secreting plasmablasts *in vitro*, and to determine if this process occurs independently of other soluble or cellular factors.

Differentiation of plasmablasts after DENV stimulation *in vitro*

In this study, I first aimed to identify if DENV can drive a general production of plasmablasts in healthy donors that reached the robust levels observed in PBMCs isolated from acute patients. For this purpose, I isolated PBMCs from donors with pre-existing immunity to DENV and examined the neutralizing and binding ability of their serum antibodies to develop a profile of their spontaneous DENV specific ASCs. The purpose for this was to indirectly identify the potential cross-reactivity and specificity of the resting memory B-cell population present in PBMCs. I found that regardless of their neutralizing antibody profile, all donors produced an average plasmablast response that was stronger or equivalent when stimulated with DENV-2

versus DENV-3 *in vitro*. This suggests, for reasons unknown, that DENV-2 is a stronger stimulator *in vitro*, but this could be related to the epidemiological observations which suggest that DENV-2 is the more virulent serotype [46, 78]; hence, it is linked to more severe disease outcomes. It was interesting to note that donors with neutralizing antibodies to only DENV-3 produced a similar plasmablast response *in vitro* when exposed to DENV-2. This indicated that *in vitro* their general plasmablast recall response against the original infecting serotype was no better than against a heterotypic serotype.

When examining the overall potency of the plasmablast response in culture, the responses only made up ~1 to ~12 percent of the $CD19^{+}$ peripheral B-cell population, which is very low compared to the average 47 percent observed during clinical studies [67, 68]. However, this *in vitro* plasmablast response reached the low levels observed during vaccine studies with influenza virus or yellow fever virus vaccine [68]. Although *in vitro* DENV was not able to recapitulate the robust response observed during acute infection, DENV was able to drive a general plasmablast response that reached comparable levels seen in other viral infections in some donors. Therefore, this data suggests that overall the robust plasmablast response seen in acute patients may not completely depend on the reactivation of DENV specific memory B-cells, but may also rely on the plasmablast subset produced by the GCs formed in response to the ongoing infection. This hypothesis is also supported by the observation that when identifying the specificity of the plasmablasts during infection at their peak, they predominately secrete IgG against the current infecting serotype [67, 68].

I also examined the effects DENV had on donors without a DENV specific memory B-cell subset. According to these results, DENV could stimulate a plasmablast response in these donors comparable or greater than the individuals with pre-existing immunity. For reasons that I

did not further investigate, I could not decrease the level of this non-specific response to reach background or very low levels. However, I did establish that this response was not DENV specific. This response had evidence of a slight IgG ASCs response, but I did not test the IgM ASC population, which would have indicated if DENV was simply non-specifically activating the naïve B-cell population in these donors.

Stimulation of the quiescent DENV specific memory B-cells

There have not been many studies looking at DENV-induced activation of long-term memory B-cells and how this may drive them to differentiate into plasmablasts. Most investigators studying this population examine the Ig secreting cells during acute infections or isolate a few DENV specific memory B-cells from previously exposed donors to look at individual clones [69, 79, 80]. All these studies concentrate on the cross-reactivity of the antibodies secreted by these cells rather than how this circulating population may represent the long-term memory B-cell population present during the early stages of infection. It is important to work towards identifying the founding subset that drives the eventual potent plasmablast response seen four to seven days after infection. The specificity of the peak plasmablast response to the infecting serotype suggests that a primary response by naïve B-cells may play a role, but is it a skewed or strong cross-reactive memory B-cell population that drives the manifestation of this response.

To look at the frequency and the specificity of the DENV specific memory B-cells present in the PBMCs drawn directly from the donors, I first used a strong mitogen to reactivate B-cells in general. Then using an ELISPOT assay to detect the presence of ASCs that are DENV specific, I identified that all 3 DENV immune donors had higher than expected frequencies of DENV-specific memory B-cells, 0.72 to 3.70 percent of the IgG ASCs. A previous study using

the exact same mitogen cocktail looked into the frequency of long-lived memory B-cells specific for vaccinia virus $(VV)_{WR}$ in individuals who were vaccinated for small pox up to fifty years before the study. This study found that ~0.1 percent of the IgG secreting cells were VV-specific [73]. In a separate study, another group also identified the same frequency of tetanus-specific classed switched, memory B-cells using a different detection system [66]. The donors with previous clinical histories of DENV-3 infections produced, not surprisingly, a dominant frequency of DENV-3 specific IgG secreting cells. This data suggests that for reasons not yet fully investigated, DENV specific memory B-cells in individuals who have not been re-challenged with DENV for over a decade can maintain higher than average frequencies in circulation. In addition to this, the frequency of this population, although cross-reactive in nature, is still dominantly specific for the previous infecting serotype. If these individuals were re-infected with DENV and the heterotypic virus was able to reactivate even a small portion of the circulating memory B-cells, then the starting overall percent of re-activated DENV specific B-cells would be higher compared to the average recall response to another viral infection.

In order to further examine this population, I took this ELISPOT assay one step forward and looked for the frequency of the DENV specific IgG secreting cells after stimulating the same DENV immune donors with DENV. This aim was to detect if -as in acute patient samples- >70 percent of the IgG secreting plasmablasts produced *in vitro* were also DENV specific. The outcome observed was that when these donors were stimulated with DENV-2, only 0 to 5 percent of the plasmablasts produced in culture were specific for any DENV serotype. Not surprisingly, the donor with neutralizing antibodies against DENV-2 -hence previous exposure to DENV-2- was able to produce the highest frequency of DENV specific IgG ASCs. Unexpectedly, when looking at the frequency of DENV specific IgG ASCs in donors with

previous exposures to DENV-3, after their PBMCs were re-exposed to DENV-3, they still failed to produce any ASCs that were specific for DENV-3 (data not shown). Their overall DENV-2 response was also weaker, supporting the previous observation that in culture DENV-3 is the weaker stimulator of the two tested serotypes.

In conclusion, although DENV specific memory B-cells were reactivated by DENV to become DENV specific IgG secreting plasmablasts, this interaction alone is not sufficient to drive the robust plasmablast response observed in culture. Therefore, future study is needed. Such a study would look more closely at whether or not these plasmablasts have a skewed cytokine profile that may downstream influence how the naïve B-cells mount a plasmablast response in reaction to the infection serotype. This investigation would require a mixture of cell culture and animal model work. In cell culture, the phenotype of the plasmablasts produced after the memory B-cells differentiations can be examined while the dynamics between the cross-reactive recall response and the primary response to the heterotypic serotype should be examined with animals.

Monocytes are important in driving the differentiation of plasmablasts

As described in the introduction, monocytes are the primary targets of DENV infections both *in vivo* and *in vitro.* As the central cell believed responsible for driving severe disease manifestations [81], it is necessary to establish a possible link between the monocyte population and the differentiation of plasmablasts. To study this role, I first determined that after exposing PBMCs to DENV, monocytes become infected and the monocytes with previous DENV exposures were not more susceptible. Regardless of immune status, all donors tested had a similar frequency of infected monocytes forty-eight hours post stimulation. Using matched experiments, this frequency of infection did not seem to correlate to the ability of these

individuals to mount an above or below average plasmablast response. These results suggest that the efficiency of infection is not a sufficient factor, which is surprising since the ADE is based on the hypothesis that higher viremia, thus, more severe disease, is linked to a higher frequency of infected monocytes [48]. Using another approach to test the direct role of monocytes, $CD14^+$ cells were depleted from PBMCs before simulating with DENV. The population without $CD14^+$ had a 50 percent reduction in the amount of plasmablast produced compared to the whole PBMC control condition. These results suggest monocytes do play a role in the differentiation of plasmablasts in culture, but their removal isn't sufficient to eliminate the development of plasmablasts.

Recent studies have also found that monocytes are important in the development of plasmablasts *in vitro*. In this study, I only looked for monocytes in general, identifying them by $CD14^+$, but two groups have produced publications stating that monocytes infected with dengue favor a $CD14^+CD16^+$ inflammatory monocyte phenotype [77, 80]. This particular monocyte subset in culture secretes important cytokines such as IL-6, BAFF/APRIL and IL-10 which are cytokines known to promote activation, survival and proliferation in B-cells [77, 81]. *In vitro,* IL-6 is important for promoting survival. IL-10 is required for B-cells to proliferate; this cytokine also enhances the proliferation abilities of B-cells when their BCR is engaged [59]. BAFF and APRIL are the classic B-cell survival and differentiation factors [82, 83]. The study reference above showed that co-culturing DENV infection monocytes ($CD14^+CD16^+$) with purified B-cells drove the proliferation and differentiation of resting B-cells, particularly with the help of IL-10 and BAFF [77]. Therefore, in the absence of monocytes, particularly the infected monocyte population, these cytokines would not be readily available in culture assisting and driving the proliferation of B-cells upon reactivation. Hence the plasmablast population would be

lower, yet not abolished, as activation due to antigen-BCR engagements could be strong enough to drive differentiation yet not proliferation.

After observing that monocytes are not the only cell type important for driving the differentiation of plasmablasts after DENV stimulation, I ran a small study depleting $CD3^+$ cells. Although the experiment did not conclusively determine the role $CD3^+$ cells play, it is suspected that they play an important role in survival and help push the activated memory B-cells to differentiate. A review by Nature regarding immunological memory states that both naïve and memory B-cells both require cognate T-cell help, but the requirements are lower for a memory B-cell [62]. Therefore, it is likely T-cells are required for the regulation of initiating secondary responses. In addition, when comparing the overall viability of the cells with and without T-cells, it was evident that when CD3 cells were depleted the overall survival rate decreased drastically. Activated T-cells are primary producers of IL-2, which is an important survival cytokine for B-cells, as well as other cell types [84]. IL-2 was also added to the co-culture system described above suggesting that T-cell help is important. More testing needs to be completed to definitively establish the precise roles of each cell type, but previous research, as well as our own, indicates that the CD14 and CD3 cells are required for the production of DENV specific plasmablasts [77].

Overall Conclusions

As a whole these observations suggest that the DENV specific memory B-cell population is not solely responsible for the robust plasmablast response during acute infections. Most likely, the cross-reactive DENV specific memory B-cells reactivated during the early events of infection play their role either by the antibodies or cytokines they produce. At the worst, the antibodies produced early on during the infection could help drive ADE; at the best, they could provide

some protection until the primary response against the heterotypic infection kicks in. Future studies need to investigate if there is a link between first response plasmablasts originating from the original DENV specific memory B-cell subset and the primary responding B-cells. The resulting peak plasmablast response at day four to seven most likely consists of plasmablasts from both the secondary and primary immune responses. In addition to this, future studies need to investigate more definitively how monocytes influence plasmablasts during infections. *In vitro* studies suggest that the cytokines secreted by these cells during infection play an important role [77, 81].

To further examine these possibilities, the ability to draw blood samples long-term after recovery -early in a known secondary infection and at the critical points of peak plasmablast production- may help build a more complete profile. Such a profile could help determine if there is a connection between the DENV specific memory B-cell population before infection and the resulting plasmablast response. Unfortunately, this proves difficult because most patients do not present themselves to hospitals for treatment until the manifestation of symptoms, at which time the early events of interest have already taken place. The next best model is an animal model where there is a known history, known time of infection and known timeframe of responses. *In vitro* studies can be used in a closed system to parse out the factors that influence the phenotypes and the characteristics of the generated plasmablasts. Reconstruction of an environment that favors a high percent of DENV specific plasmablasts where either DENV specific memory B-cells or naïve B-cells with the potential to recognize the virus are exposed to DENV may provide clues as to how these cells fit into the overall pathogenesis model of DENV.

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