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Crystal Lane

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THE PLATELET-VIRUS INTERACTION: ANALYSIS OF HUMAN PLATELET
PHENOTYPE AND FUNCTION IN THE CONTEXT OF DENGUE VIRUS EXPOSURE

By

Crystal Lane
Master of Science

Graduate Division of Biological and Biomedical Science
Immunology and Molecular Pathogenesis

Guey Chuen Perng
Advisor

Aron Lukacher
Committee Member

Brian Evavold
Committee Member

Accepted:

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

Date

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ABSTRACT

THE PLATELET-VIRUS INTERACTION: ANALYSIS OF HUMAN PLATELET PHENOTYPE AND FUNCTION IN THE CONTEXT OF DENGUE VIRUS EXPOSURE

By: Crystal Lane

Dengue is a problematic mosquito-borne disease that is prevalent in most tropical and sub tropical countries. However, recent outbreaks in Key West, Florida, and Brazil serves as a vivid reminder that Dengue is an emerging public health threat to our continent. One of the hallmark complications in dengue virus infection is thrombocytopenia, or low platelet count. Dengue virus can interact directly with platelets, causing activation and subsequent release of soluble platelet factors. The experiments presented in this thesis explore the phenotypic and functional characteristics of human platelets in the context of prolonged dengue virus exposure *in vitro*. Specifically, analyses show that dengue virus exposed platelets display activation-related characteristics that differed from thrombin induced classical activation. Further experiments verified the release and function of biomolecule polyphosphate from dengue virus exposed platelets, which induced apoptosis in myeloma cells resulting in lower levels of antibody production. Together, these studies show that dengue virus exposure causes activation of platelets and subsequent release of a soluble factor that may be physiologically harmful. These findings may be important in linking dengue virus associated thrombocytopenia to the development of dengue pathogenesis in patients. These investigations also bridge the gap on how platelets may shape immunity in the course of infection and provide a new avenue to further understand human immunology during the course of infection.

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Chapter I: Introduction

Introduction:

Dengue is an acute, vector-borne disease that has become a major international public health concern over recent decades. The disease has increasingly spread throughout the tropical and sub-tropical regions of the world, predominantly in urban areas. According to the World Health Organization, severe dengue disease is the leading cause of childhood morbidity and mortality in some Asian countries (1). However, the on-going dengue outbreak in Key West, FL reminds us that it's spread is an immediate threat to public health in the United States (2).

Dengue disease is caused by infection with the dengue virus, which is primarily transmitted through bites of the *Aedes* mosquito. Infection can be asymptomatic or cause a variety of illnesses ranging from mild-fever to life-threatening complications such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Unfortunately, there is no specific drug treatment or vaccine against dengue virus infection (3).

Among the key clinical findings in dengue patients are thrombocytopenia (low platelet count), abrupt long-lasting fever, and high viremia despite the presence of neutralizing antibodies. Thrombocytopenia has been attributed to peripheral platelet destruction and/or reduction in production from megakaryocyte precursors. However, the association of these parameters has not been clearly demonstrated.

This thesis investigates the phenotype and function of platelets exposed to dengue virus directly. Understanding the role of platelets in dengue virus pathogenesis could potentially lead to the design of new therapeutic approaches to manage and perhaps prevent the more severe manifestations of dengue virus infection.

A. Overview of dengue

1. The dengue virus

The dengue virus belongs to the Flaviridae family of viruses along with others such as Japanese Encephalitis, West Nile and Yellow Fever viruses (4). The viral particle is composed of an icosahedral nucleocapsid encased by a lipid envelope. The viral genome is made-up of positive-sense single-stranded RNA approximately 11kB in length. Genomic RNA is translated as a single polyprotein, which is then cleaved by cell and viral proteases eventually yielding three structural proteins (Core, pre-Membrane, Envelope) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) (5, 6).

Several pathways have been linked to virus entry into cells. The leading conceptual pathway is believed to occur by receptor-mediated endocytosis and fusion at acidified endosomal membranes via the E-glycoprotein, however, the mechanism is not fully elucidated. Replication of dengue virus occurs exclusively within the cytoplasm of infected cells. Immature virions are initially assembled at the endoplasmic reticulum membrane, and matures as the particles are transported in vesicles through the trans-Golgi network to be released by exocytosis at the plasma membrane (5). However, the actual paths leading to the release of dengue virus from the infected cells remain unclear in spite of intense investigations.

There are four distinct serotypes of dengue virus – DENV-1, DENV-2, DENV-3, and DENV-4. All have been associated with outbreaks and are capable of co-circulating in endemic regions; however, DENV-2 has been the etiologic cause of recent outbreaks in the Americas. DENV-2 is also the most well-characterized strain of the viruses. Infection with one serotype provides long-lived homotypic immunity, while only providing transient or partial heterotypic immunity against the remaining serotypes (6).

2. Global burden of dengue disease

Dengue is one of the most common vector-borne infections with two-fifths of the world's population being at risk of infection (Figure 2). Worldwide it is estimated that 50-100 million infections occur annually, with over 500,000 cases of life-threatening disease, dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS), and over 22,000 deaths (3). Prior to 1970, only nine countries were reported to have DHF epidemics; however, the disease is now endemic in more than 100 countries (Figure 1). In 2007, there were over 800,000 reported cases of dengue in the Americas alone, with 26,000 cases of fatal DHF (3). The spread of dengue virus is attributed to multiple factors including urbanization, global travel, and the expanding distribution of its mosquito vectors (3).

3. Transmission of dengue virus

Dengue is transmitted to humans through the bites of *Aedes* mosquitoes, primarily the *Aedes aegypti* species, carrying the infectious dengue virus. Mosquitoes can acquire the virus while feeding on the blood of an infected person. It is generally believed that the virus incubates inside of a mosquito for eight to ten days; afterwards the insect is capable of transmitting the virus for the rest of its life (3, 6). However, evidence also suggests that the virus can be transmitted to a new host without an incubation period due to multiple feeding behavior of *Aedes* spp.

Infected humans are the main reservoirs of the dengue virus in urban cities. A newly infected person is viremic for two to seven days during which mosquitoes can acquire the virus during a blood-meal (6). Thus, the density of mosquitoes is the critical factor to account for the dengue outbreak and endemic.

4. Clinical features of dengue virus infection

Dengue virus infection can be asymptomatic or produce a spectrum of clinical illnesses ranging from mild dengue fever (DF) to potentially lethal complications of DHF or DSS. DF is a flu-like illness characterized by high fever, severe headaches, retro-orbital pain, muscle and joint pain, nausea, vomiting, and rash lasting approximately two to seven days. Upon subsequent heterotypic infection, patients are 40-80 times more likely to develop complications than patients with primary infections. DHF is defined by hemorrhagic manifestations, thrombocytopenia, often with hepatomegaly but rarely with splenomegaly, and plasma leakage. DSS is a more severe complication where extensive plasma leakage leads to prolonged shock and disseminated intravenous coagulation. The pathophysiology of severe dengue disease is poorly understood, but it is generally accepted that the complications are immune-mediated (3, 4, 6).

5. Treatment and prevention of dengue infection

Currently, there is no specific drug or vaccine against dengue virus infection. While DF is a self-limiting illness with a mortality rate of less than 0.01%, it is estimated that 25-37% of symptomatic cases will require hospitalization. DHF and DSS can be managed with proper supportive treatment and fluid balance. When treated appropriately the mortality rate for DHF can be reduced from approximately 20-40% to as low as 1% (3, 7).

Currently there are two front-runner vaccine candidates being tested in residents of endemic regions, however, the lag in development of an effective vaccine lies in the challenge to provide protection against all four viral serotypes. Moreover, there is limited understanding of the virus-host interactions due to the lack of a perfect laboratory animal model of dengue disease. To date the most effective prevention against dengue involves the proper control of the mosquito vector by elimination of open water sources and insecticide treatment of *Aedes* spp. larvae (3, 6).

B. Pathogenesis of dengue disease

1. Cellular targets of dengue virus

Dengue virus can infect many cell types *in vitro* including monocytes, macrophages, and dendritic cells. But *in vivo*, the cells accounting for the viremia remain elusive. However, in theory, cells with phagocytic ability such as platelets, monocytes, macrophages, and dendritic cells are considered the likely targets for viral replication and dissemination in an infected host. When activated these cells can release an abundance of biomolecules and pro-inflammatory mediators, which may in-turn promote vascular permeability, hypotension, and shock during dengue disease (8).

Many groups have demonstrated that *in vitro* infection of dendritic cells is far more permissive than infection of monocytes and macrophages. However the susceptibility of infection in monocytes and macrophages can be enhanced greatly in the presence of non-neutralizing antibodies. It has been postulated that, *in vivo*, enhanced viral replication occurs inside of macrophages during re-infections due to the presence of pre-existing dengue specific antibodies. Thus, an amplified re-infection could contribute to the complications associated with DHF and DSS. This phenomenon has been described as antibody-dependent enhancement (ADE) (8-10).

2. Complications of dengue virus infection: DHF

The mechanisms leading to severe dengue disease are not well-understood, but literature suggests immune-mediated pathogenesis. The primary pathophysiology observed in DHF is increased vascular permeability, which results in plasma leakage, rise of hematocrit concentration, and decreased blood pressure. While there is some *in vitro* evidence for dengue virus induced endothelial cell apoptosis, the vascular bed is not characterized by necrotic or inflammatory lesions *in vivo*. Furthermore the plasma leakage associated with shock is short-lived, indicating that the function of soluble

biomolecules or immune mediators may play a more critical role in the transient vascular permeability. Studies of acute dengue virus infection have revealed that patients who progress to DHF are likely to have high levels of plasma cytokines earlier in the course of infection as compared to asymptomatic dengue patients. Such indicator cytokines include IL-6, IL-1, and IFN γ (6, 9). However, the involvement of soluble biomolecules in plasma leakage has not been well investigated.

As for the immune mediators derived from immune cells, several components of the immune system seem to be important for dengue virus induced immunopathology. One thing to keep in mind is that dengue is a progressive disease and the time at which sample collection occurs may dictate the research outcomes. Nonetheless, it is thought that promiscuous activation of T-lymphocytes and enhancement of virus infection in macrophages via ADE produce abundant amounts of cytokines to promote systemic plasma leakage. However, there are other factors that may be associated with the plasma leakage, such as viral strains, individual's genetic background, and nutrition status. Moreover, the onset of plasma leakage begins during defervescence, several days after the peak viremia has been considerably reduced or cleared completely (4, 9). Altogether, these evidences suggest that there are unidentified factors circulating in the peripheral blood of dengue patients contributing to the plasma leakage.

One of such sources may be derived from activated platelets. Circulating platelets are the second most numerous peripheral blood cells in humans. Upon disease development, their counts drop from 200-300 $\times 10^6$ to under 10,000 per ml of blood. The amounts of biomolecules released from the activated platelets should be physiologically overwhelming, and yet its impact in the pathophysiology in disease development within the capillary micro-environment has not been the center of attention. This thesis

investigates the likely biomolecule(s) released from activated platelets that may contribute to the pathogenesis of dengue.

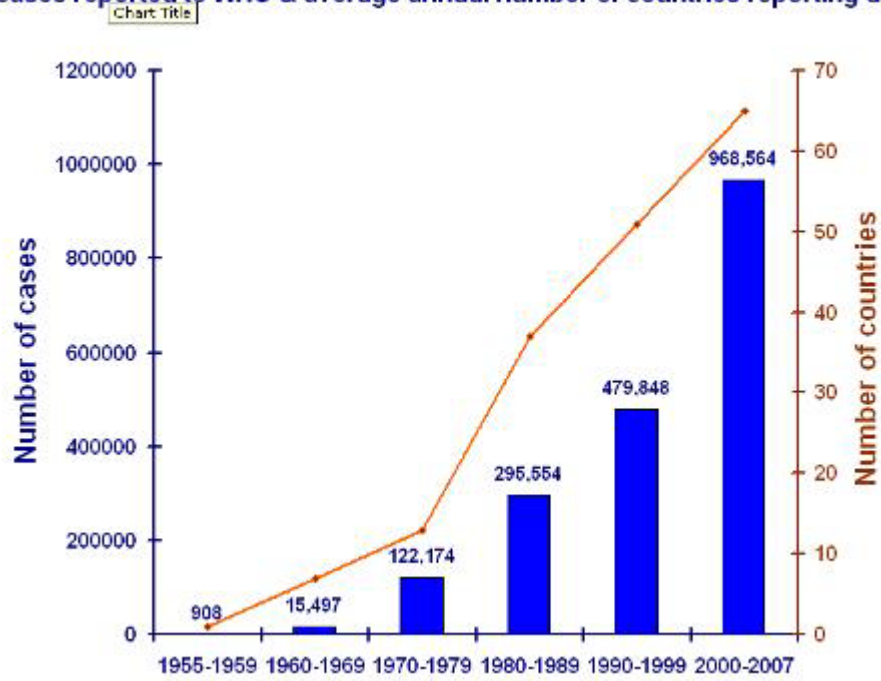
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Figure 1: Average annual number of DF/DHF cases reported to WHO, and average annual number of countries reporting dengue. During the 19th century, dengue was considered a sporadic disease that caused epidemics at long intervals, a reflection of the slow pace of transport and limited travel at that time. Today, dengue ranks as the most problematic mosquito-borne viral disease in the world. In the last 50 years, incidence has increased 30-fold. An estimated 2.5 billion people live in over 100 endemic countries and areas where dengue viruses can be transmitted. Up to 50 million infections occur annually with 500,000 cases of DHF and 22,000 deaths mainly among children. Prior to 1970, only 9 countries had experienced cases of DHF; since then the number has increased more than 4-fold and continues to rise (3)

Figure 2: Emergence of DEN/DHF. Prior to 1970, only 9 countries had reported cases of DHF; since then the number has increased more than 4-fold and continues to rise. Modified from (3).

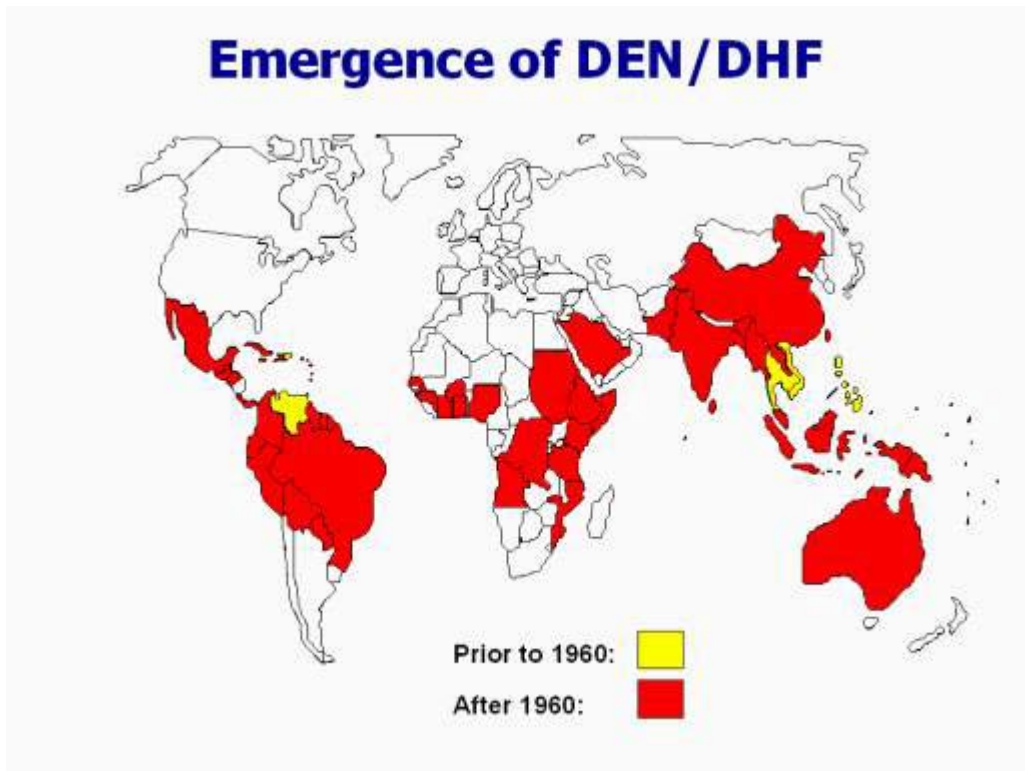
Figure 1.

Average annual number of DF/DHF cases reported to WHO & average annual number of countries reporting dengue



World Health Organization

Figure 2.



World Health Organization

**Chapter II:
Phenotypic Analysis of Dengue Virus Exposed Platelets**

Abstract:

Aberrant platelet activation and clearance is one of many proposed mechanisms leading to thrombocytopenia during DHF. Herein we show by monoclonal antibody staining and fluorescence-activated cell sorter (FACS) analysis that dengue virus exposed platelets upregulate surface glycoprotein CD41 and express degranulation markers CD62P, CD154, and CD107. These results are consistent with previous reports that dengue virus type-2 (DV2) exposure caused expression of CD62P (11). In contrast, fluorescence microscopy examination showed that DV2 exposed platelets did not display classical activation-related morphology as seen in thrombin activated cells. This phenomenon has been previously reported; in which platelet response to different reagents generate varied morphological changes. Taken together these experiments reveal that dengue virus exposure may cause sub-typical activation of platelets.

Introduction:

Thrombocytopenia, a common complication of viral infections, is a hallmark feature of dengue disease that is characterized by low blood platelet counts. There are several proposed mechanisms that contribute to dengue-associated thrombocytopenia: immunoglobulin-mediated destruction of platelets, hyper-activation and clearance of platelets, and impaired production of platelets. Despite the effort to understand this phenomenon, less research has focused on the direct interaction between dengue virus and platelets (12-18).

Platelets are small, anuclear cells that are derived from the cytoplasmic fragmentation of the megakaryocyte precursor cell in the bone marrow. Each megakaryocyte is capable of shedding 5,000 to 10,000 platelets, producing an average of 1×10^{11} platelets per liter of human blood (19).

Although small, platelets are primarily granulocytic, serving as a natural source of cytokines such as growth factors, chemokines, haemostatic factors, and pro-inflammatory molecules. As the primary mediators of hemostasis and thrombus formation, platelets circulate throughout the blood in an inactive state until they are exposed to damaged or activated endothelium or stimulants. Upon activation, granule contents are released into the surrounding extracellular space. There are three main types of platelet granules: dense bodies that contain molecules such as ATP, alpha-granules that are rich in anti-microbial peptides and clotting factors, as well as lysosomal-like granules. Platelets also undergo morphological changes when activated, developing pseudopodia which allows for aggregation and adherence to other platelets, leukocytes, and endothelial cells.

Recently, platelets have been suggested to play a more of active role in the innate immune response. Platelets have been demonstrated to internalize both bacteria

and viruses in addition to expressing functional toll-like receptors. Moreover, numerous platelet receptors have been implicated in virus binding, suggesting that platelets may be targets for infection as is the case with many other leukocytes (20-23).

Several lines of evidence have confirmed that dengue virus can interact directly with human platelets. *In vitro* experiments have revealed that platelets exposed to dengue virus undergo activation-related morphological changes as well express P-selectin (CD62P) (11). More importantly, electron microscopy studies have discovered the presence of dengue viral-like particles inside of platelets isolated from the blood of dengue patients (24).

Experiments here show that platelets with prolonged exposure to dengue virus express surface activation markers CD62P, CD107b, and CD154 as detected by monoclonal antibody staining and fluorescence-activated cell sorter (FACS) analysis. However, the level of activation is sub-maximal compared to thrombin activated platelets. Furthermore, dengue virus type-2 (DV2) exposed platelets did not display the characteristic activated morphology. Thus, dengue virus exposure may cause only sub-typical platelet activation. The significance of the differential phenotypes in activated platelets is currently unknown. The experiments presented here may provide important information about the interaction between platelets and dengue virus.

Materials and methods:

Cell lines: Vero cells were grown in complete medium [RPMI 1640 medium (GIBCO) supplemented with 10% fetal-calf serum (FCS), 10mM L-glutamine, 100 IU penicillin, and 100 ug/mL streptomycin]. All cells were incubated in a humidified tissue culture chamber at 37°C and 5% CO₂.

Isolation of human platelets: Human blood was collected by venipuncture in a 3.2% buffered sodium citrate solution. Platelets were isolated on an iodixanol barrier (Optiprep) according to the manufacturer's protocol. Briefly, five volumes of Optiprep (Axis-Shield) were diluted with twenty-two volumes of diluent (.85% (w/v) NaCl, 20 mM Hepes-NaOH, 1 mM EDTA, pH 7.4) to produce a 1.063 g/mL solution. In a 15 mL conical tube, 4-5 mL of blood was layered over 5 mL of the 1.063 g/mL solution and centrifuged at 350g for 15 minutes at 22°C in a swinging-bucket rotor with no brake. The plasma layer and underlying platelet band were harvested separately. The platelets were then washed once with sterile phosphate-buffered saline or PBS pH 7.4 by centrifugation at 1200 rpm for 5 minutes at 22°C and discarding the supernatant.

Platelet activation: Isolated platelets were activated with human alpha-thrombin (thrombin) (HTC). Cells were incubated with a high concentration (10 U/mL) of thrombin in complete medium for at least 30 minutes in a tissue culture chamber.

Virus strain and titration: Dengue virus type-2, strain 16681 (a gift from Dr. Gubler, University of Hawaii Asia-Pacific Institute of Tropical Medicine and Infectious Diseases) was used in all experiments. Titration of virus stock was carried out by foci-assay in Vero cells (a permissive cell line). To prepare a monolayer of cells for infection, Vero cells were plated at a density of 2×10^4 cells per well in a total volume of 100 uL in 96-well microtiter plates and incubated overnight in a tissue culture chamber. Serial 10-fold dilutions (10^{-1} to 10^{-7}) of the virus stock were prepared in complete MEM medium

(GIBCO). 70 uL of media was removed from each well and replaced with 50 uL of the diluted virus suspension. The plates were then incubated in a tissue culture chamber for 2 hours. Afterwards, 100 uL of overlay medium (MEM supplemented with 2% FCS, 2 mM L-glutamine, 1.2% penicillin-streptomycin, 2% methylcellulose) was added to each well. The plates were then incubated in a tissue culture chamber for 2-3 days or until cytopathic effect could be observed. To stain foci, the cell monolayer was washed three times with PBS, fixed with 3.7% formaldehyde/PBS, and permeabilized with 1% Triton X-100 (Fisher). 50 uL of primary antibody, 4G2 monoclonal antibody culture supernatant, was added to each well and incubated in a tissue culture chamber for 30 minutes. Wells were then washed three times with PBS to remove unbound antibody. Next 50 uL of the secondary antibody, rabbit anti-mouse immunoglobulin (Ig) conjugated to horse-radish peroxidase (Dako); diluted 1:1000 in PBS-T (PBS supplemented with .05% Tween-20 (Fisher) and 2% FCS) was added to each well. The secondary antibody was incubated for 30 minutes in a tissue culture chamber. Wells were then washed three times with PBS-T to remove residual antibody. Lastly, 50 uL of DAB substrate (Fisher) was added to each well and allowed to develop for 10-15 minutes at room temperature. Plates were rinsed with tap water and allowed to air dry. Infected foci were counted under a light microscope and the focus-forming unit per milliliter was calculated.

Dengue virus exposure: Cells were resuspended in 200 uL of complete medium containing the appropriate concentration of virus for the number of cells used. The cell-virus suspension was incubated in a 15 mL conical tube in a tissue culture chamber for 2 hours. Afterwards, cells were washed twice with media by centrifuging at 1200 rpm for 5 minutes at 22°C and discarding the supernatant.

Antibody labeling for FACS and fluorescence microscopy: Diluted whole blood or isolated platelets were stained with a panel of cell surface markers and subjected to

FACS analysis. Multicolor FACS analysis was performed on an LSRII machine (BD Biosciences) using BD FACSDiva software (BD Biosciences). Subsequent data analysis was performed with FlowJo software (TreeStar). The following fluorescent antibodies were used for cell surface staining: CD61-APC (Caltag), CD61-FITC (Geneway), CD41-A647 (Serotec), CD62P-PE (BD Pharmingen), CD154 (5C8 clone), and CD107b (Southern Biotech). The CD154 and CD107b antibodies were labeled using the Zenon Mouse IgG A488 Labeling Kit (Invitrogen) according to manufacturer's protocol. Platelets were labeled with .1-.25 ug of each antibody in 100 uL of PBS supplemented with 2% FCS for 20 minutes at room-temperature. Next cells were fixed with 1% formalin for 15 minutes at room-temperature. If FACS was not performed right away, cells were kept at 4°C until analysis.

Results:

DV2 exposed platelets upregulate surface glycoprotein CD41 and express degranulation markers CD62P, CD154, and CD107b.

Although it is speculated that dengue virus may directly contribute to aberrant platelet activation during infection, little work has been done to characterize the phenotype of dengue exposed platelets. A study by Gnosh et al. demonstrated that after 30 minutes, platelets exposed to DV2, but not Japanese encephalitis virus, showed activation-related morphological changes (11). However, the experiments shown here characterize the change in surface markers and morphology of DV2 exposed platelets over extended periods of time. Monoclonal antibody labeling and FACS of non-permeabilized cells was used to analyze DV2 induced changes in platelet surface markers. Platelets freshly isolated from human whole blood was exposed to DV2 (MOI 1), thrombin (10 U/mL), or complete medium (mock) for various lengths of time. FACS analysis showed that at 2 hours, CD41 expression was 1.6 times higher on DV2 exposed cells than on mock exposed cells. In comparison, thrombin activated cells expressed 4.6 times more CD41 than mock cells (Figure 1a). The expression of CD62P, an alpha-granule membrane protein, was also increased among DV2 exposed and thrombin activated cells, 1.6 and 9.3 times higher than mock, respectively (Figure 1b). These trends were consistent at 1, 6, 9, and 12 hours post-exposure (data not shown). Platelets were also labeled for additional degranulation markers, CD154 and CD107b (present on lysosomes and dense bodies). DV2 exposed cells showed an average 4.6 fold increase in surface CD154 expression up to 12 hours post-exposure when compared to cells fixed immediately after isolation. However, when compared to mock cells, there was no difference in the average CD154 expression at 2 hours post-exposure and slightly higher levels of CD154 expression at 12 hours post-exposure.

Thrombin activated cells displayed an initial spike and subsequent decrease in surface CD154 expression, which is consistent with what is known about CD154 shedding when platelets are activated with high doses of thrombin (Figure 2a). The average CD107b expression on DV2 exposed cells was comparable to mock cells over time. In contrast, thrombin activated platelets displayed higher levels of CD107b expression at 2 hours post-exposure compared to mock cells, but this difference was not maintained over time (Figure 2b). The results here suggest that DV2 exposure causes platelet degranulation via a mechanism different from that of thrombin exposure. This can be confirmed with morphology of DV2 exposed platelets, which have a noticeable morphology with milder difference to resting cells.

Morphology of DV2 exposed platelets.

To visualize platelet morphology, freshly isolated cells were labeled with a monoclonal antibody against the CD61 surface glycoprotein. Fluorescence microscopy examination showed that at 2 hours post-exposure the morphology of DV2 exposed cells was slightly different from cells fixed immediately after isolation. In contrast, thrombin activated cells were characterized by membrane extensions (Figure 3). These observations indicate that DV2 exposed platelets, despite upregulating activation markers (Figures 1-2), do not undergo classical activation after 2 hours of DV2 exposure.

Discussion:

Thrombocytopenia is a hallmark hematological feature of dengue disease. There have been multiple hypotheses explaining the mechanisms contributing to low platelet counts including aberrant activation and clearance of platelets as well as decreased thrombopoiesis. Although it has been known that viruses could interact with platelets, the direct effect of dengue virus exposure on platelet phenotype and function has not been studied in detail.

Prior work has demonstrated that dengue viral-like particles could be visualized inside of platelets isolated from the blood of dengue patients and that infectious virus was recovered from co-cultivation of the platelets with a permissive cell line (24). Furthermore, electron microscopy studies have revealed that dengue virus exposed platelets displayed activation-related morphological changes as well as increased CD62P expression detected by FACS (11).

The pilot experiments discussed here used a more detailed FACS analysis to evaluate the presence of platelet activation markers in the context of prolonged dengue virus exposure. Overall, dengue exposed platelets expressed surface CD62P, CD107b, and CD154 albeit in lower levels than thrombin activated platelets. However, fluorescence microscopy did not reveal classical activation-related morphological changes in DV2 exposed platelets. Overall no definitive conclusions can be made since these experiments do not distinguish between background activation of platelets possibly due to cell manipulation and the possibility of delayed cell activation due to DV2 exposure. Furthermore advanced microscopy techniques, such as electron microscopy, are needed to provide superior insight into exposure related morphological changes. Lastly, there was a great amount of individual variability between experiments that may have been masked when platelets were pooled together. Thus, in future studies,

improved blood donor sampling will be needed to reduce confounding variables between donors.

Figure Legends

Figure 1: DV2 exposed platelets upregulate surface glycoprotein CD41 and degranulation marker CD62P. FACS analysis of CD41 and CD62P expression on non-permeabilized platelets exposed to DV2 (MOI 1), thrombin (10 U/mL), or complete medium (mock) for 2 hours and then stained with anti-CD41-A647 or anti-CD62P-PE. A) The fold increase in mean CD41 expression of each exposure group. B) The fold increase in mean CD62P expression of each exposure group. The results are expressed as MFI (Mean Fluorescence Intensity), which is an arbitrary value expressing the fluorescence intensity. The mean of CD41 and CD62P in each group is shown as a histogram bar. The results are representative of two independent experiments performed in triplicate where each experiment utilized pooled platelets from 3 blood donors.

Figure 2: DV2 exposed platelets upregulate degranulation markers CD154 and CD107b. FACS analysis of CD154 and CD107b expression on non-permeabilized platelets exposed to DV2 (MOI 1), human α -thrombin (10 U/mL), or complete medium (mock) for 2 or 12 hours and then stained with anti-CD154-A488 or anti-CD107b-A488. A) The mean CD154 expression of each exposure group. B) The mean CD107b expression of each exposure group. The results are expressed as MFI (Mean Fluorescence Intensity), which is an arbitrary value expressing the fluorescence intensity. The mean of CD154 and CD107b in each group is shown as a histogram bar. The results are representative of three independent experiments performed in triplicate where each experiment utilized platelets from 1 blood donor.

Figure 3: Morphology of DV2 Exposed Platelets. Fluorescence microscopy showing the morphology of platelets exposed to DV2 or thrombin for 2 hours and then labeled with anti-CD61-FITC. As a control, platelets were fixed immediately after isolation. Cells were mounted onto glass slides via single cell suspension droplet. Platelets were

visualized at 100X in oil immersion. CD61: green, DAPI: blue

Figure 1.

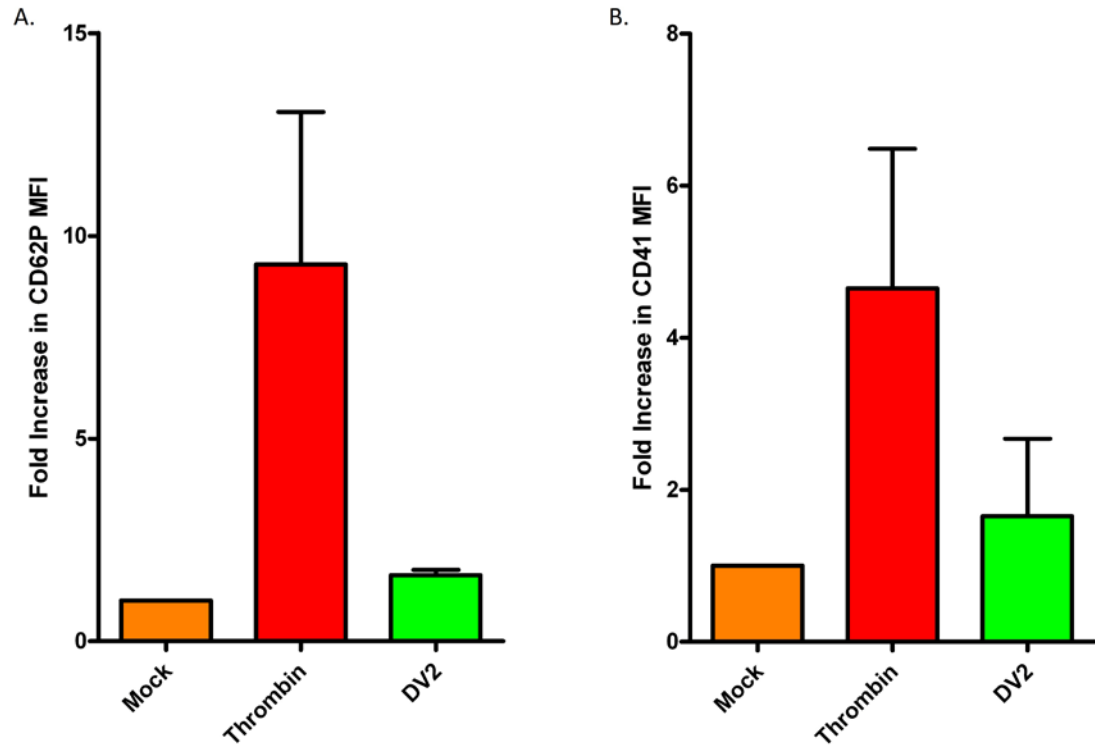


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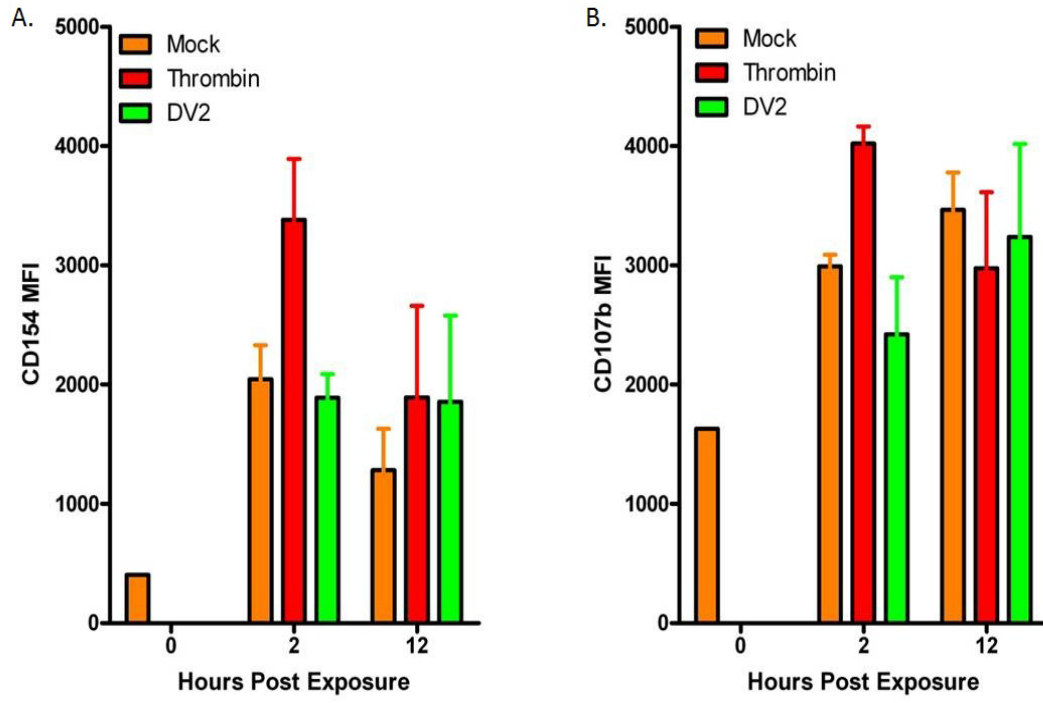
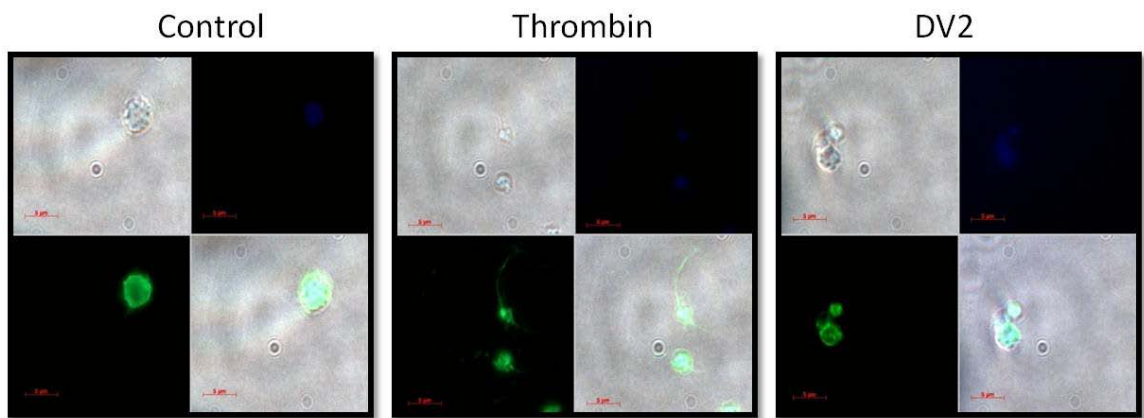


Figure 3.



**Chapter III:
Functional Analysis of Dengue Virus Exposed Platelets:
Secretion of Soluble Mediators**

Abstract:

Platelets are an abundant source of soluble factors including various vasoactive components, biomolecules, chemokines, and inflammatory cytokines. Experiments here show by metachromatic reaction and enzyme-linked immune-absorbent assay (ELISA) analysis that dengue virus exposed platelets secrete biomolecule, polyphosphate, which inhibits myeloma cell immunoglobulin (Ig) secretion. These results are consistent with previous reports that synthetic inorganic polyphosphate is toxic to plasma and myeloma cells (25). Taken together these experiments reveal that dengue virus interaction with platelets cause the release of factors that are potentially pathogenic.

Introduction:

Severe dengue disease is characterized by short-lived vascular permeability. The common belief is that hyperactivation of immune cells results in an increase in soluble inflammatory mediators. Despite the presence of thrombocytopenia, little research has focused on the contribution of dengue exposure on the release of platelet-derived soluble factors. Here we characterize the function of polyphosphate released from dengue virus exposed platelets.

Platelets release an abundance of bioactive molecules important for hemostasis and inflammatory responses. These soluble factors include various vasoactive components, chemokines, and pro-inflammatory cytokines. Platelet-derived polyphosphate has recently been described as a new class of mediator having a role in platelet-driven inflammation. Muller et al., was able to demonstrate that platelet-derived polyphosphate led to fibrin formation and vascular leakage in mice (26). Moreover, it has been previously demonstrated that synthetic polyphosphate can inhibit Ig secretion and stimulate apoptosis in plasma cells and malignant plasma cell (myeloma) lines (11).

Polyphosphate is an inorganic polymer of 60-100 phosphate residues linked by phosphoanhydride bonds. Polyphosphate is abundant in nature and has been studied extensively in prokaryotes and lower eukaryotes where it functions in metabolism, stress responses, and as a structural component. Despite its widespread presence, not much is known about the *in vivo* role of polyphosphate in mammalian cells. Moreover, it is reported that the dense granules of platelets contain 10-20 times more polyphosphate than other human tissues (25-27).

The following experiments examined the effect of dengue virus exposure on the release and function of platelet-derived polyphosphate. To detect polyphosphate, platelet culture supernatants were reacted with a toluidine blue (TBO), while functionality

of platelet-derived polyphosphate was determined by its effects on U266 myeloma cells. Experiments shown here demonstrated that DV2 exposure leads to the release of polyphosphate from platelets. Moreover, myeloma cells cultured in medium containing platelet-derived polyphosphate secreted less IgE. Altogether these studies reveal that dengue virus exposure causes platelets to release soluble factors, which in turn may contribute to the complications observed during severe dengue disease.

Materials and Methods:

Cell lines: U266 myeloma cells (a gift from Dr. Ehrhardt, Emory University School of Medicine) were grown in complete medium [RPMI 1640 medium (GIBCO) supplemented with 10% FCS, 10mM L-glutamine, 100 IU penicillin, and 100 ug/mL streptomycin]. All cells were incubated in a humidified tissue culture chamber at 37°C and 5% CO₂.

Detection of polyphosphate: A metachromatic assay with toluidine blue (TBO) was used to quantify polyphosphate. The TBO method was based on decrease in absorbance at 620 nm by metachromatic reaction of TBO solution with polyphosphate. Briefly, platelet culture supernatant was diluted 1:3 in carbonate/bicarbonate buffer (3.03 g Na₃CO₃, 6 g NaHCO₃, 1L H₂O, pH 9.6) and distributed as 100 uL per well of a 96-well plate. 100 uL of TBO assay solution (.05 mg/mL TBO in .1N acetic acid) was added to each well, incubated for 15 minutes, and the OD 620 nm was measured within 30 minutes.

Indirect co-culture assay: An indirect co-culture assay was used to investigate the function of platelet-derived polyphosphate. Platelets were separated into the following exposure groups: medium only, thrombin (10 U/mL), and dengue virus (MOI of 1). All platelet samples were adjusted to a concentration of 1 x 10⁷ cells/mL and incubated in a tissue culture chamber. Cell-free supernatant was collected at 2 or 12 hours post-exposure. All supernatant was stored at -80°C until further use. U266 myeloma cells were cultured overnight in the platelet-derived cell-free supernatant (platelet supernatant) at a concentration of 1-2 x 10⁵ cells/ in 96-well microtiter plates. The indirect ratio of platelets to U266 myeloma cells was 10:1. The samples were set-up as such in triplicate:

Sample	Stimulants		
U266 + platelet supernatant (2hr)	Medium only	Thrombin	Dengue virus

U266 + platelet supernatant (12hr)	Medium only	Thrombin	Dengue virus
U266 controls (fresh medium)	Medium only	Thrombin	Dengue virus

Secretion of IgE: U266 myeloma cell IgE secretion was determined by direct enzyme-linked immune-absorbent assay (ELISA). After culturing U266 myeloma cells overnight, cell-free supernatant was collected and transferred to a new microtiter plate for analysis. 100 uL of undiluted supernatant was coated onto wells for 4 hours in a tissue culture chamber. Wells were then washed twice with PBS. The remaining protein-binding sites in the coated wells were blocked by the addition of blocking buffer (5% nonfat dry milk/PBS) for at least 2 hours at room temperature. Excess blocking buffer was removed by washing the wells twice with PBS. Next, 100 uL of mouse anti-human IgE monoclonal antibody conjugated to horse-radish peroxidase (Southern Biotech), diluted 1:500 in blocking buffer, was added to each well and incubated for 2 hours in a tissue culture chamber. Excess antibody was removed by washing the wells thrice with PBS-T. To detect bound antibody, 100 uL of TMB substrate (Fisher) was added to each well. After sufficient color development, 100 uL of stop solution (.16M sulfuric acid) was added to each well. The optical density of each well was read at 405 or 450 nm using a spectrophotometer (BioTek).

Giemsa Staining: Bright-field microscopy was used to visualize and count cells with fragmented DNA and apoptotic bodies. Briefly, U266 cells were mounted onto glass slides via cytopspin and fixed in methanol for 5 minutes. The cells were covered with dilute Giemsa stain (1:20 in deionized water) for one hour then rinsed with water. Cells were visualized at 100X in oil-immersion.

Annexin-V labeling for FACS: Apoptosis was determined by cell labeling using an annexin-V-FITC apoptosis detection kit (BD Pharmingen) according to the

manufacturer's protocol. Briefly, triplicate samples were pooled together, washed with PBS, and resuspended in 100 uL of 1X annexin-V binding buffer with 5uL of annexin-V-FITC per sample. The sample was incubated for 15 minutes at room-temperature, washed with PBS, and fixed in 2% formaldehyde/PBS until analyzed by FACS.

Results:

DV2 exposed platelets secrete bioactive polyphosphate into supernatant.

Experiments were initially performed using pooled platelets from multiple donors. Supernatants from DV2 exposed platelets, on average, bound 50% more TBO than control supernatants. Likewise supernatants from thrombin activated platelets, on average, bound 40% more TBO than control supernatants. These results indicate that polyphosphate is being secreted from platelets exposed to DV2 (Figure 1). Furthermore, U266 myeloma cells cultured in DV2-exposed or thrombin-activated platelet supernatant secreted on average 50% less IgE than cells cultured in mock-exposed platelet supernatant (Figure 2). IgE secretion was not affected by the presence of DV2 or thrombin alone (data not shown).

To assess the variability of an individual's difference in response to dengue virus exposure, experiments were also performed using single donor exposed to various stimuli over time. Here, supernatants from DV2 exposed platelets, on average, bound 10-12% of TBO. In contrast, thrombin-activated platelet supernatants, on average, bound up to 22% TBO. There was some reactivity of the 12 hr culture of mock-exposed platelet supernatant with TBO (Figure 3). This observed reactivity in mock samples is probably indicative of basal level activation of platelet. However, it has been demonstrated that platelets are capable of secreting soluble factors independent of classical activation (28).

The detection of polyphosphate loosely corresponded with an observed inhibition of IgE secretion. U266 myeloma cells cultured in supernatant from DV2 exposed platelets secreted approximately 40-60% less IgE than control samples (U266 culture supernatant). This observed inhibitory effect was dependent on time of DV2 exposure. However, the inhibition was comparable to what was observed from U266

cells cultured in mock-exposed platelet supernatant. In contrast, cells cultured in supernatant from short and long-term thrombin activated platelets secreted on average 60% less IgE than control samples (Figure 4). IgE secretion was not inhibited by the presence of thrombin or DV2 alone (data not shown).

Together these results indicate that DV2 exposed platelets release bioactive polyphosphate and are consistent with previous reports that synthetic inorganic polyphosphate inhibits U266 myeloma cell function. In these experiments, there may be some basal level of polyphosphate release, but it does appear that polyphosphate release is enhanced by DV2 exposure. However we cannot rule out the possibility that the experimental manipulation of platelets induces low levels of activation and other soluble factors released from activated platelets may have an enhancement effect.

Apoptosis of U266 Myeloma Cells.

It has been shown that inorganic polyphosphate can specifically induce apoptosis in plasma and myeloma cells. Thus, Giemsa stain or annexin-V staining was used to assess the amount of apoptosis in U266 myeloma cells after culture in platelet supernatants from aforementioned exposure groups. Giemsa staining of pooled platelets demonstrated that supernatant from DV2-exposed and thrombin-activated platelets induced apoptotic body formation in U266 myeloma cells, which corresponded to the increase in polyphosphate detection as well as the decrease in IgE secretion. DV2 exposure alone or mock supernatant did not affect cell viability (Figure 5). Moreover, FACS results indicated that the percentage of increase in annexin-V MFI corresponded to the increase in polyphosphate detection as well as the decreased in IgE secretion (Figures 3, 4, 6). The percentage of annexin-V positive cells in all experiments did not surpass 10% (data not shown), suggesting that the contribution of cell death to the decrease in the observed IgE secretion in plasma cells warrants more investigations.

Discussion:

Platelets, anuclear cells, are the second most abundant cells circulating in peripheral blood of human body. Although the known role of platelets is maintenance of hemostasis, recent knowledge reveals that platelets can participate in inflammation by sharpening adaptive immunity; however, their contribution to these events largely remains unexplored (29).

Dengue is one of the most problematic vector-borne human diseases. One of the hallmarks of the clinical manifestations is thrombocytopenia or low platelet count. The low platelet count can result from the direct engagement of the dengue virus with platelets. Moreover, blood transfusion incidents reveal that the involvement of dengue virus in platelet function is a very early event, prior to the onset of clinical signs (30). The early engagement of dengue virus with platelets may be one of the mechanisms by which the virus evades the host immune system since majority of populations in endemic regions are serological positive for dengue antigens. Furthermore, the interaction of dengue virus with platelets likely alters the functions of the platelets. One of such functional changes is activation, resulting in the release of biomolecules into the surrounding.

Since plasma cells are the dominant source of antibody production, the quantity and quality of the antibody is very important in pathogen control (31). The quality and quantity of antibody production in plasma cells can be influenced by several factors, in particular within the microenvironment of capillary. One of the intriguing clinical pathological findings from postmortem autopsy is that majority of specimens indicate that few inflammatory events occur in spleen or lymph node, suggesting that the involvement of secondary immune system may be hampered by dengue virus infection. However, the causes of these observations are very difficult to investigate and remain largely

unknown, primarily because there is no animal model that perfectly mimics the cardinal features of dengue disease. Importantly, plasma cells in dengue virus infection have not been studied well. Although antibodies from dengue affected individuals show a wide range of cross-reactivity, they are largely non-neutralizing. The wide-range cross-reactivity property of dengue antibodies may be due to a defect in plasma cells.

With the observation that human platelets contains high amounts of polyphosphates in its storage pool and that inorganic polyphosphate is capable of selectively inducing apoptosis in plasma cells, this thesis set out to investigate the biomolecules released from the interaction of platelets with dengue virus *in vitro*.

The results presented here indicated that the biomolecules, likely polyphosphate, released from platelets exposed to dengue virus, had an effect on malignant plasma (myeloma) cells, which include inhibition of antibody production and induction of apoptosis observed by bright-field microscopy. However, the levels of inhibition of antibody production did not correlate with annexin-V staining in myeloma cells. Since the use of annexin-V staining assays only detects the early apoptosis; the cells could still be functional. Thus, the inclusion of a secondary marker could have discriminated between the early and late apoptotic cells; allowing for a detectable correlation between cell death and apoptosis.

Figure Legends:

Figure 1. DV2 Exposed Platelets Secrete Polyphosphate into Supernatant.

Polyphosphate was detected using the TBO method. TBO solution was mixed with supernatants from platelets exposed to DV2 (MOI 1), thrombin (10 U), or complete medium (control) for 12 hours. The OD was read at 620 nm. The results are expressed as the percentage of increase in polyphosphates, compared to the control group. The mean percentage of increase in polyphosphate for each group is shown as a histogram bar. The results are representative of one experiment performed in triplicate using pooled platelets from 3 blood donors.

Figure 2. DV2 Exposed Platelet Supernatant Reduces U266 Myeloma Cell IgE

Secretion. IgE secretion was detected by direct ELISA of U266 myeloma cell supernatant after overnight culture in cell-free platelet-derived supernatant. Supernatant was derived from platelets exposed to either DV2 (MOI 1), thrombin (10 U), or complete medium (control) for 12 hours. The results are expressed as percentage of antibody production, with the control supernatant set to 100%. The mean percentage of antibody production for each group is shown as a histogram bar. The results are representative of one experiment performed in triplicate using pooled platelets from 3 blood donors.

Figure 3. DV2 Exposed Platelets Secrete Polyphosphate into Supernatant Over

Time. Polyphosphate was detected using the TBO method. TBO solution was mixed with supernatants from platelets exposed to DV2 (MOI 1), thrombin (10 U), or complete medium (mock) for 2 and 12 hours. TBO solution was used as a control. The OD was read at 620 nm. The results are expressed as the percentage of increase polyphosphate, compared to the control group. The mean percentage of increase in polyphosphate for each group is shown as a histogram bar. The results are

representative of 3 independent experiments performed in triplicate each using platelets from 1 blood donor.

Figure 4. DV2 Exposed Platelet Supernatant Reduces U266 Myeloma Cell IgE

Secretion. IgE secretion was detected by direct ELISA of U266 myeloma cell supernatant after overnight culture in cell-free platelet-derived supernatant. Supernatant was derived from platelets exposed to either DV2 (MOI 1), thrombin (10 U), or complete medium (mock) for 2 and 12 hours. U266 myeloma cell culture supernatant was used as a control. The results are expressed as percentage of antibody production, with the control supernatant set to 100%. The mean percentage of antibody production for each group is shown as a histogram bar. The results are representative of 3 independent experiment performed in triplicate each using platelets from 1 blood donor.

Figure 5. Analysis of Apoptosis by Giemsa Stain. U266 myeloma cells were cultured overnight in supernatant from platelets exposed to DV2 (MOI 1), thrombin (10 U), of complete medium (mock) for hours. U266 myeloma cells cultured in fresh medium was used as a control. The effect of DV2 on cell viability was also assessed. Giemsa stain was used to visualize DNA fragmentation and apoptotic body formation. A) Bright-field microscopy image of U266 myeloma cells at 100X in oil-immersion. B) Percentages of cells with apoptotic bodies are indicated by histogram bars. Cells were counted from twenty fields chosen at random. These results are representative of one experiment performed in triplicate using pooled platelets from 3 blood donors.

Figure 6. Analysis of Apoptosis by Annexin-V Stain. U266 myeloma cells were cultured overnight in supernatant from platelets exposed to DV2 (MOI 1), thrombin (10 U), of complete medium (mock) for 2 and 12 hours. U266 myeloma cells cultured in fresh medium was used as a control. Apoptosis was determined by annexin-V-FITC labeling and FACS. The results are expressed as the percent increase in annexin-V

mean fluorescence intensity (MFI), as compared to the control. MFI is an arbitrary value expressing the fluorescence intensity. The mean percent increase of MFI in each group is represented by histogram bars. The results are representative of 3 independent experiments performed in triplicate.

Figure 1.

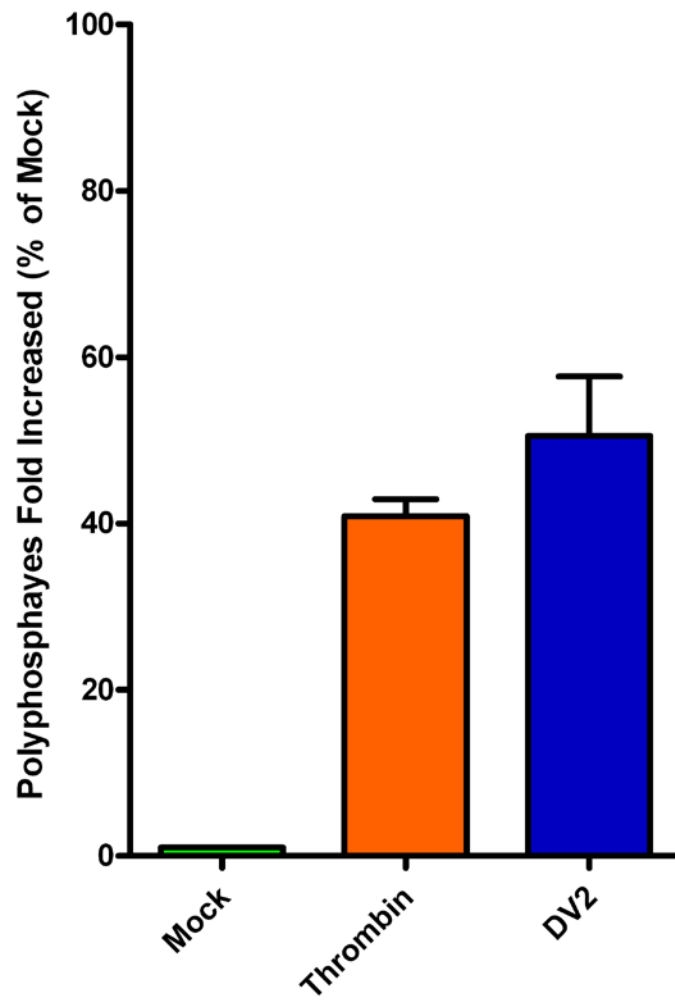


Figure 2.

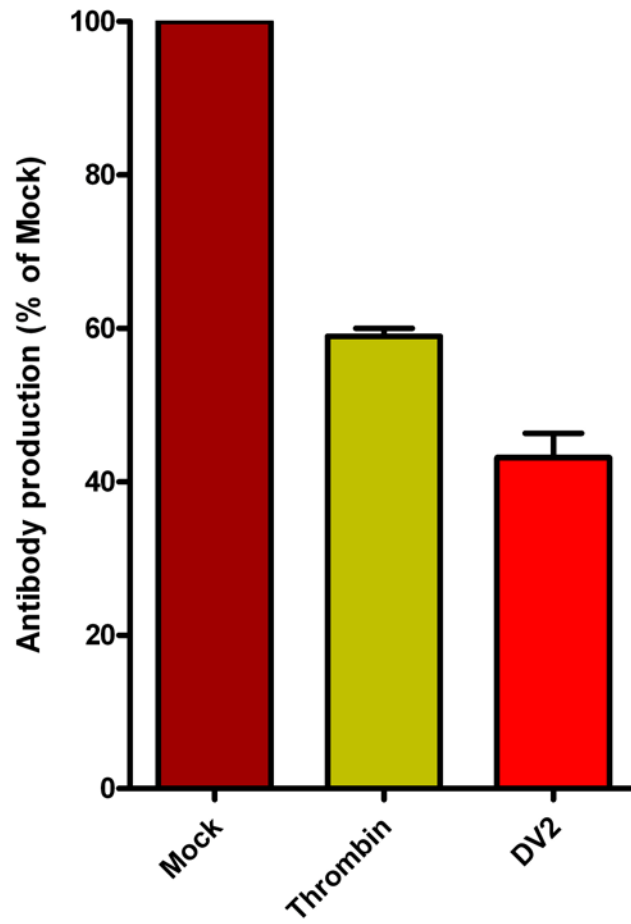


Figure 3.

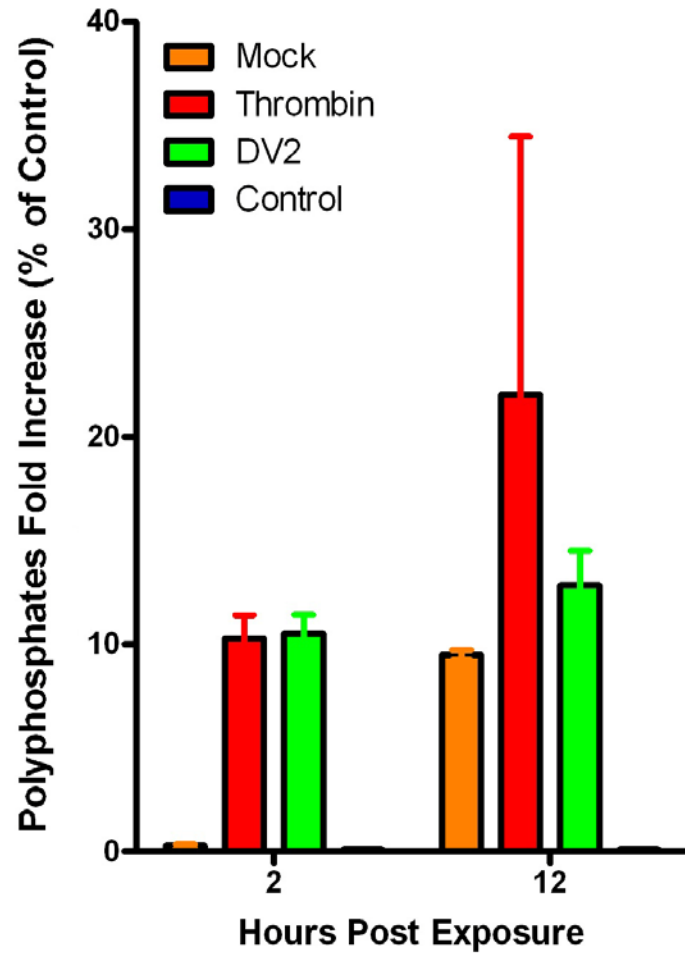


Figure 4.

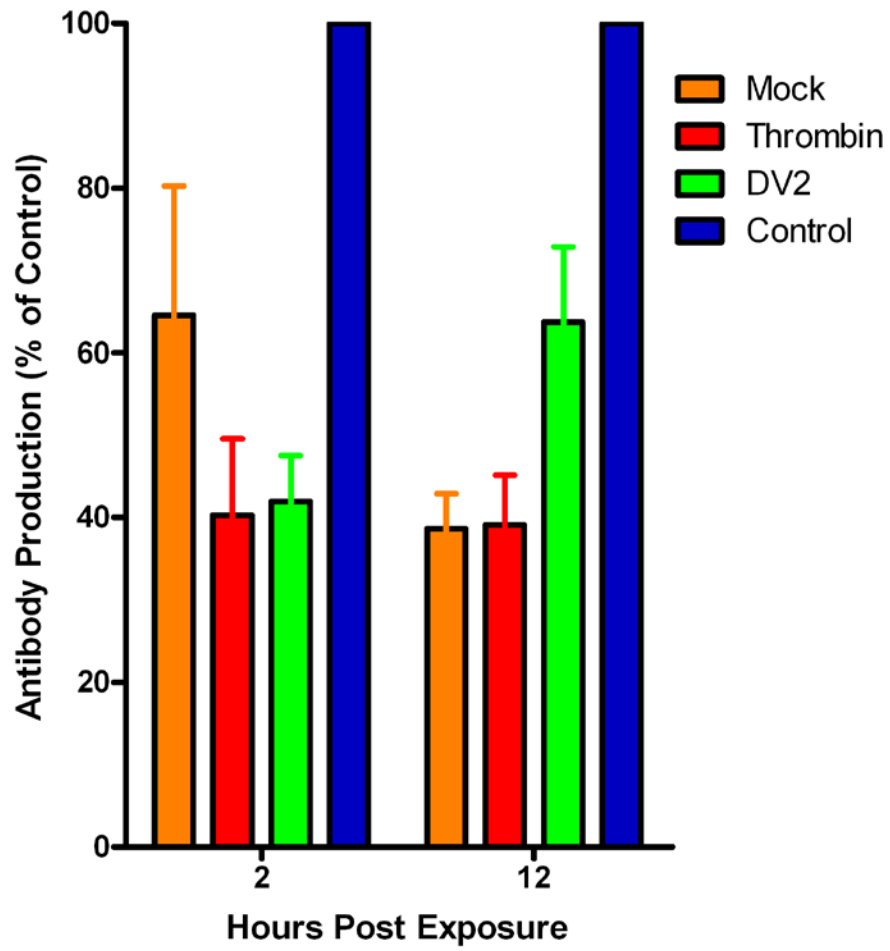


Figure 5.

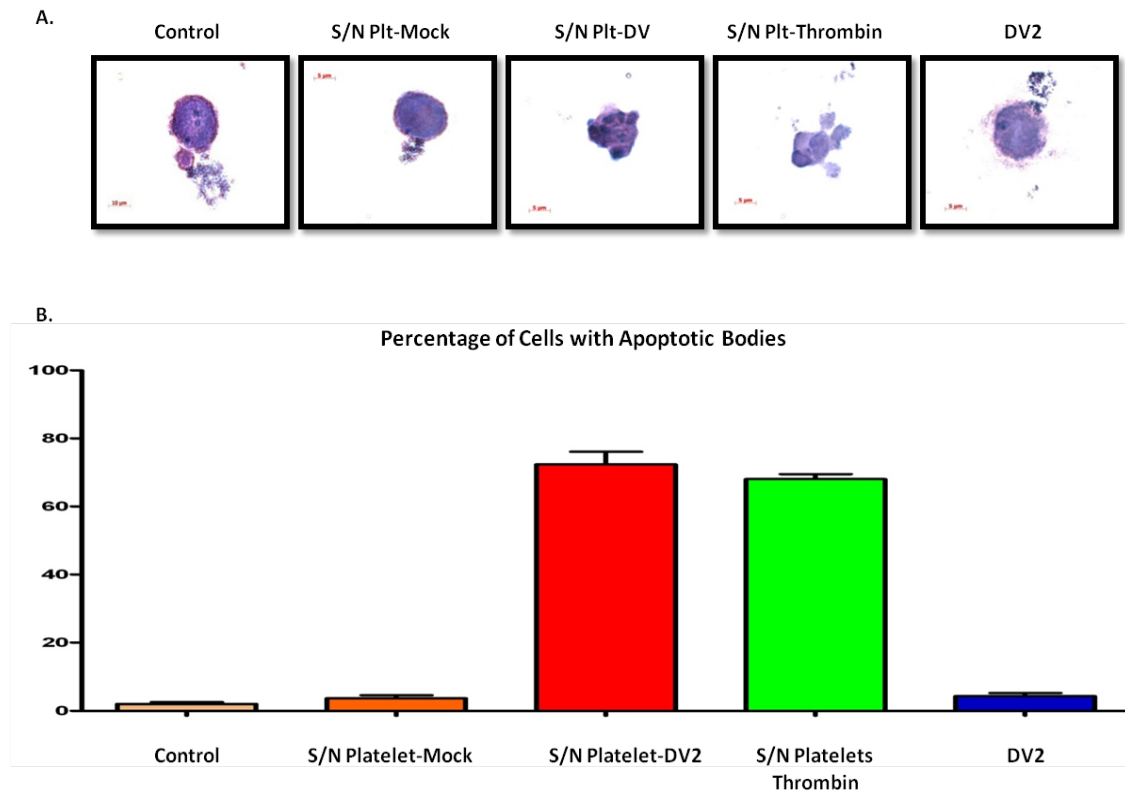
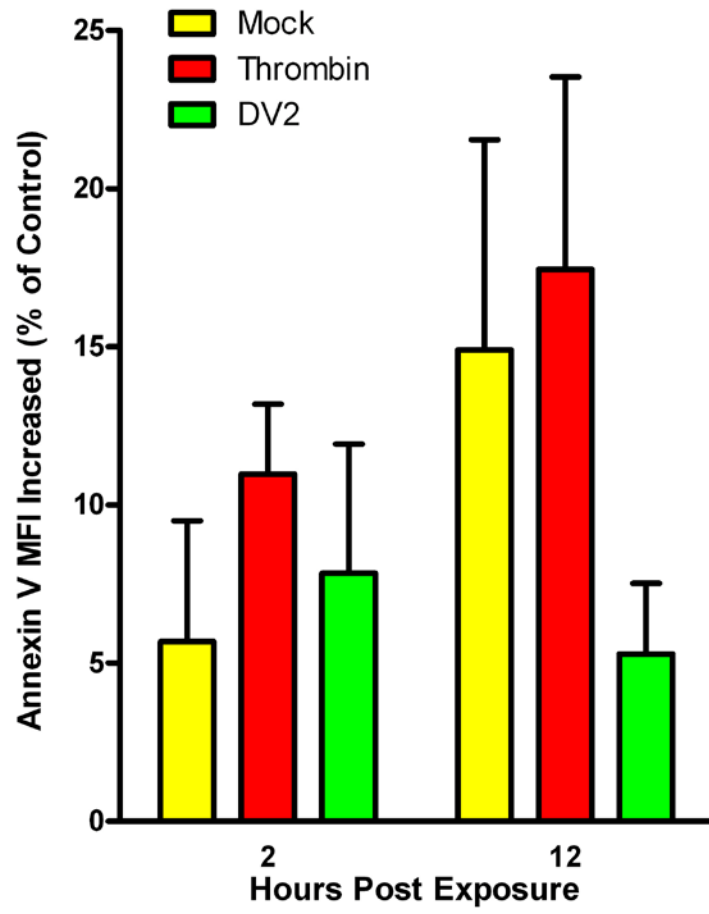


Figure 6.



Chapter IV: Conclusion

Concluding Remarks:

Dengue is one of the most common vector-borne infections with two-fifths of the world's population being at risk of infection. Worldwide it is estimated that 50-100 million infections occur annually, with over 500,000 cases of life-threatening disease, dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS), and over 25,000 deaths. Unfortunately, there are no specific preventative or therapeutic drugs for dengue infection (3).

Dengue virus infection can be asymptomatic or cause a variety of illnesses ranging from mild-fever to life-threatening complications such as DHF and DSS. Severe dengue disease is characterized by short-lived vascular permeability. The common belief is that the observed pathophysiology is immune-mediated with much focus on hyperactivation of T-lymphocytes. Despite the presence of severe thrombocytopenia, little research has focused on the pathologic contribution of platelet-derived soluble factors.

The experiments presented in this thesis demonstrate that dengue virus exposure causes activation of platelets and subsequent release of polyphosphate, which has been previously demonstrated to have harmful immunological effects (25, 26). Specifically, polyphosphate appears to induce apoptosis and subsequent reduction of IgE secretion from U266 myeloma cells. Altogether, these findings may be important in linking dengue virus associated thrombocytopenia to the development of dengue pathogenesis in patients. Moreover, understanding the role of platelets in dengue virus pathogenesis could potentially lead to the design of new therapeutic approaches to manage or prevent the more severe manifestations of dengue virus infection

References:

1. World Health Organization (WHO). Dengue: guidelines for diagnosis, treatment, prevention and control. 2009.
2. CDC. Locally Acquired Dengue--Key West, Florida, 2009-2010. *MMWR*. 2010 May 21, 2010;59(19):577-81.
3. World Health Organization (WHO). Dengue. 2010.
<<http://www.who.int/topics/dengue/en>>
4. Nielsen D. The relationship of interacting immunological components in dengue pathogenesis. *Virology Journal*. 2009;6(1):211.
5. Navarro-Sánchez E, Desprès P, Cedillo-Barrón L. Innate Immune Responses to Dengue Virus. *Archives of Medical Research*. 2005;36(5):425-35.
6. Teo D, Ng LC, Lam S. Is dengue a threat to the blood supply? *Transfusion Medicine*. 2009;19(2):66-77.
7. World Health Organization (WHO). Dengue, Dengue haemorrhagic fever and Dengue shock syndrome in the context of the Integrated Management of Childhood Illness. *Discussion Papers on Child Health*. 2005.
8. Torrentes-Carvalho A, Azeredo EL, Reis SR, Miranda AS, Gandini M, Barbosa LS, et al. Dengue-2 infection and the induction of apoptosis in human primary monocytes. *Memorias do Instituto Oswaldo Cruz*. 2009;104:1091-9.
9. Green S, Rothman A. "Immunopathological mechanisms in Dengue and Dengue hemorrhagic fever". *Current Opinion in Infectious Disease*. 2006;19(5):429 - 36.
10. Wu S-JL, Grouard-Vogel G, Sun W, Mascola JR, Brachtel E, Putvatana R, et al. Human skin Langerhans cells are targets of dengue virus infection. *Nat Med*. 2000;6(7):816-20.

11. Ghosh K, Gangodkar S, Jain P, Shetty S, Ramjee S, Poddar P, et al. Imaging the interaction between dengue 2 virus and human blood platelets using atomic force and electron microscopy. *J Electron Microsc (Tokyo)*. 2008 June 1, 2008;57(3):113-8.
12. Cheng HJ, Lei HY, Lin CF, Luo YH, Wan SW, Liu HS, et al. Anti-dengue virus nonstructural protein 1 antibodies recognize protein disulfide isomerase on platelets and inhibit platelet aggregation. *Mol Immunol*. 2009 Dec;47(2-3):398-406.
13. Honda S, Saito M, Dimaano EM, Morales PA, Alonzo MTG, Suarez L-AC, et al. Increased Phagocytosis of Platelets from Patients with Secondary Dengue Virus Infection by Human Macrophages. *Am J Trop Med Hyg*. 2009 May 1, 2009;80(5):841-5.
14. Kaushansky K. Determinants of platelet number and regulation of thrombopoiesis. *Hematology*. 2009 January 1, 2009;2009(1):147-52.
15. Krishnamurti C, Peat R, Cutting M, Rothwell S. Platelet adhesion to dengue-2 virus-infected endothelial cells. *Am J Trop Med Hyg*. 2002 April 1, 2002;66(4):435-41.
16. Lin CF, Lei HY, Liu CC, Liu HS, Yeh TM, Wang ST, et al. Generation of IgM anti-platelet autoantibody in dengue patients. *J Med Virol*. 2001 Feb;63(2):143-9.
17. Matondang AV WD, Zulkarnain I, Rengganis I, Trihandini I, Inada K, Endo S; Suhendro. The correlation between thrombopoietin and platelet count in adult dengue viral infection patients. *Acta medica Indonesiana*. 2004;36(2):62-9.
18. SAITO M, OISHI K, INOUE S, DIMAANO EM, ALERA MTP, ROBLES AMP, et al. Association of increased platelet-associated immunoglobulins with thrombocytopenia and the severity of disease in secondary dengue virus infections. *Clinical & Experimental Immunology*. 2004;138(2):299-303.
19. Sowa J, Crist S, Ratliff T, Elzey B. Platelet influence on T- and B-cell responses. *Archivum Immunologiae et Therapiae Experimentalis*. 2009;57(4):235-41.

20. Andonegui G, Kerfoot SM, McNagny K, Ebbert KVJ, Patel KD, Kubes P. Platelets express functional Toll-like receptor-4. *Blood*. 2005 October 1, 2005;106(7):2417-23.
21. Blair P, Rex S, Vitseva O, Beaulieu L, Tanriverdi K, Chakrabarti S, et al. Stimulation of Toll-Like Receptor 2 in Human Platelets Induces a Thromboinflammatory Response Through Activation of Phosphoinositide 3-Kinase. *Circ Res*. 2009 February 13, 2009;104(3):346-54.
22. Cognasse F, Hamzeh H, Chavarin P, Acquart S, Genin C, Garraud O. Evidence of Toll-like receptor molecules on human platelets. *Immunol Cell Biol*. 2005;83(2):196-8.
23. Semple JW, Freedman J. Platelets and innate immunity. *Cell Mol Life Sci*. 2010 Feb;67(4):499-511.
24. Noisakran S, Gibbons RV, Songprakhon P, Jairungsri A, Ajariyakhajorn C, Nisalak A, et al. Detection of dengue virus in platelets isolated from dengue patients. *Southeast Asian J Trop Med Public Health*. 2009 Mar;40(2):253-62.
25. Hernandez-Ruiz L, Gonzalez-Garcia I, Castro C, Brieva J, Ruiz F. Inorganic polyphosphate and specific induction of apoptosis in human plasma cells. *Haematologica*. 2006 September 1, 2006;91(9):1180-6.
26. Müller F, Mutch NJ, Schenk WA, Smith SA, Esterl L, Spronk HM, et al. Platelet Polyphosphates Are Proinflammatory and Procoagulant Mediators *In vivo*. *Cell*. 2009;139(6):1143-56.
27. Ruiz FA, Lea CR, Oldfield E, Docampo R. Human Platelet Dense Granules Contain Polyphosphate and Are Similar to Acidocalcisomes of Bacteria and Unicellular Eukaryotes. *Journal of Biological Chemistry*. 2004 October 22, 2004;279(43):44250-7.
28. Hamzeh-Cognasse H, Cognasse F, Palle S, Chavarin P, Olivier T, Delezay O, et al. Direct contact of platelets and their released products exert different effects on human dendritic cell maturation. *BMC Immunology*. 2008;9(1):54.

29. Leslie M. Cell biology. Beyond clotting: the powers of platelets. *Science*. 2010 Apr 30;328(5978):562-4.
30. Tambyah PA, Koay ES, Poon ML, Lin RV, Ong BK. Dengue hemorrhagic fever transmitted by blood transfusion. *N Engl J Med*. 2008 Oct 2;359(14):1526-7.
31. kuby J. *Kuby Immunology*. San Francisco: W.M. Freeman, 2007.