DENGUE VIRUS INFECTION OF B LYMPHOCYTES IN VITRO

by

Frances M. Still

B.A. in Biology, Colby College, 2010

Submitted to the Graduate Faculty of

Infectious Diseases and Microbiology

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2011

UNIVERSITY OF PITTSBURGH

Graduate School of Public Health

This thesis was presented

by

Frances M. Still

It was defended on

December 16, 2011

and approved by

Jared D. Evans,PhD
Assistant Professor, Department of Microbiology & Molecular Genetics
School of Medicine, University of Pittsburgh

Ernesto T.A. Marques Jr.,MD, PhD
Associate Professor, Department of Infectious Diseases and Microbiology
Graduate School of Public Health, University of Pittsburgh

Charles R. Rinaldo Jr, PhD
Chairman and Professor, Department of Infectious Diseases and Microbiology
Graduate School of Public Health, University of Pittsburgh

Thesis Advisor: Giovanna Rappocciolo, PhD
Research Assistant Professor, Department of Infectious Diseases and Microbiology
Graduate School of Public Health, University of Pittsburgh

DENGUE VIRUS INFECTION OF B LYMPHOCYTES IN VITRO

Frances M Still, M.S.

University of Pittsburgh, 2011

Dengue virus infection of certain subsets of the immune system, especially dendritic cells and macrophages, is well-established in the literature. On these cells, the virus can use DC-SIGN as a receptor. Infection of B lymphocytes is debated, and since B cells upregulate DC-SIGN upon activation, we hypothesize that this receptor may also be used for DENV infection of B cells.

In this thesis, I show that DENV-2 infects primary aB and does not infect resting B cells, while DENV-1 infects neither resting nor aB. DENV-2 infection of aB is DC-SIGN dependent. Infection of Raji/DC-SIGN cells with DENV-2 is DC-SIGN dependent, while Raji/0 infection is observed. Infection is detected via flow cytometry using DENV-specific antibodies: 2H2 for DENV-2 infection, and 3H5.1 for DENV-1 infection.

Statement of Public Health Impact

Dengue virus infection incidence has increased in recent decades, and poses a particular burden on children in South and Central America. It is known that cells of the immune system are directly involved with disease pathogenesis, and so further investigating infection of specific immune cells is crucial to understanding how best to prevent negative outcomes of DENV infection. B lymphocytes are a key part of the adaptive immune system, and may be infected by DENV. My thesis directly investigates this question, and also examines if the C-type lectin DC-SIGN is the receptor used by the virus for infection.

TABLE OF CONTENTS

LIST OF FIGURES

PREFACE

I would like to thank the support of my advisors, Dr. Rinaldo and Dr. Rappocciolo, for supporting my research and giving me invaluable advice and recommendations. Additionally, I would like to acknowledge Emilee Knowlton, Lauren Lepone, and Diana Campbell for their advice and pleasant conversation. I'd also like to thank Reuben and my family for their support and encouragement.

Many labs provided me with reagents for use. Dr. Tianyi Wang and Kevin McCormick gave initial pools of virus (data not reported in thesis) as well as the 2H2 antibody used for IFA. Dr. Jared Evans and Dr. Sean McBurney provided me with the DENV-1 and DENV-2 used in this work. Dr. Simon Barratt-Boyes and Amanda Smith provided DENV-2 and DENV-3 stocks (DENV-2 used in thesis), as well as methodology and advice for performing the focus forming unit assay.

1.0 INTRODUCTION

Dengue virus (DENV), an enveloped single-stranded RNA virus, causes the most arthropod-borne infections worldwide (50-100 million per year)[1]. Approximately 20,000 deaths per year are caused by DENV[2]. Dengue is spread by the *Aedes* species of mosquito, and unlike other flaviriruses that require a non-human animal reservoir to sustain transmission to humans, dengue virus is highly adapted to humans and can maintain a mosquito-human-mosquito transmission cycle in urban areas[2]. The *Aedes aegypti* mosquito is the principal urban vector for dengue, and is highly adapted to humans. In addition to preferentially laying eggs in artificial containers near homes, the female mosquito often feeds from more than one human in the gonotrophic cycle, potentially transmitting virus to each[2].

The dengue virus is present in four serotypes, DENV-1, -2, -3, and DENV-4, and the immune response that develops to infection is serotype-specific: no cross-protective immunity develops. This makes vaccine development extremely difficult; vaccines to other flaviriuses have been successfully developed[3 4 5], while much work has been performed on a DENV vaccine without success[6 7].

The illness that results from human DENV infection is typically subclinical and asymptomatic infection, while dengue fever, as well as the more serious diseases of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) can develop. DF is an acute febrile illness that most commonly occurs in older children and adults, and convalescence can last for

several weeks. DHF/DSS are vascular leak syndromes that are thought to be the result of an immunological cytokine cascade[2] which causes increased vascular permeability, leakage, hypovolemia, shock, and death if not corrected. There is no specific drug to treat DENV infection; treatment is in response to symptomology. If properly managed, DHF/DSS cases have a fatality rate of less than 1%[8]. The main risk factors for severe disease include the strain of the virus, previous infection with a heterotypic DENV strain, age, and genetic background of the person[2].

Dengue fever itself is not a new disease, but there has been a marked rise in global epidemic dengue in the past 25 years[2]. Contributing to this rise are concurrent human population growth, especially in tropical urban centers, increased movement of dengue-infected individuals via modern transportation, and a lack of effective and sustained mosquito control strategies[2]. As the rate of virus evolution has increased[9], and homologus recombination has been observed[10 11 12 13], there is the possibility for the rise of strains with epidemic potential.

1.1 DENGUE VIRUS

DENV is a member of the *Flaviridae* family of viruses. Besides DENV, other notable flaviviruses are West Nile virus, yellow fever virus, and hepatitis C virus. All flaviriruses are positive-sense, single stranded RNA viruses, each with a genome of approximately 11,000 nucleotides in length[14]. The spherical virion is composed of three proteins, and is enclosed by a lipid envelope[15]. Ten proteins are encoded by the genome in a single open reading frame, of which the three structural genes-the capsid (C), premembrane (prM), and envelope (E)-are encoded immediately after a brief noncoding region at the 5' end of the genome. The capsid

protein serves to form the nucleocapsid shell that protects the viral genome, while the premembrane and envelope proteins are both embedded in the viral envelope. The seven nonstructural genes (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are encoded in the latter two thirds of the viral genome. Together, NS3 and NS2B act as a protease, while NS5 serves as the RNA-dependent RNA polymerase.

Upon entry into the host cell, the DENV positive-sense genome is transcribed by host cell ribosomes associated with the endoplasmic reticulum[16]. The resulting polyprotein is then cleaved by the NS3-NS2B protease[17] between the NS proteins, and the virus components perform replication of the DENV genome from a negative-sense intermediate. Assembly of the virion occurs in the membranes of the endoplasmic reticulum, where signal peptidase[18] cleaves at the C-prM, M-E, and E-NS1 junctions. The virions are transported to the Golgi complex, where virus maturation occurs. Here, the prM protein is cleaved into M + pr by furin in the acidic trans-Golgi network[19]. In release from the host cell, prM stabilizes the E protein, preventing fusion of the newly-synthesized virion with the host cell. Upon release from the cell, the pr segments dissociate from the virion. The release of fully mature virion is dependent on the cell in which the virus replicated. Virus propagated on Vero cells, an epithelial cell line, release mainly prM-containing virions due to inefficient prM cleavage in late-stage maturation[20], although prM containing virions have many of the same characteristics as the fully mature M-containing virions.

The 5’ and 3’ UTR ends of the genome contain highly structured segments, and are approximately 100 nucleotides[21] and 600 nucleotides in length, respectively[22]. Two stem-loop structures are found within the capped[23] 5’ UTR – the larger stem loop A (SLA) is the promoter recognized by viral RNA polymerase for RNA synthesis[24]. The smaller stem loop B (SLB)

contains the 5'UAR (*U*pstream *A*UG *R*egion) which is complimentary to a region at the 3' end of the genome[22]. This complementarity is crucial to virus replication, since circularization of the genome is required for successful replication[24]. The 3' UTR contains a highly conserved 3' stem loop which is essential for virus replication[25].

The E protein is a class II fusion receptor, and is also responsible for attachment to host-cell receptors and virus-mediated cell-membrane fusion[26]. The E protein is organized as homodimers on the surface of the virion, and is involved in interaction with cellular receptors as well as the membrane fusion process[27]. Each E protein monomer contains three domains – the N-terminal domain I, the dimerization domain II, and the C-terminal, Ig- like domain III[28]. Glycosylation at Asn 153 is necessary for viral infectivity, while glycosylation at Asn 67 is important for viral assembly or exit, as well as for interaction with DC-SIGN[29].

1.1.1 Dengue infection of human cells

Flaviviruses enter cells by clathrin-mediated endocytosis, rolling over the surface of the cell to a pre-existing clathrin-coated pit[27]. This endocytosis is mediated by at least two virus:cell interactions. First, DENV nonspecifically binds to glycosaminoglycan heparin sulfate[30 31] or the structural analogue heparin[27 32] serves to concentrate virus at the membrane surface for interaction with a second, high-affinity receptor[33]. Since heparin sulfate is found on the surface of cells in many organs[34], and the pathology of dengue manifests itself in specific organs, such as the liver, other specific receptors must be involved in organ infection.

DENV has been shown to interact with numerous cellular molecules besides heparin and heparin sulfate. In mosquito cells, DENV has been shown to interact with Hsp70, R80, R67, and a 45-kDa protein[35]. In human cells, other receptors besides heparin/heparin sulfate have been

identified: Hsp90, CD14, GRP78/BiP, and on cells of the myeloid lineage, C-type lectin receptors (DC-SIGN, mannose receptor, and CLEC-5)[35].

Of the cells of the immune system, the cells of the mononuclear lineage – dendritic cells (DCs), macrophages, and monocytes, including Langerhans cells (skin-resident DCs)- are the primary target for DENV infection[36 37 38]. Upon infection, monocytes and macrophages are responsible for the dissemination of virus, and the presence of heterotypic antibodies at subneutralizing titers serves to increase uptake of the virus-antibody complex by these cells[39] in a process termed antibody-dependent enhancement (ADE).

In the antibody response, any antibody directed against DENV will enhance virus infection to some extent[40]. Due to the sequence divergence among DENV strains, antibodies are of low enough avidity to the novel DENV strain so that complete neutralization is not possible[42]. IgM antibodies, those produced against an initial DENV infection, are thought to contribute to disease pathogenesis by activating complement through interaction with C3R[41 40]. The IgG antibodies that develop in a secondary infection can bind to the FcR, causing opsonization and an increase of virus uptake into the host cell. FcγRIA is found solely on macrophages and dendritic cells and preferentially binds monomeric IgG, while the FcγRIIA binds immune complexes and is more broadly distributed[40].

Mononuclear-lineage cells serve to initiate the innate immune response against DENV through the production of interferons (IFNs). Upon infection, both type I (IFN-α, -β) and type II (IFN-γ) are secreted upon virus:cell interaction through pattern recognition receptors (i.e., C-type lectins [DC-SIGN][43], Toll-like receptors[35]). Other immunomodulatory factors are involved in DENV clinical progression. The proinflammatory cytokine TNF-α peaks in production at the

same time virus production peaks in vitro[44], and TNF-α secretion by infected macrophages is correlated with clinical disease severity[45].

There is some controversy surrounding whether B cells are targets for DENV infection. Some reports have indicated that B cells are not infected in pools of cells in vitro – either PBMCs[46] or splenic mononuclear cells[47]. In both of these reports, cells of the monocyte lineage were the only infected cells. Alternatively, purified primary B cells were able to be productively infected by DENV-2 in one report[48]. Different levels of infection were also observed between blood donors. DC-SIGN expression is not uniform between individuals, so the differing levels of infection could be due to different amounts of receptor availability on each donor's B cells. In vivo, no DENV-infected B cells have been observed during acute infection.

1.2 B LYMPHOCYTES AND DISEASE PATHOGENESIS

B lymphocytes are the antibody-secreting cells of the adaptive immune system, secreting pathogen-specific antibodies after clonal expansion and subsequent stimulation. Antibodies have a crucial role in protection against viral disease. Virus-specific antibodies can bind to the virus directly and prevent their entry into cells, neutralizing infectivity[49], and they can also coordinate various effector functions through the Fc (crystallizable fragment) region of the antibody heavy chain[50 51].

In addition to their antibody-secreting role, B cells also act as antigen-presenting cells. Upon encountering antigen, display of antigens internalized via binding to the B cell receptor (BCR) is favored over display of antigens acquired via phagocytosis or pinocytosis[51]. Internalization of antigen via the BCR also leads to an upregulation of MHCII expression[52 53],

facilitating the efficient presentation of antigen to $CD4^+$ T cells by activated B cells. Pathogens can thus subvert the antigen-presenting nature of B cells in order to alter the action of T cells. Several virus infections - HIV[54] and SIV[55], as well as EBV[56] and HHV-8[57] – downregulate either MHC I or II. DENV infection of either K562 or THP-1 cells causes the upregulation of MHC I[58], allowing for infected cells to evade detection by NK cells.

Since B cells are not lymph-node restricted after activation, they can serve as a vehicle for virus transport around the body. In hepatitis C virus (HCV) infection, B cells bound by virus in a DC-SIGN dependent manner can infect hepatoma cells *in trans*[59].

1.2.1 DC-SIGN

The C-type lectin DC-SIGN (*D*endritic *C*ell - *S*pecific *I*ntercellular adhesion molecule 3-*G*rabbing *N*onintegrin) plays an important role in pathogen-dendritic cell and DC-T cell interactions, as well as in DC migration and pathogen uptake[60]. Since DC-SIGN is expressed on DCs at the initial site of infection, it is likely an important molecule for DENV infection of cells. DC-SIGN is important for the internalization of other pathogens, such as Ebola virus[61], *Leishmania amastigotes*[62], hepatitis C virus[63], human herpesvirus 8[64], and *Mycobacterium tuberculosis*[65]. DC-SIGN is also important for the trafficking of HIV through the immune system. The virus binds to DC-SIGN on mucosal DCs, which then migrate to secondary lymphoid organs and transfer the virus *in trans* to $CD4^+$ T cells[66]. DC-SIGN, which is upregulated on IL-4, CD40L stimulated B cells[67], also mediates the *trans* infection of $CD4^+$ T cells[68].

The DC-SIGN carbohydrate recognition domain (CRD) binds to DENV-2 E glycoproteins at Asn 67, and binds to 2 of 3 E molecules in complex on the mature virion,

leaving the other E protein free to bind with other receptors[28]. Relating to clinical disease, polymorphisms within the DC-SIGN promoter are associated with disease severity[60].

2.0 STATEMENT OF THE PROBLEM

DENV exerts much of its pathologic effects via infection of cells of the immune system, but little work has been done directly on isolated primary B cells to determine if they are a target for infection. I hypothesize that B cells are a target for DENV infection in vitro. The majority of the literature surrounding DENV infection of human cells does not show infection in B cells[46 47], but one group has shown infection. Lin et al[48] notes a difference in the infection of B cells between blood donors, and since DC-SIGN levels are known to vary amongst individuals, DC-SIGN expression may be the cause for this observation. B cells may also be a method for DENV traffic through the body, as is observed in HCV infection[59], contributing to the pathogenesis of disease. In this thesis, I will investigate the role of B cells and DC-SIGN in DENV infection.

2.1 AIM 1- INFECTION OF CELL LINES AND PRIMARY B CELLS BY DENV-2

Given the controversy surrounding the infection of primary human B cells, I will investigate whether B cells are target for DENV-2 infection. Initially, I will perform experiments using the Raji cell line, which is a cell line derived from Burkitt lymphoma cells, and also Raji cells that have been stably transfected with a DC-SIGN expression plasmid. I show that DC-SIGN is important for infection of Raji/DC-SIGN as well as activated B cells. Infection of resting B cells is not observed.

2.2 AIM 2 – DENV-1 DIFFERENCES IN B CELL INFECTION

Since DENV is present in 4 serotypes, I next chose to examine if DENV-1 infection of B cells differs from that of DENV-2. Using the Hawaii strain[69], a prototype DENV-1 strain, and again using activated and resting B cells, I show that DENV-1 infects neither cell type.

3.0 MATERIALS AND METHODS

3.1 B CELL ISOLATION

Human blood from anonymous donors was obtained from the Central Blood Bank (Pittsburgh PA) and processed within 18 hours of delivery. Blood was spun initially to obtain the buffy coat layer, which was then diluted extensively and placed over Ficoll-Hypaque (GE Healthcare). PBMCs were then isolated, counted, and frozen in heat-inactivated fetal bovine serum (FCS, GemCell) with 10% DMSO (Sigma). Upon use, frozen cells were quickly thawed, washed once in either RPMI-1640 (Gibco) +10% FCS + antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL gentamicin, 0.5 μg/mL amphotericin B) or Hank's Balanced Salt Solution (Gibco) and purified. B cells were then isolated using negative selection using the B Cell Isolation Kit II (Miltenyi Biotec), or by negative selection using the CD14 Microbeads (Miltenyi Biotec), and then positive selection using CD19 Microbeads (Miltenyi Biotec). After isolation, B cells were counted and either used immediately as resting B cells, or activated for 48 hours in RPMI+10% FCS + antibiotics supplemented with CD40L (1 μg/mL, Enzo) and recombinant human IL-4 (rH IL-4, $1*10^3$ U/mL, R&D Systems) for use as activated B cells. Cells were incubated in a humid chamber at 37°C supplemented with 5% CO_2.

3.2 CELL CULTURE

Vero cells (ATCC CCL-81) were cultured in Eagles Minimum Essential Media (EMEM, Lonza) supplemented with 10% FCS. Cells were split upon reaching confluence using 0.05% trypsin/EDTA (Gibco), and were re-seeded at a concentration of $4*10^4$ cells/cm^2 in a T-75 flask (Corning).

Raji cells were obtained from ATCC, Virginia USA. Raji/DC-SIGN cells were a kind gift of Dan Littman, NYU School of Medicine. DC-SIGN expression is under the control of MLV promoters (plasmid pMX DC-SIGN, NIH AIDS Research & Reference Program). This plasmid does not have a selectable marker for eukaryotic cells; an ampicillin resistance cassette is encoded for selection of transformed bacteria.

Cells were maintained in RPMI-1640 supplemented with 10% FCS, and were kept in a humidified chamber (37°C, 5% CO_2).

3.3 INFECTION OF CELLS AND BLOCKING OF DC-SIGN

For infection of cells, virus (DENV-1 Hawaii strain or DENV-2 strain 16681) was added to cells resuspended in a minimum volume of media at MOI of either 1.2 (B cell experiments) or 2.4 (cell lines). The lower viral titer for primary cells was used in order to continue using the same pool of virus through the experiment – increasing the titer would have resulted in multiple pools being used. Virus stocks were a kind gift of J. Evans and S. McBurney. The virus-cell mixture was then incubated with gentle shaking every 15 minutes for a total of 1 hour. After infection, cells were pelleted and supernatant samples were placed at -20° for at least 2 hours, then at -80°

indefinitely. The cells were then washed twice and used either immediately or placed in the incubator for analysis at later time points.

When DC-SIGN was blocked prior to infection, cells were pelleted, resuspended in a minimum volume of cold media, and 20 μg/mL anti-human DC-SIGN (R&D Systems) was added. The cell mixture was then placed at 4° for 1 hour, and cells were washed using an excess volume of cold media. Cells were then used for infection.

For infection of adherent Vero cells, virus (DENV-2 strain 16681, gift of Dr. Barratt-Boyes) was added to 75% confluent Vero cells in a muli-chambered slide. Virus was added in a minimal volume, and was allowed to incubate at 37° for 1 hour, at which point cells were washed. Fresh media was placed over the cells, and immunofluorescence assay was conducted 24 hours post infection.

3.4 FLOW CYTOMETRY

50,000 cells suspended in PBS were placed in a 96-well V-bottom plate and centrifuged. They were then stained with 50 uL of LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen) and incubated in the dark for 30 minutes. After washing with monoclonal wash buffer (0.22 μm filtered PBS + 2% (v/v) FCS + 0.1% (mass/volume) NaN_3), cells were resuspended in 100 uL of 1x permeabilizing solution (BD FACS Permeabilizing Solution 2, prepared in PBS) and again allowed to incubate. After washing, cells were resuspended in 200 uL Super Blocking Buffer (Pierce, in PBS) and allowed to incubate. After centrifugation, cells were resuspended in 50 uL wash buffer, and antibodies were added.

For analysis of infection of Raji or Raji/DC-SIGN cells, the following antibodies were added: PE-CD209, Zenon AF680-3H5.1, and Zenon AF594-2H2. Isotype control antibodies were also used: PE-IgG2b, Zenon AF680-IgG1, and Zenon AF594-2H2.

For analysis of infection of resting or activated B cells, the following antibodies were used: PE-CD209, Zenon AF680-3H5.1, Zenon AF594-2H2, APC-CD23, and FITC-CD19. Isotype control antibodies were also used: PE-IgG2b, Zenon AF680-IgG1, Zenon AF594-2H2, APC-IgG1κ, and FITC-IgG1.

Again, after 30 minutes incubation in the dark, cells were washed, and resuspended in 200 uL of 1% paraformaldehyde. The plate was then placed in a covered box at 4° until analysis.

Compensation controls were prepared on the day of running the samples as follows. One drop each of either a negative or positive control bead (BD Biosciences) was added to a 96-well V-bottom plate, and 5 uL of antibody was added to the wells containing positive control beads: V500-IgG1κ (control for viability dye), PE-CD209, AF700-IFNγ (control for AF680-labeled Abs), PE-Texas Red (control for AF 594-labeled Abs), and, for analysis of B cells, APC-CD19 and FITC-CD23. After incubation for 10 minutes in the dark, the beads were washed, pelleted, and resuspended in monoclonal wash buffer. Samples were read on a BD LSRII flow cytometer, and data was analyzed using FlowJo (TreeStar Inc.). Figures and statistics (Student's two-sample paired *t* test) were prepared using Microsoft Excel.

Antibodies were obtained from the following sources: BD Pharmigen (PE-IgG2b, AF700-IFNγ, APC-CD23, FITC-CD19, APC-IgG1κ), BD Biosciences (FITC-IgG1), BD Horizon (V500-IgG1κ), R&D Systems (PE-CD209), Invitrogen (Zenon labeling kits), Millipore (3H5.1, 2H2), and Sigma-Aldrich (Mouse IgG1 and IgG2a).

3.5 IMMUNOFLUORESCENCE ASSAY

At the same time as isolation for flow cytometry, approximately $5*10^4$ cells were placed onto a trypsin-coated glass slide. The cell spot was allowed to dry, and then the slide was placed in PBS at 4°C for less than one week. Upon immunostaining, slides were removed from PBS and fixed in 2% paraformaldehyde for 15 minutes, washed in PBS, and then permeabilized using 0.1% Triton-X 100 (Sigma) for 15 minutes. After washing in PBS, nonspecific sites of the cell were blocked using Super Blocking Buffer (Pierce) for 45 minutes, the slide was washed, and then 50 uL of a 1:10 dilution of hybridoma-produced 2H2 antibody (kind gift from T. Wang) was added to each spot. The antibody was allowed to incubate for 1 hour, at which point the slide was washed as usual, and then a mixture of 1:500 Alexa Fluor 568 $F(ab')_2$ fragment of goat anti-mouse IgG (H+L) (Invitrogen) and FITC-CD209 (BD) were mixed in equal volumes, and approximately 50 uL of this mix was added to each cell spot and allowed to incubate, again for 1 hour, in the dark. After washing, slides were mounted using SlowFade Gold with DAPI (Invitrogen), and the coverslip was sealed using clear nail polish. Slides were imaged using a Nikon Eclipse E600 microscope, and images were collected and layered using MetaMorph software (Molecular Devices). Images visualization was minimally enhanced using Photoshop (Adobe).

3.6 FOCUS FORMING UNIT ASSAY

Vero cells (ATCC CCL-81) below the 10th passage were plated in a 12-well plate at $7.5x10^4$ cells per well in 2 mL of EMEM (Lonza) supplemented with 2% FCS. After overnight

incubation (5% CO_2, 37°), serial dilutions of saved supernatant samples from experiments were prepared in EMEM without FCS. Media was removed from the cell plates, and the cells were rinsed once with 2 mL PBS. 300 uL of each virus dilution was added to wells in duplicate, and the plate was incubated at 37°, 5% CO_2 for 1 hour with gentle shaking every 15 minutes. After 1 hour incubation, the virus dilutions were aspirated from the cells, the plate was washed 2X with PBS, and 2 mL of 0.2% methyl cellulose media (500 mL EMEM, 15 mL heat-inactivated FCS, 5 mL penicillin/streptomycin [100 U/mL penicillin, 100 μg/mL streptomycin], 4 grams methyl cellulose) was added to each well. The plates were incubated for 5 days, after which time the medium was removed and wells were rinsed 2X with PBS. Cells were then fixed in a methanol/acetone mixture (1:1) for 30 minutes, and were then dried for at least 2 hours. Wells were then rehydrated with PBS, blocked using a 3% FCS in PBS solution for 30 minutes, and 250 μL of a 1:450 dilution of 2H2 antibody (Millipore) was then added to each well. Plates were placed in a humid chamber on a rotating platform overnight. The following morning, plates were rinsed with PBS, and 250 uL of a 1:500 dilution of goat anti-mouse IgG-HRP (Millipore, prepared at 0.5 mg/mL) was added to each well and incubated for 1 hour on a rotating platform. The wells were rinsed, and 250 μL of TrueBlue Peroxidase Substrate (KPL) was added to each well. The plates incubated for 10 minutes in darkness on the rotating platform before removal of the substrate, and were then fixed with 1 mL water for 5 minutes. The liquid was removed, and plates were allowed to dry overnight before calculating the FFU titer as follows: FFU/mL = ((#spots in well 1 + #spots in well 2)/2)/((dilution factor)(volume plated (0.3 mL)). Spots were counted manually.

4.0 RESULTS

4.1 OPTIMIZATION OF PROTOCOLS

In initial experiments (data not shown), concern was raised about the ratio of the Zenon kit-labeled anti-DENV antibody 2H2 to the number of cells. This antibody binds to the prM protein of all DENV serotypes. To test this, I performed a dilution series of the labeled antibody on DENV-2 infected Vero cells (Figure 1). The antibody preparation was diluted in PBS after conjugation. Like in infection of cells and cell lines, infection was performed for 1 hour. After infection, cells were washed with PBS and allowed to incubate for 24 hours. No negative control was performed for this experiment.

Although not tested on Vero cells, the 3H5.1 antibody, a DENV-2 E protein specific Ab, is also used to detect DENV-2 infection by flow cytometry after labeling with a Zenon kit. From these results, 1 μg of Zenon-labeled antibody, either AF680-2H2 or AF594-3H5.1, was used in future experiments to detect infection by flow cytometry. Since the Zenon kit instructions are written to stain $1x10^5$ cells, and the number of cells used per well for flow cytometry is $0.5x10^5$, I used one-half of the Zenon kit's final volume for staining.

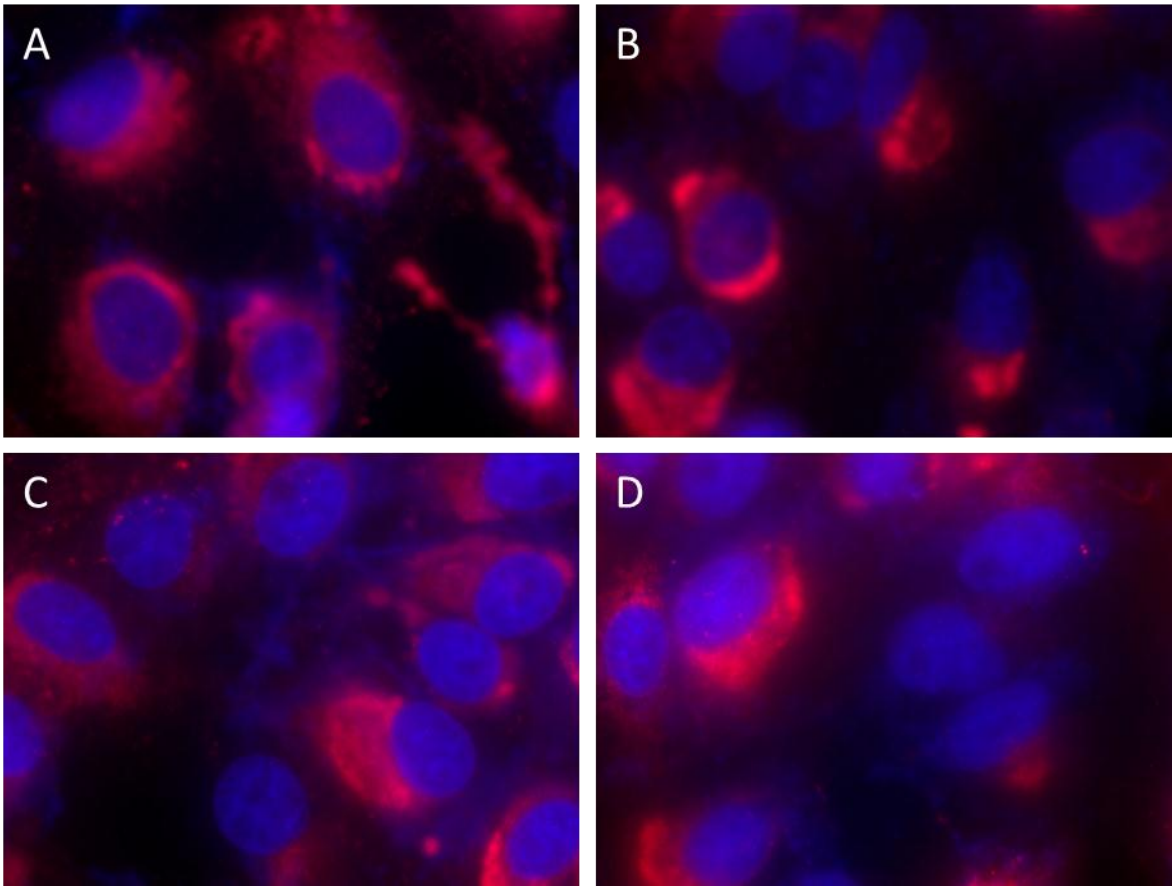

Figure 1. 1 µg of Zenon AF680-labeled 2H2 antibody gives optimum detection of DENV-2 infection of Vero cells.
DENV-2 infected Vero cells were stained with decreasing amounts of Zenon AF680-labeled 2H2 antibody (red) for one hour at 24 hours post-DENV infection. Blue = DAPI. A) 1 µg labeled 2H2, B) 0.5 µg labeled 2H2, C) 0.25 µg labeled 2H2, D) 0.125 µg labeled 2H2.

Infections were then performed on Raji cells that have been stably transfected with a DC-SIGN expression plasmid (Raji/DC-SIGN cells). The cells were infected with DENV-2 for 1 hour, and were assayed at 0, 24, and 48 hours post-infection (hpi) by flow cytometry (Figure 2). Mock-infected cells do not stain positive for the DENV-specific antibodies at a level above that of the isotype controls (Figure 2A). Cells infected with DENV-2 (Figure 2B) do not stain positive for the DENV-specific antibodies at 0 and 24 hpi, but do stain positive for both 2H2 and 3H5.1 at 48 hpi. The majority of DENV-positive cells (positive for 3H5.1 antibody) also express DC-SIGN (Figure 3). DC-SIGN levels slightly decrease in infected cells when compared with

their mock-infected counterparts. The combination of 2H2 and DC-SIGN (CD209) was not tested.

Although not directly tested, this experiment indicates that the infection of Raji/DC-SIGN cells by DENV-2 may be DC-SIGN dependent. Given these results, the time-points of 0 and 48 hpi were used for all future experiments. The DC-SIGN expression for the cells was relatively low: 42±4.8 percent of cells expressed DC-SIGN. For future work with Raji/DC-SIGN cells, another lineage of cells was used that had an average of 80% DC-SIGN expressing cells.

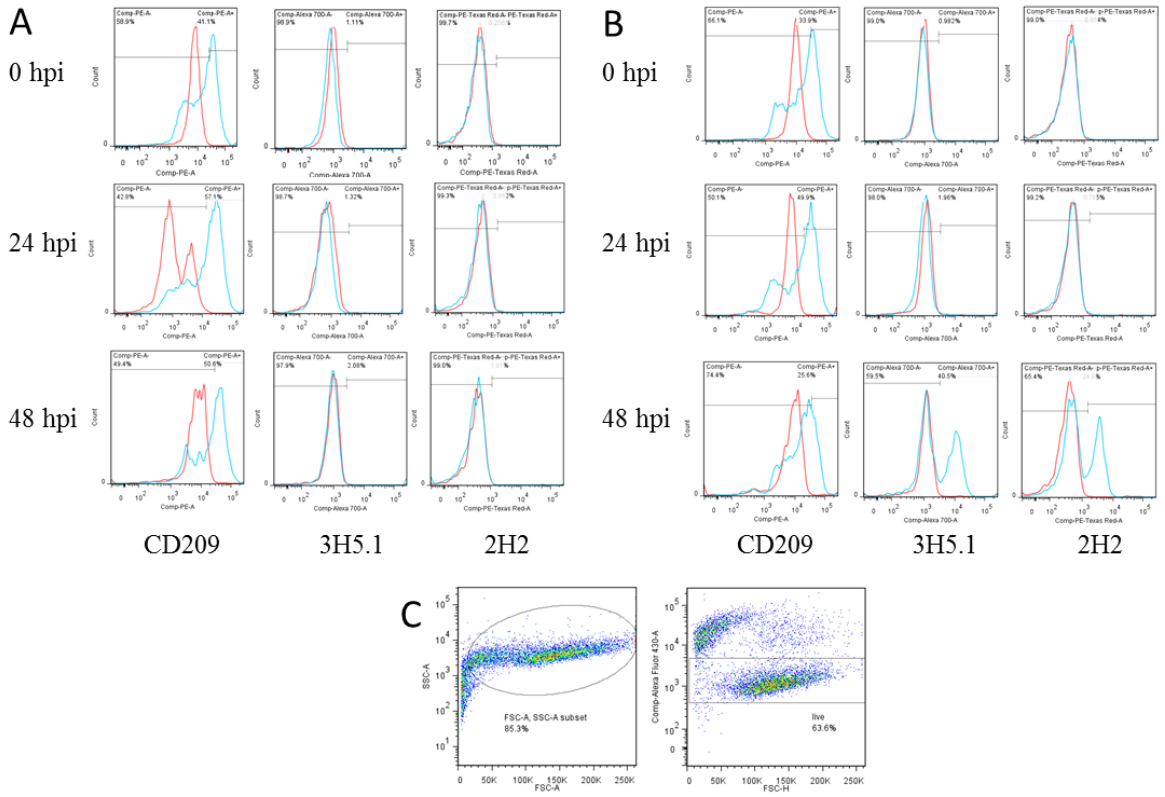

Figure 2. DENV-2 infection of Raji/DC-SIGN cells is detected at 48 hpi.
A) DENV-specific antigens are not detected in mock-infected cells. B) Both the DENV-2 specific 3H5.1 and pan-DENV 2H2 antibodies bind to DENV-2 infected cells (MOI=2.4) at 48 hours post infection. CD209=DC-SIGN. Red, isotype control. Blue, specific antibody. C) Gating strategy – first on singlets (left), then on live cells (right). N=1

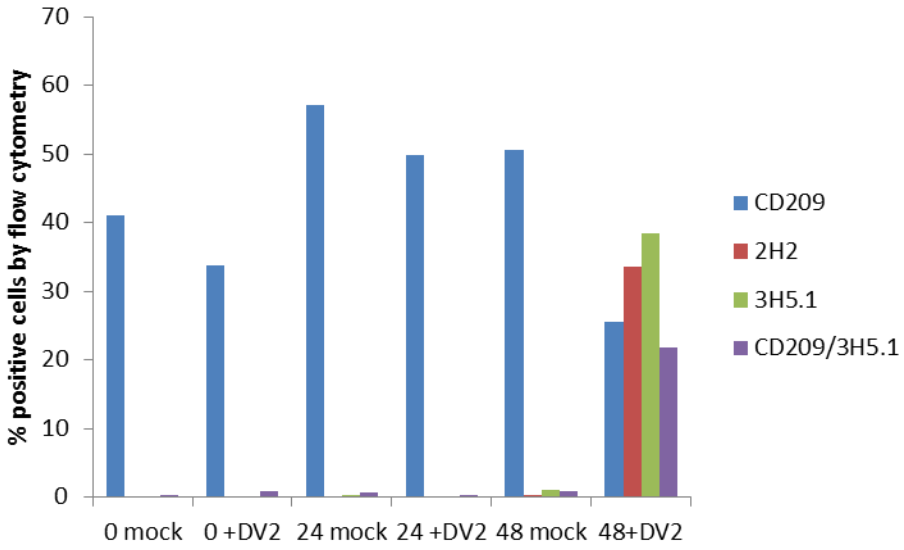

Figure 3. DENV-2 infection of Raji/DC-SIGN cells is DC-SIGN dependent.
Summary table of Figure 2. Values presented = (% positive specific stain - % positive isotype control). CD209=DC-SIGN. N=1.

4.2 INFECTION OF RAJI/0 CELLS

To investigate our hypothesis that the infection of Raji/DC-SIGN cells is in fact DC-SIGN dependent, I first infected Raji/0 cells – Raji cells that have not been transfected with a DC-SIGN expression plasmid (Figure 4). The mock-infected Raji/0 cells (Figure 4A, Raji/0) have significantly different appearances at 48 hpi in regards to the specific antibodies (3H5.1 and CD209). Since it appears that the double-negative population at 0 hpi shifts completely to the X-axis at 48 hpi, no fluorescence is being detected for this cell population, most likely due to incorrect addition of antibodies during the staining step of the flow cytometry procedure. This experiment was repeated, in order to both confirm the results seen in the DENV-2 infected cell population and also to ensure that antibodies were correctly added to all wells. However, analysis of these samples was not performed due to all of the cells being dead based on the Live/Dead cell

stain kit. Before the flow staining protocol, viability of the cells was approximately 50% by the trypan blue exclusion method.

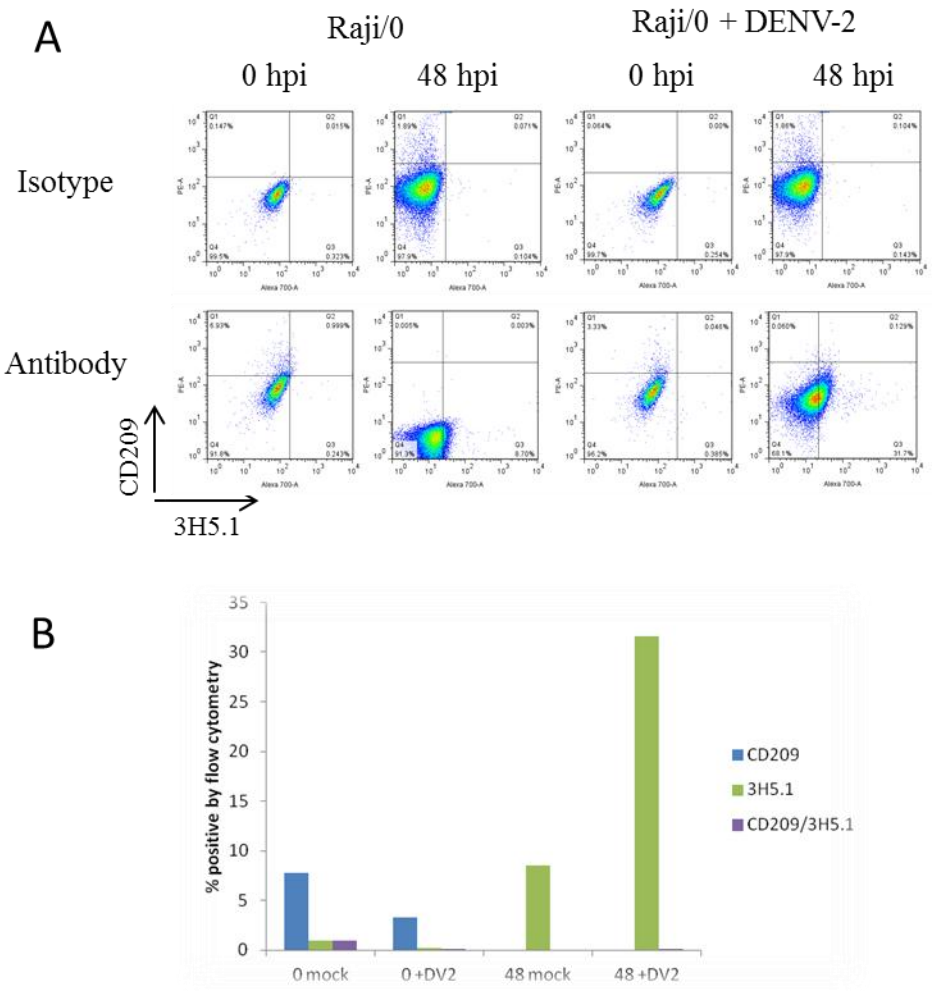

Figure 4. Infection of Raji/0 cells may be DC-SIGN independent.
A) A) Mock-infected Raji/0 cells (left) or DENV-2 infected Raji/0 cells (right) were stained with isotype control antibodies (top row) or specific antibodies (bottom row) against CD209 (DC-SIGN) or the DENV-2 specific 3H5.1. B) Graph summarizing the results of A. Values presented = (% positive specific antibody) – (% positive isotype control antibody). N=1.

From these results, the infection of Raji/0 cells may be DC-SIGN independent, since there are no 3H5.1-positive cells that also are positive for DC-SIGN. At 0 hpi, DENV-2 infection causes a decrease in DC-SIGN expression. If DENV uses DC-SIGN as a receptor, then the binding of a virus and subsequent internalization of virus and receptor would then result in the

reduction of DC-SIGN detection by flow cytometry. The larger issue with this data, however, is that nearly 7% of Raji/0 cells are expressing DC-SIGN at 0 hpi. Since the cell line has theoretically never come into contact with a DC-SIGN expression plasmid, and Raji cells do not express DC-SIGN endogenously, there must have been some contamination of Raji/0 cells with Raji/DC-SIGN cells at some point in time. There is no way to selectively eliminate DC-SIGN expressing cells from the culture without using cell sorting, so any future work on Raji/0 cells should ensure using cells that have had their DC-SIGN expression validated before infection.

4.3 BLOCKING DC-SIGN PRIOR TO INFECTION OF RAJI/DC-SIGN CELLS

In order to investigate whether the infection of Raji/DC-SIGN cells is DC-SIGN dependent, and since the infection of Raji/0 cells is possible, I performed a blocking experiment on Raji/DC-SIGN cells. Before infection, cells were incubated with an anti-human DC-SIGN monoclonal antibody, and were then used for infection (Figure 5).

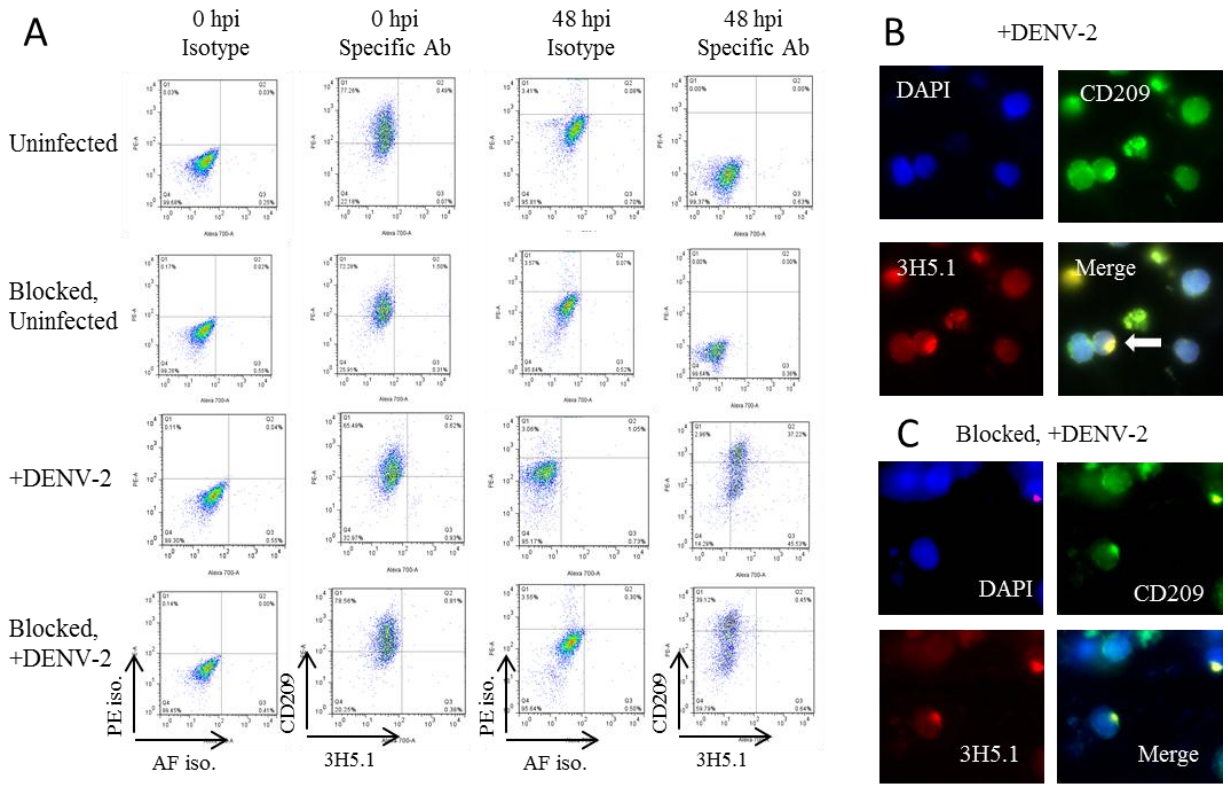

Figure 5. Infection of Raji/DC-SIGN cells by DENV-2 is DC-SIGN dependent.
A) Flow cytometric plots showing the infection of Raji/DC-SIGN cells at 0 and 48 hpi with blocking DC-SIGN prior to infection. Gating is derived from isotype controls. B) and C) IFA of mock-blocked, DENV-2 infected cells (B) and blocked, DENV-2 infected cells (C) at 48 hpi. Arrow=infected cell. N=1.

Blocking DC-SIGN with a monoclonal antibody prior to infection greatly reduced the detection of DENV-2-specific antigen by the 3H5.1 antibody. At 48 hpi, 80.9% of cells are infected by DENV-2, while less than 1% of cells that have been blocked prior to infection are DENV-2 positive. This indicates that the infection of Raji/DC-SIGN cells is DC-SIGN dependent. Since literature has already been published on this finding[70], this result validates my laboratory methods.

As was seen with Raji/0 cells (Figure 4), the detected DC-SIGN levels decrease between 0 and 48 hpi. Putting aside the discussion of why Raji/0 cells are expressing DC-SIGN in the first place, the Raji cells may be losing the DC-SIGN expression plasmid with time, since no selection marker exists within the plasmid.

4.4 DENV-2 INFECTION OF PRIMARY RESTING AND ACTIVATED B CELLS

Since my experimental methodology had been validated in the Raji/DC-SIGN cell line, I next moved to using primary resting or activated B cells. The B cells were activated with a cocktail of CD40L and IL-4, mimicking $CD4^+$ T activation in the germinal center.

DENV-2 infection was performed on both resting and activated B cells. As in previous experiments, analysis was conducted at 0 and 48 hpi. Three independent experiments were performed (Figure 6) using B cells from three different blood donors (groups 1, 2, and 3 in Figure 6).

The DC-SIGN expression of rB and aB does not differ significantly ($p=0.36$) when pooled together. Individually, blood donors 1 and 2 do upregulate DC-SIGN upon activation – Donor 1 increases DC-SIGN expression in aB compared to rB by 9.1%, Donor 2 by 7.8%. Donor 3 is unique in that there is very high DC-SIGN expression in rB at 0 hpi (23.8% positive), which is decreased at all future timepoints and with activation. Extensive literature has been published using the method of activating B cells using the CD40L and IL-4 cocktail that I use here. By another marker of activation, CD23, the aB are, in fact, activated when compared with rB ($p=0.299*10^{-8}$, Figure 7). A different blood donor was used as a source of B cells for each of the three experiments that were conducted.

In infection of resting B cells (rB) and activated B cells (aB) with DENV-2, resting B cells are not infected, while activated B cells are infected. The detection of DENV-2 in activated B cells is not statistically significant between the two time-points tested. The difference in 3H5.1

expression between infected aB and rB at 48 hpi is mildly significant (p=0.07). Using the focus forming unit assay, DENV-2 does not productively replicate in aB, since there is no virus detected at 48 hpi in supernatants from infected cells. Another method to analyze the infection of cells would be to test the supernatants for an increase in the copy number of (positive-sense) genomes by quantitative PCR. Resting B cells are not infected by DENV, and there is no statistically significant difference between 3H5.1 detection at 0 hpi and 48 hpi.

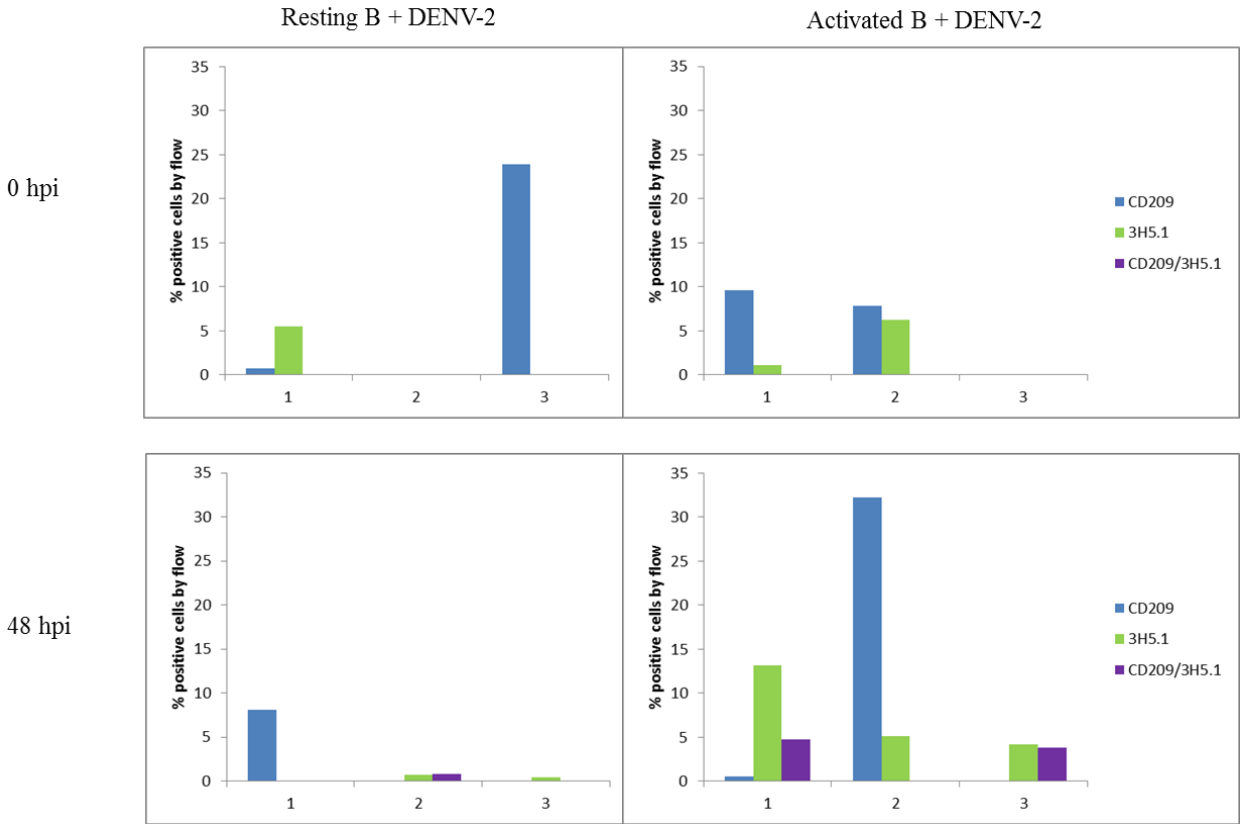

Figure 6. DENV-2 does not infect resting B cells, but does infect activated B cells.
Left column, resting B cells; Right column, activated B cells. Numbers along X-axis represent the 3 separate independent experiments conducted, each with a different blood donor. For 3H5.1 and 3H5.1/CD209, values presented are % positive over the mock-infected control values for the same antibody. For CD209, values presented are the % positive over isotype controls. CD209=DC-SIGN.

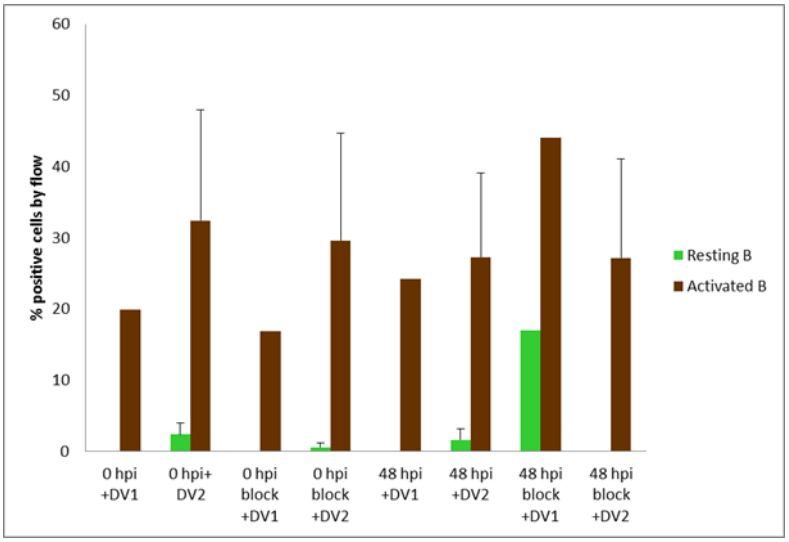

Figure 7. CD23 is upregulated with B cell activation.
CD23 expression, a marker of B cell activation, in resting B cells (green) and activated B cells (brown) at 0 and 48 hpi with various treatments and virus infections. Bars represent the standard error of the mean.

To fully investigate the role of DC-SIGN as a receptor for DENV-2 infection, I performed an experiment in which the cells were pre-incubated with a monoclonal anti-DC-SIGN antibody. This incubation causes the receptor to be endocytosed, thus making it unavailable to both a pathogen and other DENV-specific antibodies. In one experiment, DC-SIGN was successfully blocked with a monoclonal antibody prior to infection (Figure 8). Ideally, the DC-SIGN expression on any DC-SIGN expressing cell would be close to zero percent after blocking.

In this experiment, a low level of DENV was detected at 0 hpi, and this detection increased with the blocking of DC-SIGN prior to infection. PRrevious experiments (Figure 5) indicated that DC-SIGN was important to DENV-2 infection of Raji/DC-SIGN cells, while the increased detection at 0 hpi in aB could indicate that a receptor other than DC-SIGN is also important for infection.

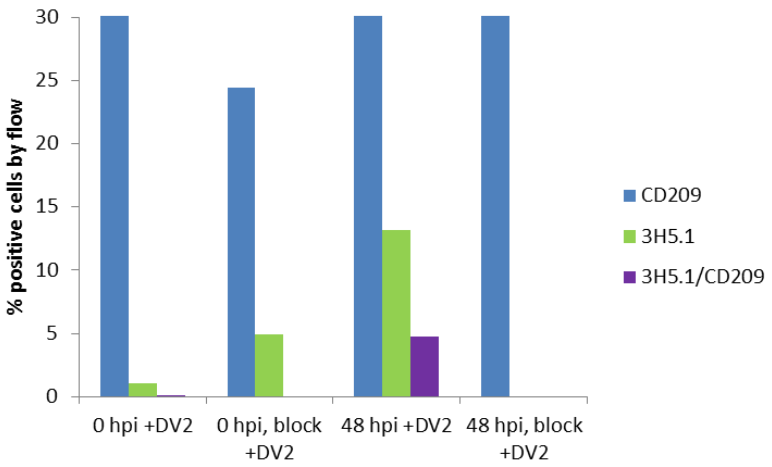

Figure 8. DC-SIGN is an important receptor for DENV-2 infection of activated B cells. Activated B cells express DC-SIGN (CD209), which is reduced upon blocking with an anti-DC-SIGN antibody. Data are from one experiment with blood donor number 1.

Here, although DC-SIGN is only reduced by approximately 10%, the downregulation is still notable. This observation is due to the detection of endocytosed or newly-synthesized DC-SIGN that is made available to the flow cytometry antibody by permeabilization. Performing the stain with the anti-DC-SIGN antibody prior to permeabilization would allow for a true quantification of the DC-SIGN levels after blocking. This would also allow for a definitive statement to be made about if DENV-2 infection of aB is DC-SIGN dependent. As the data stands, DC-SIGN is important to DENV-2 infection.

In order to validate these data, the same blocking experiment was independently repeated twice (Figure 9).

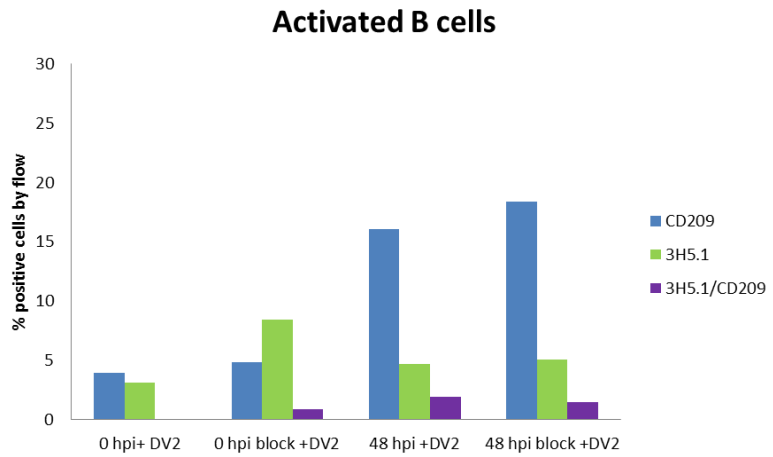

Figure 9. In two experiments, blocking DC-SIGN prior to the infection of activated B cells did not reduce DC-SIGN detection as expected.
Values presented are the average of two independent experiments, using blood donors 2 and 3. For 3H5.1 and 3H5.1/CD209, values presented are % positive over the mock-infected control values for the same antibody. For CD209, values presented are % positive over isotype control. N=2.

Staining for DC-SIGN after blocking at 0 hpi, approximately 2 hours post-blocking, should result in a strong reduction in DC-SIGN levels when compared to non-blocked cells. This is not observed (Figure 9), and DC-SIGN levels are actually slightly higher in blocked cells at 0 hpi than in the mock blocked cells (4.8% as compared to 3.9% DC-SIGN positive cells, respectively). There is not a significant difference in 3H5.1 detection between the two timepoints, indicating that the DENV-2 infected cells do not support replication of the virus.

4.5 DENV-1 INFECTION OF RESTING AND ACTIVATED B CELLS

In order to compare the differences between DENV serotypes in the infection of B cells, infections were also performed using the DENV-1 strain Hawaii (Figure 10). Infections were

performed as previously described in two independent experiments. Infections were not performed on Raji/DC-SIGN cells before conducting experiments on primary rB and aB.

DENV-1 does not infect rB cells at 0 hpi, and the detection of the DENV antigen 2H2 does not differ significantly between 0 and 48 hpi ($p=0.13$). DENV-1 does not infect aB, and 2H2 levels do not significantly increase as they should if infection was present at 48 hpi ($p=0.15$). DC-SIGN expression does not differ significantly between rB and aB ($p=0.25$). The same blood donors for these experiments were used in the infections with DENV-2, and so the lack of DC-SIGN upregulation is expected. As in the DENV-2 infections, CD23 expression does increase significantly with activation of B cells ($p=0.01$, Figure 7).

DC-SIGN expression between infection with DENV-2 (Figure 6) and DENV-1 (Figure 10) differs. The same blood donors were used in each experiment, and infections with the different serotypes of virus were conducted at the same time. Viability between the cell populations did not differ. One explanation for this observation could be that the receptor is being endocytosed with viral binding and entry, but this is not reflected in the percentage of DENV-1 positive cells.

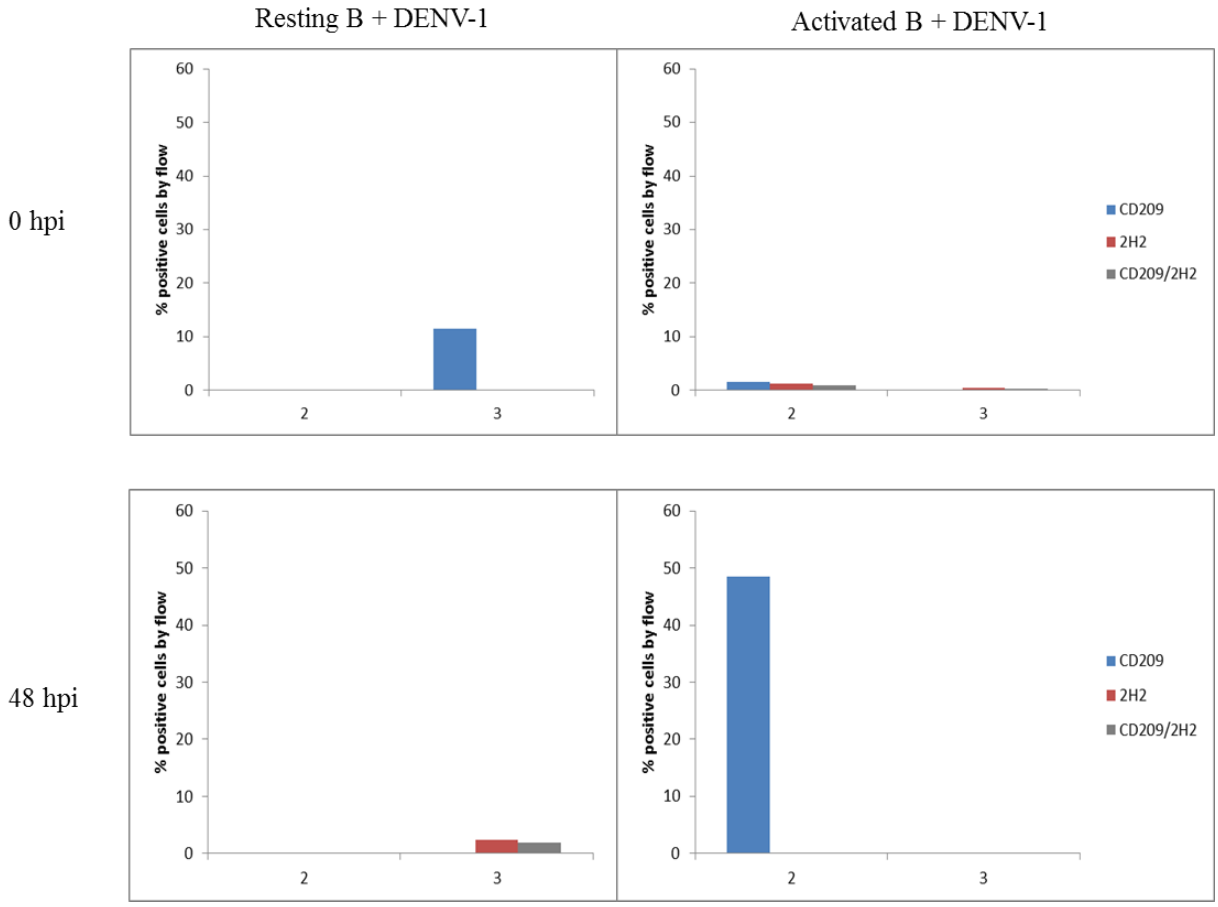

Figure 10. DENV-1 does not infect resting or activated B cells.
Resting B cells, left column; Activated B cells, right column. Values along X-axis represent the two independent experiments conducted with blood from donors 2 and 3. For 2H2 and 2H2/CD209, values presented are % positive over the mock-infected control values for the same antibody. For CD209, values presented are % positive over isotype control. CD209=DC-SIGN.

5.0 DISCUSSION

The majority of my thesis comprised of methods validation. Initially, I established the correct volume of Zenon kit to visually quantitate DENV-2 infection of Vero cells, and this volume was used for all other experiments. I also established a timepoint at which infection was detected in the model system of Raji/DC-SIGN cells, as well as confirming that DENV infection of Raji/DC-SIGN cells may be DC-SIGN dependent.

I also performed experiments on Raji/DC-SIGN cells using DENV-2 to establish protocols within the model system. DENV has been shown to fuse to the endosome within 5 minutes of endocytosis[71], and others have used various times of incubation (1 hour[72 73 74], 90 minutes[46 38 47 48], 2 hours[75 76], 4 hours[70]). Incubating the virus with cells for 1 hour is a standard procedure in studies of virus infectivity, and was therefore chosen in this study as well. Raji/DC-SIGN infection by DENV-2 was detected at 48 hpi, and this timepoint was used for all future experiments. The infection of Raji/DC-SIGN cells was eliminated when cells were pre-blocked with a monoclonal anti-DC-SIGN antibody. Images of this experiment (Figure 5) demonstrate that infection can be visualized via IFA, and that DENV-2 infected cells are also DC-SIGN expressing cells. It is interesting to note that there is a low percentage (approximately 25%) of DENV-infected cells by IFA, while by flow cytometry, 80.9% of cells were DENV-positive. Fundamentally, the detection methods do not differ significantly between these two analysis methods, so the same percentage of DENV-infected cells should be noted with each method.

Flow cytometry is a more accurate method of protein quantification per cell than IFA, so this method is the predominant one presented. Analysis of infection by another method, such as detecting nonstructural proteins by Western blot, would have given a clear indication of the extent of infection.

It was observed that DC-SIGN expression decreases with time within the Raji/DC-SIGN cell population. DENV-2 infection may cause the downregulation of DC-SIGN expression. Receptor downregulation occurs in other viral systems, either as a part of immune evasion (HIV[77]) or as a consequence of infection (measles[78]). DENV may downregulate DC-SIGN in order to prevent re-infection of cells that it has just been released from.

Since Raji cells are used as a model for B lymphocytes, I expected to see similar results regarding DENV-2 infection in aB as I did in Raji/DC-SIGN cells. My results indicate that DENV-2 infects aB, but that the infection is not statistically significant when compared to rB. This may be due to the low DC-SIGN expression in general of the aB. If DENV-2 is indeed entering B cells using DC-SIGN as a receptor, as it does in Raji/DC-SIGN cells, then the low receptor availability may explain the low percentage of infected aB observed. This supports my hypothesis that the infection of B cells is DC-SIGN dependent. In these experiments up to 80% of Raji/DC-SIGN cells expressed DC-SIGN, while an average of 15% of aB expressed DC-SIGN. Since a large part of my research questions are reliant on aB expressing DC-SIGN, this relatively low DC-SIGN expression (when compared to Raji/DC-SIGN cells) dampens any results observed.

Within the larger investigation of the DC-SIGN dependence of DENV infection, it was observed that blocking did not work (significantly reduce DC-SIGN detection by flow) as expected in two out of three B cell experiments, while it was moderately successful with

blocking on Raji/DC-SIGN cells. When the receptor was successfully blocked, either in aB or Raji/DC-SIGN cells, DC-SIGN was shown to be important for DENV-2 infection. Multiple other experiments should be conducted to fully investigate the extent to which DC-SIGN mediates DENV infection of B cells. First, the flow cytometry staining protocol should be changed so that the antibody against DC-SIGN is added before permeabilization of cells. This would detect only surface DC-SIGN, and with blocking using monoclonal anti-DC-SIGN, the total percent of DC-SIGN positive cells should significantly decrease. Single-cell qRT-PCR could also be used to detect the copy number of DC-SIGN in relation to the number of copies of DENV. This would investigate if there is a minimum number of DC-SIGN molecules needed per cell for infection.

Both tested serotypes of DENV (1 and 2) do not infect rB, indicating that rB may not be a natural target for DENV infection. Further experiments need to be done on rB with other serotypes of DENV (DENV-3 and DENV-4) before making a statement about if they are truly not infected by DENV. DENV-1 infects neither resting nor aB. In this regard, this serotype differs from DENV-2, which infects aB. Since the blood donors used for the experiments were the same, and DC-SIGN expression did not significantly differ between resting and aB, the reason for the difference in infection of aB between serotypes remains unclear. I show that DC-SIGN is important for DENV-2 infection of aB, but it is highly likely that one or more other receptors are a part of the infection of aB. Trypsinization of cells before infection would establish if the infection of aB is due to virus:receptor interactions, or whether the cells are nonspecifically taking up the virus.

This research is novel since it uses purified primary resting and aB as a target for DENV infection. One paper[48] has shown infection in purified resting B cells. Others who have researched DENV infection *in vitro* have used pools of cells – either splenic mononuclear cells[47]

or peripheral blood mononuclear cells[46] – have not found B cells to be a target for infection. The circulating PBMCs would contain rB, while B cells in the spleen are transitional B cells[79]. Since rB cells typically express low levels of DC-SIGN, my finding that DENV-1 and DENV-2 do not infect rB is expected, while it contradicts Lin et al's paper. Lin et al used laboratory-adapted strains of virus passaged on *Aedes albopictus* C6/36 cells, as I did in this thesis.

The literature has not yet reflected infection of DENV within the secondary lymphoid organs, where the majority of aB would be found. My research is novel in this respect, and I have found that DENV-2 infects aB, and that DC-SIGN is important in this infection.

6.0 FUTURE DIRECTIONS

In order to fully validate the results described here, several issues need to be considered. First, the blocking of DC-SIGN using a monoclonal antibody should be validated. Another vial for the antibody should be used, and the affinity of the antibody for DC-SIGN should be established on Raji/DC-SIGN cells before use on B lymphocytes.

With regards to the infection of cells, performing infection with UV-inactivated DENV would allow for the investigation as to whether cells are nonspecifically picking up virus. Performing infection on trypsinized cells, thus removing all of the proteins from the surface of cells, would also investigate if there were other receptors aiding in the endocytosis of the virus.

The infection of cells needs to be analyzed by other methods than ones reliant on fluorescence. Numerous methods[80 81 82] have been published for the quantification of DENV by qRT-PCR, and using primers specific for the negative strand genome would allow for quantification of the replicative genome.

Given the negative findings, I believe it can be concluded that neither DENV-2 nor DENV-1 infects rB. Infection of aB by DENV-2 should be further investigated using many additional blood donors, and it may be worthwhile to extend the investigation to DENV-1 and DENV-4 infection.

BIBLIOGRAPHY

1. Guzmán, M.G. & Kourí, G. Dengue: an update. *Lancet Infect Dis* **2**, 33-42 (2002).
2. Mackenzie, J.S., Gubler, D.J. & Petersen, L.R. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. *Nat. Med.* **10**, S98-109 (2004).
3. Barrett, A.D.T. & Teuwen, D.E. Yellow fever vaccine - how does it work and why do rare cases of serious adverse events take place? *Curr. Opin. Immunol.* **21**, 308-313 (2009).
4. Heinz, F.X., Holzmann, H., Essl, A. & Kundi, M. Field effectiveness of vaccination against tick-borne encephalitis. *Vaccine* **25**, 7559-7567 (2007).
5. Halstead, S.B. & Thomas, S.J. Japanese encephalitis: new options for active immunization. *Clin. Infect. Dis.* **50**, 1155-1164 (2010).
6. Whitehead, S.S., Blaney, J.E., Durbin, A.P. & Murphy, B.R. Prospects for a dengue virus vaccine. *Nat. Rev. Microbiol.* **5**, 518-528 (2007).
7. Guy, B. *et al.* Preclinical and clinical development of YFV 17D-based chimeric vaccines against dengue, West Nile and Japanese encephalitis viruses. *Vaccine* **28**, 632-649 (2010).
8. WHO | Dengue haemorrhagic fever: diagnosis, treatment, prevention and control. 2nd edition. Geneva : World Health Organization. *WHO* at <http://www.who.int/csr/resources/publications/dengue/Denguepublication/en/>
9. Bennett, S.N. *et al.* Selection-Driven Evolution of Emergent Dengue Virus. *Molecular Biology and Evolution* **20**, 1650 -1658 (2003).
10. Tolou, H.J. *et al.* Evidence for recombination in natural populations of dengue virus type 1 based on the analysis of complete genome sequences. *J. Gen. Virol.* **82**, 1283-1290 (2001).
11. Uzcategui, N.Y. *et al.* Molecular epidemiology of dengue type 2 virus in Venezuela: evidence for in situ virus evolution and recombination. *J. Gen. Virol.* **82**, 2945-2953 (2001).
12. Su, M.W., Yuan, H.S. & Chu, W.C. Recombination in the Nonstructural Gene Region in Type 2 Dengue Viruses. *Intervirology* (2011).doi:10.1159/000327786
13. Carvalho, S.E.S., Martin, D.P., Oliveira, L.M., Ribeiro, B.M. & Nagata, T. Comparative analysis of American Dengue virus type 1 full-genome sequences. *Virus Genes* **40**, 60-66 (2010).
14. Roehrig, J.T., Hombach, J. & Barrett, A.D.T. Guidelines for Plaque-Reduction Neutralization Testing of Human Antibodies to Dengue Viruses. *Viral Immunol.* **21**, 123-132 (2008).

15. Mukhopadhyay, S., Kuhn, R.J. & Rossmann, M.G. A structural perspective of the flavivirus life cycle. *Nat Rev Micro* **3**, 13-22 (2005).

16. Welsch, S. *et al.* Composition and three-dimensional architecture of the dengue virus replication and assembly sites. *Cell host microbe* **5**, 365-375 (2009).

17. Wu, C.-F., Wang, S.-H., Sun, C.-M., Hu, S.-T. & Syu, W.-J. Activation of dengue protease autocleavage at the NS2B-NS3 junction by recombinant NS3 and GST-NS2B fusion proteins. *J. Virol. Methods* **114**, 45-54 (2003).

18. Markoff, L. In vitro processing of dengue virus structural proteins: cleavage of the pre-membrane protein. *J. Virol.* **63**, 3345-3352 (1989).

19. Rodenhuis-Zybert, I.A. *et al.* Immature dengue virus: a veiled pathogen? *PLoS Pathog.* **6**, e1000718 (2010).

20. Wang, S., He, R. & Anderson, R. PrM- and cell-binding domains of the dengue virus E protein. *J. Virol.* **73**, 2547-2551 (1999).

21. Alvarez, D.E., Filomatori, C.V. & Gamarnik, A.V. Functional analysis of dengue virus cyclization sequences located at the 5' and 3'UTRs. *Virology* **375**, 223-235 (2008).

22. Alvarez, D.E., Lodeiro, M.F., Ludueña, S.J., Pietrasanta, L.I. & Gamarnik, A.V. Long-range RNA-RNA interactions circularize the dengue virus genome. *J. Virol.* **79**, 6631-6643 (2005).

23. Liu, L. *et al.* Flavivirus RNA cap methyltransferase: structure, function, and inhibition. *Front Biol* **5**, 286-303 (2010).

24. Filomatori, C.V. *et al.* A 5' RNA element promotes dengue virus RNA synthesis on a circular genome. *Genes Dev.* **20**, 2238-2249 (2006).

25. Zeng, L., Falgout, B. & Markoff, L. Identification of specific nucleotide sequences within the conserved 3'-SL in the dengue type 2 virus genome required for replication. *J. Virol.* **72**, 7510-7522 (1998).

26. Stiasny, K. & Heinz, F.X. Flavivirus membrane fusion. *J. Gen. Virol.* **87**, 2755-2766 (2006).

27. van der Schaar, H.M. *et al.* Dissecting the Cell Entry Pathway of Dengue Virus by Single-Particle Tracking in Living Cells. *PLoS Pathog* **4**, e1000244 (2008).

28. Pokidysheva, E. *et al.* Cryo-EM reconstruction of dengue virus in complex with the carbohydrate recognition domain of DC-SIGN. *Cell* **124**, 485-493 (2006).

29. Mondotte, J.A., Lozach, P.-Y., Amara, A. & Gamarnik, A.V. Essential role of dengue virus envelope protein N glycosylation at asparagine-67 during viral propagation. *J. Virol.* **81**, 7136-7148 (2007).

30. Gubler, D.J. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends Microbiol.* **10**, 100-103 (2002).

31. Dalrymple, N. & Mackow, E.R. Productive dengue virus infection of human endothelial cells is directed by heparan sulfate-containing proteoglycan receptors. *J. Virol.* **85**, 9478-9485 (2011).

32. Thaisomboonsuk, B.K., Clayson, E.T., Pantuwatana, S., Vaughn, D.W. & Endy, T.P. Characterization of dengue-2 virus binding to surfaces of mammalian and insect cells. *Am. J. Trop. Med. Hyg.* **72**, 375-383 (2005).

33. Cabrera-Hernandez, Arturo & Smith, Duncan R. Mammalian Dengue Virus Receptors. *Dengue Bulletin* **29**, (2005).

34. Esko, J.D. & Lindahl, U. Molecular diversity of heparan sulfate. *J Clin Invest* **108**, 169-173 (2001).

35. Rodenhuis-Zybert, I.A., Wilschut, J. & Smit, J.M. Dengue virus life cycle: viral and host factors modulating infectivity. *Cell. Mol. Life Sci.* **67**, 2773-2786 (2010).

36. Jessie, K., Fong, M.Y., Devi, S., Lam, S.K. & Wong, K.T. Localization of dengue virus in naturally infected human tissues, by immunohistochemistry and in situ hybridization. *J. Infect. Dis.* **189**, 1411-1418 (2004).

37. Wu, S.J. *et al.* Human skin Langerhans cells are targets of dengue virus infection. *Nat. Med.* **6**, 816-820 (2000).

38. Wati, S., Li, P., Burrell, C.J. & Carr, J.M. Dengue virus (DV) replication in monocyte-derived macrophages is not affected by tumor necrosis factor alpha (TNF-alpha), and DV infection induces altered responsiveness to TNF-alpha stimulation. *J. Virol.* **81**, 10161-10171 (2007).

39. Gubler, D.J. Dengue and dengue hemorrhagic fever. *Clin. Microbiol. Rev.* **11**, 480-496 (1998).

40. Nielsen, D.G. The relationship of interacting immunological components in dengue pathogenesis. *Virol. J.* **6**, 211 (2009).

41. Diamond, M.S., Pierson, T.C. & Fremont, D.H. The structural immunology of antibody protection against West Nile virus. *Immunol. Rev.* **225**, 212-225 (2008).

42. Cardosa, J.M., Porterfield, J.S. & Gordon, S. Complement receptor mediates enhanced flavivirus replication in macrophages. *J Exp Med* **158**, 258-263 (1983).

43. Fernandez-Garcia, M.-D., Mazzon, M., Jacobs, M. & Amara, A. Pathogenesis of flavivirus infections: using and abusing the host cell. *Cell Host Microbe* **5**, 318-328 (2009).

44. Espina, L.M., Valero, N.J., Hernández, J.M. & Mosquera, J.A. Increased apoptosis and expression of tumor necrosis factor-alpha caused by infection of cultured human monocytes with dengue virus. *Am. J. Trop. Med. Hyg.* **68**, 48-53 (2003).

45. Hober, D. *et al.* Serum levels of tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6), and interleukin-1 beta (IL-1 beta) in dengue-infected patients. *Am. J. Trop. Med. Hyg.* **48**, 324-331 (1993).

46. Kou, Z. *et al.* Monocytes, but not T or B cells, are the principal target cells for dengue virus (DV) infection among human peripheral blood mononuclear cells. *J. Med. Virol.* **80**, 134-146 (2008).

47. Blackley, S. *et al.* Primary human splenic macrophages, but not T or B cells, are the principal target cells for dengue virus infection in vitro. *J. Virol.* **81**, 13325-13334 (2007).

48. Lin, Y.-W. *et al.* Virus replication and cytokine production in dengue virus-infected human B lymphocytes. *J. Virol.* **76**, 12242-12249 (2002).

49. Burton, D.R. Antibodies, viruses and vaccines. *Nat. Rev. Immunol.* **2**, 706-713 (2002).

50. Nature Full Text PDF. at <http://www.nature.com/nri/journal/v8/n1/pdf/nri2206.pdf>

51. Rodríguez-Pinto, D. B cells as antigen presenting cells. *Cell. Immunol.* **238**, 67-75 (2005).

52. Cheng, P.C., Steele, C.R., Gu, L., Song, W. & Pierce, S.K. MHC class II antigen processing in B cells: accelerated intracellular targeting of antigens. *J. Immunol.* **162**, 7171-7180 (1999).

53. Zimmermann, V.S. *et al.* Engagement of B cell receptor regulates the invariant chain-dependent MHC class II presentation pathway. *J. Immunol.* **162**, 2495-2502 (1999).

54. Chaudhry, A. *et al.* HIV-1 Nef promotes endocytosis of cell surface MHC class II molecules via a constitutive pathway. *J. Immunol.* **183**, 2415-2424 (2009).

55. Schindler, M. *et al.* Down-modulation of mature major histocompatibility complex class II and up-regulation of invariant chain cell surface expression are well-conserved functions of human and simian immunodeficiency virus nef alleles. *J. Virol.* **77**, 10548-10556 (2003).

56. Li, D. *et al.* Down-regulation of MHC class II expression through inhibition of CIITA transcription by lytic transactivator Zta during Epstein-Barr virus reactivation. *J. Immunol.* **182**, 1799-1809 (2009).

57. Sirianni, M.C. *et al.* Control of human herpes virus type 8-associated diseases by NK cells. *Ann. N. Y. Acad. Sci.* **1096**, 37-43 (2007).

58. Hershkovitz, O. *et al.* Dengue virus replicon expressing the nonstructural proteins suffices to enhance membrane expression of HLA class I and inhibit lysis by human NK cells. *J. Virol.* **82**, 7666-7676 (2008).

59. Stamataki, Z. *et al.* Hepatitis C virus association with peripheral blood B lymphocytes potentiates viral infection of liver-derived hepatoma cells. *Blood* **113**, 585-593 (2009).

60. Wang, L. *et al.* DC-SIGN (CD209) Promoter -336 A/G polymorphism is associated with dengue hemorrhagic fever and correlated to DC-SIGN expression and immune augmentation. *PLoS Negl Trop Dis* **5**, e934 (2011).

61. Full Text PDF. at <http://jvi.asm.org/content/76/13/6841.full.pdf>

62. Colmenares, M., Puig-Kröger, A., Pello, O.M., Corbí, A.L. & Rivas, L. Dendritic cell (DC)-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN, CD209), a C-type surface lectin in human DCs, is a receptor for Leishmania amastigotes. *J. Biol. Chem.* **277**, 36766-36769 (2002).

63. Ludwig, I.S. *et al.* Hepatitis C virus targets DC-SIGN and L-SIGN to escape lysosomal degradation. *J. Virol.* **78**, 8322-8332 (2004).

64. Rappocciolo, G. *et al.* Human herpesvirus 8 infects and replicates in primary cultures of activated B lymphocytes through DC-SIGN. *J. Virol.* **82**, 4793-4806 (2008).

65. Geijtenbeek, T.B.H. *et al.* Mycobacteria target DC-SIGN to suppress dendritic cell function. *J. Exp. Med.* **197**, 7-17 (2003).

66. Geijtenbeek, T.B. *et al.* DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* **100**, 587-597 (2000).

67. He, B. *et al.* HIV-1 envelope triggers polyclonal Ig class switch recombination through a CD40-independent mechanism involving BAFF and C-type lectin receptors. *J. Immunol.* **176**, 3931-3941 (2006).

68. Rappocciolo, G. *et al.* DC-SIGN on B lymphocytes is required for transmission of HIV-1 to T lymphocytes. *PLoS Pathog.* **2**, e70 (2006).

69. Schlessinger, W. & Frankel, J.W. Adaptation of the New Guinea B strain of dengue virus to suckling and to adult swiss mice; a study in viral variation. *Am. J. Trop. Med. Hyg.* **1**, 66-77 (1952).

70. Alen, M.M.F. *et al.* Antiviral activity of carbohydrate-binding agents and the role of DC-SIGN in dengue virus infection. *Virology* **387**, 67-75 (2009).

71. PLoS Full Text PDF. at <http://www.plospathogens.org/article/fetchObjectAttachment.action?uri=info%3Adoi%2F10.1371%2Fjournal.ppat.1000244&representation=PDF>

72. Huang, K.-J. *et al.* The dual-specific binding of dengue virus and target cells for the antibody-dependent enhancement of dengue virus infection. *J. Immunol.* **176**, 2825-2832 (2006).

73. Chase, A.J., Medina, F.A. & Muñoz-Jordán, J.L. Impairment of CD4+ T cell polarization by dengue virus-infected dendritic cells. *J. Infect. Dis.* **203**, 1763-1774 (2011).

74. Rodriguez-Madoz, J.R., Bernal-Rubio, D., Kaminski, D., Boyd, K. & Fernandez-Sesma, A. Dengue virus inhibits the production of type I interferon in primary human dendritic cells. *J. Virol.* **84**, 4845-4850 (2010).

75. Tassaneetrithep, B. *et al.* DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J. Exp. Med.* **197**, 823-829 (2003).

76. Navarro-Sanchez, E. *et al.* Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses. *EMBO Rep.* **4**, 723-728 (2003).

77. Dubé, M., Bego, M.G., Paquay, C. & Cohen, É.A. Modulation of HIV-1-host interaction: role of the Vpu accessory protein. *Retrovirology* **7**, 114 (2010).

78. Schneider-Schaulies, J. *et al.* Receptor usage and differential downregulation of CD46 by measles virus wild-type and vaccine strains. *Proceedings of the National Academy of Sciences* **92**, 3943 -3947 (1995).

79. Allman, D., Srivastava, B. & Lindsley, R.C. Alternative routes to maturity: branch points and pathways for generating follicular and marginal zone B cells. *Immunol. Rev.* **197**, 147-160 (2004).

80. Warrilow, D., Northill, J.A., Pyke, A. & Smith, G.A. Single rapid TaqMan fluorogenic probe based PCR assay that detects all four dengue serotypes. *J. Med. Virol.* **66**, 524-528 (2002).

81. Poloni, T.R. *et al.* Detection of dengue virus in saliva and urine by real time RT-PCR. *Virol. J.* **7**, 22 (2010).

82. Gurukumar, K.R. *et al.* Development of real time PCR for detection and quantitation of Dengue Viruses. *Virol. J.* **6**, 10 (2009).