EVALUATION OF DENGUE VIRUS INFECTION IN IMMUNE CELLS OF HUMAN SKIN

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ABSTRACT

Dengue virus (DENV) is a reemerging infectious agent causing an estimated 50-100 million cases annually and is endemic in more than 100 countries. Recently there has been an increase in the geographic spread, the overall number, and the severity of disease cases, making DENV an increasing threat to public health. The most prevalent of the arboviruses, DENV is introduced into human hosts during a vector mosquito's blood meal; here, virus is deposited into the epidermal and dermal layers of the skin. Therefore, elucidating the cutaneous immune response to DENV is critical to our understanding of the immune mechanisms involved in protection and pathogenesis of DENV and to the development of effective treatments and vaccines. In addition to resident T- and B-cells, the skin immune system includes three major populations of innate immune cells, dermal dendritic cells (dDCs), dermal macrophages (dMØs), and epidermal Langerhans' cells (LCs). These immune cells are potential targets of DENV infection. The receptor profile of dDCs and dMØs indicates that they are susceptible to DENV infection, however evidence of this is lacking. LCs are infected following *ex vivo* inoculation of human skin explants, yet their role in DENV pathogenesis remains unclear. Upon exposure to a pathogen, cutaneous immune cells mature and migrate to the cutaneous draining lymph node *via* the draining lymphatics; these functions are critical to the induction of adaptive immune responses and to pathogen clearance. It remains unclear how DENV infection affects the maturation, migration and survival of cutaneous immune cells. Herein, we use a combination of *in vitro* studies and *ex vivo* inoculation of healthy human skin explants to explore the role of cutaneous immune cells in DENV infection. We investigated the degree to which cutaneous immune cells become infected with DENV and measured upregulation of activation markers, migration out of the skin, and the life span of infected cells. We discovered that populations of cutaneous DCs, as well as dMØs, become infected with DENV upon scarification of skin with

virus. *In vitro*, we confirmed that DENV infection partially inhibits maturation of DCs and potentially MØs. Additionally, DENV infection induces DC apoptosis in a caspase-dependent manner. These results help to clarify the role of cutaneous immune cells in the immunopathogenesis of DENV.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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ABBREVIATIONS

Dengue Virus (DENV), dengue fever (DF), dengue hemorrhagic fever (DHF), dengue shock syndrome (DSS), DC-specific intracellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), mannose receptor (MR), C-type lectin domain family 5 member A (CLEC5), precursor membrane protein (prM), endoplasmic reticulum (ER), dendritic cell (DC), monocytes-derived dendritic cell (mdDC), myeloid (mDC), plasmacytoid (pDC), Toll-like receptor (TLR), monocytes (MO), macrophage (MØ), dermal macrophage (dMØ), dermal dendritic cell (dDC), Langerhans' cell (LC), major histocompatibility complex-II (MHC-II), pathogen recognition receptor (PRR), Birbeck granules (BG), contact hypersensitivity (CHS), tumor necrosis factor (TNF), interferon (IFN), cell-mediated immunity (CMI), multiplicity of infection (MOI), New Guinea C (NGC), baby hamster kidney (BHK), Human Leukocyte Antigen DR-1 (HLA-DR).

1.0 INTRODUCTION

Dengue (DENV) is the most prevalent arthropod-borne viral disease worldwide causing an estimated 50-100 million cases annually, according to the World Health Organization. While a majority of these cases are asymptomatic or manifest as a self limiting febrile illness known as dengue fever (DF), approximately 500,000 cases progress to a severe forms of the disease, known as dengue hemorrhagic fever (DHF). A fraction of DHF patients develop dengue shock syndrome (DSS), which has a case fatality rate of 20% (1). Currently DENV is endemic in more than 100 countries located primarily in tropical and sub-tropical regions of the world. However, the potential for farther geographic spread is great as it depends solely on the habitable range of the vectors, *Aedes aegypti and Aedes albopictus* (2, 3). The primary vector, *A. aegypti*, thrives in rural and urban settings, while *A. albopictus,* an increasingly capable secondary vector, is an urbanized species that thrives in densely populated regions. Since DENV persists in a mosquito-human-mosquito transmission cycle, dense populations increase the efficiency of DENV transmission and the likelihood of co-circulating serotypes (4). Epidemiological data suggest that secondary infection with a heterotypic serotype of DENV is the primary risk factor for development of DHF and DSS (5). It is estimated by public health authorities that two billion of the world's population live at risk of acquiring dengue infection. As of July 2009 the two mosquito vectors could be found in 28 of the United States, which puts an additional 170 million Americans at risk for infection (2, 3). Despite the escalating disease threat there is currently no licensed vaccine for DENV, although there is a candidate vaccine in clinical trials, and the ill-defined disease pathogenesis complicates the development of a vaccine that is both safe and effective (6). Due to the vector-borne nature of the virus, elucidating the effects of DENV on the primary targets of infection, thought to be the immune sentinels of the skin, will be vital to this effort.

1.1 DENV BACKGROUND

1.1.1 DENV: classification, structure, and life cycle

Dengue virus is a member of the *Flavivirdae* family, genus Flavivirus, along with many other mosquito-borne viruses that are a disease threat to humans such as; West Nile Virus, Japanese encephalitis, and yellow fever virus (7). Mature virions have a classic icosahedral shape and are approximately 30nm in diameter (8). Upon cell egress the virion acquires a host-cell derived lipid by-layer envelope in which 180 copies of the viral membrane (M) and envelope (E) protein are embedded (7). This envelope surrounds a viral nucleocapsid formed by multiple copies of the viral capsid protein (C); the nucleocapsid houses a single-stranded positive sense RNA genome that is approximately 10.7 kilobases in length (9). Interaction of DENV with human myeloid cells has been found to involve C-type lectin receptors such as the DC-specific intracellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), mannose receptor (MR), and C-type lectin domain family 5 member A (CLEC5 or MDL-1) (9-11). However, a wide range of cells are permissive to DENV infection, including many mammalian and mosquito cell lines, indicating that viral infection can be mediated by multiple receptors or by virion binding to a ubiquitously expressed surface molecule.

Cell entry relies on clathrin mediated endocytosis and results in virions being internalized into a Rab5 positive early endosome; maturation and acidification of the endosome leads to membrane fusion, uncoating of the nucleocapsid, and release of viral RNA (12, 13). The genome is translated into a single polyprotein that is cleaved by cellular and viral proteases, into three structural proteins (capsid, C; precursor membrane and membrane, prM/M; envelope, E) and seven nonstructural proteins (NS1, 2a, 2b, 3, 4a, 4b and 5) (14). As with other flaviviruses, replication occurs in association with the endoplasmic reticulum (ER). There, particle formation occurs in ER-organelles (lipid droplets) when the newly synthesized genome is encapsidated (15). Association of the prM and E proteins, embedded in the lumen of the ER, is believed to cause a structural change that guides virion budding to the golgi (8, 16). pH changes encountered during egress through the trans-golgi network result in reorganization of prM and E such that the E protein is protected from undergoing conformational changes. This reorganization also creates

a cleavage site for the cellular endoprotease furin to cleave prM; removal of pr peptide is the final step in the formation of a mature virion (9).

There are four serotypes of DENV (DENV1-4) which share great sequence homology. Within each serotype, nucleotide variation allows for the identification of genotypes. Analysis of DENV genotypes has shown that the virus evolves during the course of an epidemic, but it is not known how this may contribute to disease severity. However, it has been shown that genetic variation of the virus and the host can have an effect on disease severity (17). Regardless, all four DENV serotypes are capable of causing asymptomatic infections, as well as a range of disease syndromes from DF to DHF and DSS (18).

1.1.2 DENV pathogenesis and host immune response

Infection with any of the four DENV serotypes can result in asymptomatic infection, as in the majority of cases, or a wide range of clinical symptoms. The least severe form of clinical disease is DF, which manifests with the rapid onset of fever, severe headache, myalgia, gastrointestinal discomfort, and a rash. In cases that resolve without complication these symptoms subside approximately 5-10 days after onset (18). Viral clearance is likely mediated by CD4 and CD8 T-cells as well as the production of virus neutralizing antibodies (19). The severe form of DENV infection (DHF) includes all of the DF symptoms as well as increased vascular permeability, liver enlargement, thrombocytopenia, hemoconcentration, and hemorrhagic manifestations (1, 18). Due to the increased vascular permeability, without appropriate treatment DHF may progress to hypovolemic shock syndrome (DSS) and death (17). The most recognized risk factor associated with the development of severe forms of DENV illness is previous infection with a heterotypic serotype of DENV(5). It is believed that the severity of secondary infection is primarily the result of host immune responses, including roles for heterologous antibody dependant enhancement of infection, activation of cross-reactive memory T-cells, excessive cytokine production, and complement activation.

Strong epidemiological data suggest that the presence of sub-neutralizing and non-protective DENV antibodies, produced during an initial DENV infection, could exacerbate disease upon secondary infection. It is believed that this occurs due to a mechanism called “antibody-dependant enhancement” (ADE), however clinical evidence of this is lacking and the hypothesis

remains controversial. It is proposed that pre-existing DENV antibodies, primarily IgG and IgM, bind DENV and mediate infection of monocytes (MOs) and macrophages (MØs) *via* Fc-receptors (19). This would lead to an increased number of infected cells and amplified virus replication; high levels of viremia early in infection is correlated with progression to DHF/DSS (20). Infection of MOs results in the production of TNF-α which increases vascular permeability. Therefore, enhanced infection and activation of MOs is likely to contribute to the hemorrhagic manifestations of DHF/DSS (20).

The induction of adaptive immune responses is thought to be critical to viral clearance during primary DENV infections. However, the presence of non-protective DENV specific memory cells during secondary infection may be detrimental (18). Memory T-cells become stimulated early in secondary infection and to very high levels. ADE results in increased infection of MOs and MØs; this leads to increased presentation of viral antigen and T-cell stimulation (21). Activated T-cells up-regulate production of pro-inflammatory cytokines such as IFN-γ and TNF-α which act directly on vascular endothelial cells (1) increasing the likelihood of plasma leakage. The presence of low avidity CD8 T-cells may contribute to increased cytokine production and fail to aid in viral clearance (1). Reactivation of memory CD8 T-cells results in the expansion of cross reactive T-cells that preferentially react to the viral epitopes from the primary infection over heterologous viral epitopes from the secondary infection. This phenomenon, termed original antigenic sin, results in CD8 T-cells that produce high levels of inflammatory cytokine but lose cytolytic activity (17).

Increased levels of cytokines including IFN-γ, TNF-α, IL-2, IL-6, IL1-β, and IL-8 in patient sera is predictive of the development of severe DENV disease (22). Furthermore, the presence of cytokines stimulates the production of additional cytokines from neighboring cells resulting in a "cytokine storm." Studies suggest that the damage of endothelial cells during DHF is caused by this cytokine storm and not by viral factors (23). Additionally, these cytokines can facilitate viral infection. One example of this is the up-regulation of Fc receptor on MOs and MØs in the presence of IFN-γ, which leads to amplification of viral replication (17).

Other mechanisms have been proposed as underlying factors in the severity of DHF. Elevated levels of complement activation factors such as C3a, C4a, and C5a in the sera of DHF/DSS patients suggests a role for complement, however the mechanisms remain unclear (22). Additionally, some DENV antibodies produced during infection are cross-reactive to self-

antigens. It is possible that cross-reactivity with endothelial cells results in increased apoptosis (17), however this remains controversial. Regardless, it is clear that the immune response to secondary DENV infections contributes to the manifestations of severe disease symptoms in DHF/DSS cases.

1.2 INNATE IMMUNE CELLS AND DENV

1.2.1 Origin and function of macrophages and dendritic cells

Antigen-presenting cells, including MØs and dendritic cells (DCs), belong to a specialized subset of leukocytes known as the mononuclear phagocyte system. These cells, along with MOs, are critical effectors and regulators of the innate immune response. Extensive research has been done to clarify the origins of these cell types and a recent review highlights the advances and short falls of that knowledge (24). All three cell types arise from hematopoietic stem cells (HCS); developmental pathways diverge in the bone marrow, likely due to varied environmental cues. Myeloid precursors (MP) and lymphoid precursors (LP) differentiate from HSCs. It is now generally accepted that MO, MØ, and DCs all arise from the MP. Two populations of DCs, myeloid (mDC) and plasmacytoid (pDC), are present at low levels in both the blood and peripheral tissues. mDCs, which leave the bone marrow as pre-mDCs, are the strongest antigen presenting cell of the innate immune system. Upon activation, often through toll-like receptors (TLRs), mDCs will assume a mature phenotype, home to the lymph node, and activate appropriate adaptive immune responses (25). pDCs, which leave the bone marrow fully differentiated, are capable of producing copious amounts of IFN-α in response to single stranded RNA, which is critical for innate immune responses against viral infections (25). MØs reside in all lymphoid and non-lymphoid tissues; these cells contribute to homeostasis by clearing apoptotic cells, necrotic cells and pathogens (24). The origin of MØs is less clear than other mononuclear phagocytes, but they are believed to differentiate from blood-borne monocytes (24). Other immune cells arise from HSCs and populate lymphoid and non-lymphoid tissues; those present in the skin are discussed further in section 1.2.2 and will be an integral part of the studies presented in chapter 3.0.

1.2.2 Immune cells of the skin

Due to the vector-borne nature of DENV, these studies will focus mainly on immune cells of the skin and monocyte derived DCs (mdDCs) as an *in vitro* model. In addition to tissue-resident T- and B-cells, the skin is populated by a number of innate immune cells, primarily dermal macrophages (dMØ), dermal dendritic cells (dDCs) and epidermal Langerhans cells (LCs). The origin, phenotype, and function of these cell types are still being elucidated by cutaneous immunologists and there is much that remains unclear and controversial.

1.2.2.1 Dermal macrophages (dMØ)

dMØ reside throughout the papillary and reticular layer of the dermis. During steady-state conditions, dMØs are believed to contribute to tissue homeostasis through the clearance of apoptotic cells and the production of growth factors (26, 27). The origin of these cells, along with other tissue-resident macrophages, is unclear. dMØs have great phagocytic capacity and express a wide array of pathogen-recognition receptors (PRRs). This phenotype allows them to recognize and clear pathogens that penetrate the protective barrier of the epidermis (24). Following pathogen uptake, dMØs are capable of producing inflammatory cytokines that aid in the recruitment of immune cells from the blood (28). Based on their expression of MHC-II dMØs likely possess some antigen-presenting capacity *in vivo*, but *in vitro* assessments of their immunostimulatory capacity (by mixed lymphocyte reactions) indicates that it is quite minimal (27). In addition to their dermal distribution and low MHC-II expression, dMØs can be identified by their expression of CD163. CD163 is a hemoglobin/haptoglobin complex-binding scavenger receptor expressed exclusively on tissue macrophages (27). *In situ* studies have shown that the dMØ phenotype includes MR (CD206) and DC-SIGN (CD209), but lacks the DC markers CD1a, CD1b, CD1c, CD11c, and langerin (CD207) (28). It is difficult to distinguish dMØs from dDCs based on morphology because dMØs range from compact and stellate to elongated and spindled in shape (28).

1.2.2.2 Dermal dendritic cells (dDC)

In contrast to dMØs, dDCs have classic DC morphology and phenotype. They are present in the papillary (upper) layer of the dermis and: (1) express high levels of MHC-II, (2) efficiently

uptake and process antigen, and (3) migrate to the cutaneous draining lymph node to present antigen to T- and B-cells (26). dDCs can be identified by expression of the nonpolymorphic MHC-like lipid antigen-presenting molecule blood DC antigen-1 (BDCA-1, also known as CD1c), with subpopulations identifiable based on the surface markers CD1a (a glycolipid presenting class-I like molecule) and CD14 (29). The three subpopulations, are: $CD1c^{+}CD1a^{high}CD14^{-}$, $CD1c^{+}$ $CD1a^{dim}CD14^{-}$, and $CD1c^{+}CD1a^{-}CD14^{+}$ dDCs (27-30). The panel of receptors on dDCs remains somewhat controversial; it is generally accepted that MR and DEC205 are on all dDC subpopulations. However, whether DC-SIGN is expressed is unclear (27-29). In response to maturation stimuli, dDCs quickly up regulate expression of CCR7, a chemokine receptor that binds CCL19 and CCL21 to direct migration of dDCs to the lymph node (31). Recent studies have identified a dermal-resident DC that expresses langerin (CD207), which was once thought to be found exclusively on epidermal LCs. In contrast to LCs, the langerin expressing subset of dDCs are radiosensitive, express the integrin CD103, and differentiate from blood monocyte precursors (32, 33). $Langerin^{+}$ dDCs are part of the $CD1a^{high}$ subset (30). Functional studies utilizing murine models suggest that this population: (1) migrates more rapidly than LCs, (2) colonizes B-cell rich areas of the lymph-node, and (3) are efficient at cross-presentation (34, 35). While their immunological role remains unclear it is important to recognize them as a distinct dermal population that develop and function independently of classic $langerin^{+}$ epidermal cells.

1.2.2.3 Langerhans' cells (LC)

Another important cell type to consider in skin immunology is the LC. It is believed that MOs, which differentiate from the MO, MØ, and DC precursor (MDP) in the bone marrow, populate the skin early in life and differentiate into LCs (24). These cells then reside and renew in the epidermis during steady-state conditions (36). Experiments using sets of congenic parabiotic mice suggest that during minor injury LCs are capable of renewing without the recruitment of blood precursors, but that extreme injury and inflammation results in the recruitment of CCR2/CCR6 expressing bone marrow derived precursors (36, 37). LCs can be defined morphologically by their classic dendritic appearance and the presence of Birbeck granules (BG); this racket shaped structure is a subunit of the endosomal recycling compartment and is thought to be involved in the uptake of antigen and the internalization of CD207 and CD1a (38).

The formation of BGs is dependent on expression of the Ca^{2+}- dependent type II transmembrane protein langerin (CD207) (26). While CD207 is a defining marker of LCs it is not limited to this cell type (32, 33). In addition to CD207, LCs have the DC markers CD1a and CD1c and lack MR and DC-SIGN (37). The role of LCs in induction of immune responses remains a controversial issue.

There is a longstanding belief that LCs are the immune sentinels of the skin with the primary function of initiating immune responses to epidermal threats (39). However, recent murine models have challenged this belief. In the presence of herpes virus, a highly localized skin tropic pathogen, $CD8\alpha^-$ LCs do not present antigen in the cutaneous draining lymph node. They transfer antigen from the skin to lymph node-resident $CD8\alpha^+$ DCs for presentation to T-cells (40). Contact hypersensitivity (CHS) is a skin inflammatory reaction indicative of a CD8 T-cell response following activation by skin immune cells. This measure has been used extensively to assess the immune function of LCs using mouse strains in which LCs have been selectively removed. These studies have revealed that LCs are not strictly necessary for the induction of CHS, which challenges their role as critical antigen-presenting cells. Extreme CHS has been observed in LC knock-out mice when compared to wild-type, suggesting an immunoregulatory function of LCs (39, 41). Unfortunately, these studies were performed prior to the discovery of $langerin^+$ dDCs, which further confounds their results. However, they do suggest that LCs may contribute to immune responses in both a stimulatory and suppressive capacity, an outcome possibly dictated by cytokines, chemokines, and other environmental immune modulating factors.

1.2.3 Role of skin immune cells in DENV infection

Current hypotheses of DENV pathogenesis suggest that the virus is injected into the blood stream during a mosquito vector's blood meal. "Spill-over" of the virus enters the epidermis and dermis. Here, DENV infection can potentially occur in keratinocytes, LCs, dMØs and dDCs. Infected immune cells mature and migrate to the cutaneous draining lymph node. In the lymph node additional macrophages and monocytes become infected; this results in viral amplification and dissemination *via* the lymphatics (17). However, data indicating that skin cells become infected with DENV are lacking. *In vivo* data are limited and skewed by the availability of

samples; data primarily come from autopsy result of fatal cases of DHF/DSS and may not be representative of the typical disease course. According to *in situ* hybridization, or a combination of PCR and virus isolation techniques (42), most cases of severe disease have DENV present in the skin. However, the populations of skin cells that are infected have yet to be identified.

The majority of data regarding infection of skin cells comes from *in vitro* or *ex vivo* studies. Exposing healthy human skin explants to DENV-2 isolates has resulted in infection of skin cells, however it is not clear what cell types become infected and what their consequent phenotype is. RT-PCR for the negative-strand DENV-RNA replication intermediate, following *ex vivo* inoculation of healthy skin, suggests that DENV is capable of replication in the skin (43). Immunolabeling for the viral non-structural protein-1 (NS1) in cryosections of inoculated skin has shown vast amounts of DENV infection in the epidermal stratum basale and very seldom infection of cells in the superficial portion of the epidermis, presumably LCs (43). Here, markers for activation, maturation, and apoptosis were up-regulated in the presence of virus. However, it is unclear whether it is DENV infected or bystander cells that acquire the mature phenotype (44). A skin biopsy obtained from the recipient of a live attenuated tetravalent DENV vaccine revealed that epidermal cells become sparsely infected with DENV (as detected by the pan-DENV 2H2 antibody) (45). Co-expression of CD1a on epidermal DENV positive cells suggests that they were LCs. Other studies have used cadaveric skin to assess the degree of infection in emigrant cells of DENV inoculated skin; these reveal that a large portion (approximately 60%) of MHC-II positive skin cells become infected with DENV upon *ex vivo* inoculation (45). Due to the complex phenotypes of cutaneous immune cells, discussed in section 1.2.2, MHC-II and CD1a are not sufficient markers to identify the subsets of cells in the dermis and epidermis. *In vitro* infection of purified skin MØs indicates that expression of DC-SIGN renders dMØ susceptible to viral entry, but that trafficking DENV to poorly acidified phagosomes limits viral replication (46). However, *in situ* studies of DENV infected dMØ are lacking. In summary, inoculation of human skin explants, both cadaveric and healthy, demonstrate that dendritic cells of the skin do become infected with DENV (43, 45). Using methods outlined in such studies, as well as other skin immunology studies (30), could clarify which immune cells become infected with DENV-2, what phenotype they portray, and thus how they contribute to disease pathogenesis.

1.2.4 Hypothesis and specific aims

We aimed to clarify the effects of DENV infection on cutaneous immune cells and **hypothesized** that DENV infects these cells, namely dMØs, dDCs, and potentially LCs, and renders them incapable of maturation, migration, and survival.

Aim 1: Determine infectivity of DENV 2 in monocyte derived DCs and the effects of infection on function and viability. Hypothesis: mdDCs are susceptible to infection with DENV 2; following infection mdDCs will fail to mature and will initiate apoptosis. Approach: Immature human monocytes will be cultured in the presence of GM-CSF and IL-4 for five days to produce immature mdDCs. Following mock or DENV-2 infection, cells will be assessed by flow cytometry for the expression of DENV protein, maturation markers, and apoptotic signals.

Aim 2: Identify cutaneous targets of DENV infection.

A) Characterize resident and emigrant immune cell populations in the presence and absence of DENV infection. Hypothesis: Immune cells of the skin, both dermal and epidermal, can be identified using a panel of antibodies to extracellular proteins. A portion of dDCs, dMØ, and potentially LCs will be infected with DENV; some infected cells will migrate out of inoculated skin while some will remain in the dermis and epidermis. Approach: Using an antibody panel containing: anti-HLA-DR, CD1a, CD1c, CD163, CD207, and anti-DENV1/2, flow cytometry analysis of emigrant skin cells will be performed. Emigrant cells will be collected 48 hours after inoculation of skin explants with DENV 2 or negative control. Infection and staining controls will be performed on BHKs. This antibody panel will also be used to perform *in situ* analysis of resident skin immune cells in cryosections of treated human skin explants.

B) Determine the effects of infection on function and viability. Hypothesis: DENV infects dDCs, dMØs, and LCs; infected cells will display an immature phenotype and early apoptotic features. Approach: Following inoculation with DENV-2 or negative control, migratory cells will be collected and analyzed by flow cytometry. Additionally, skin will be sectioned and stained for immunohistochemistry. Infection and staining controls will be performed on cytospins of BHKs.

2.0 EFFECTS OF DENV ON MONOCYTE-DERIVED DENDRITIC CELLS

2.1 INTRODUCTION AND BACKGROUND

Elucidating the effects that DENV infection has on DCs is critical to understanding the role of DCs in both innate and adaptive immune responses against DENV infection. Two of the identified DENV receptors, MR and DC-SIGN (10, 11), are expressed on immature mdDCs rendering them susceptible to DENV infection. The use of mdDCs as a model for circulating mDCs may lack biological relevance since circulating mDCs do not express MR and DC-SIGN in the absence of inflammatory cytokines (47) and have a mature phenotype when resident in the paracortex of the lymph node (48). However, the phenotypic similarities to dDCs make mdDCs an appropriate *in vitro* model for studying the effects of DENV infection of dDCs *in vivo*. During steady state, dDCs have an immature phenotype and express many of the same surface markers and receptors as mdDCs, including: CD1a, MR, and possibly DC-SIGN (27, 28, 49). mdDCs were used here as a model for dDC susceptibility to DENV and to clarify the effects that DENV infection has on DCs in regards to maturation and survival.

A large body of evidence suggests that immature mdDCs are susceptible to DENV infection; however the resultant phenotype has been a topic of conflict. Initial studies reported that infection leads to maturation of cells as measured by up-regulation of MHC-II, costimulatory molecules CD80 (B7.1) and CD86 (B7.2), the DC maturation marker CD83, and production of cytokines (TNF-α and IFN-α) (49, 50). However, populations of mdDCs exposed to DENV failed to produce IL-12, a T-cell stimulating factor required for the generation of type 1 T-cell responses in cell-mediated immunity (CMI). This finding contradicted the conclusion that DENV infected mdDCs were mature and active (49) and prompted further investigation. Subsequent studies examined separately the phenotypes of DENV infected and bystander cells. This revealed that the mature phenotype observed in DENV exposed cultures was likely the result of bystander

(uninfected cells) and not cells directly infected with DENV (44, 51). TNF-α and IFN-α are produced by DENV infected cells and are thought to result in the maturation of bystanders. Also the presence of these cytokines results in down-regulation of DC-SIGN which likely protects bystander DCs from infection. Researchers have had success reversing the immature phenotype of DENV infected mdDCs in mixed leukocyte reactions using activated T-cells, but no success with soluble stimulating factors (51, 52). Whether DENV induces cell death also remains controversial. During acute disease it has been shown that infection results in resistance to apoptosis compared to mock infection (50). Conversely, high levels of apoptosis have been observed in DENV positive mdDCs when compared to bystander and mock infected cells. However, apoptosis of DENV infected cells was only observed at very high MOIs and was equated to effects of viral burden (44).

The immune cells of the skin are thought to be primary targets of DENV infection; however there is a lack of biologically relevant evidence for this theory. This is due, in part, to the time, funding, and resource constraints that render *ex vivo* studies difficult to perform. Limited access to skin samples, from either healthy or DENV infected individuals, makes it necessary to use *in vitro* derived mdDCs as a model for DENV infection of dDCs.

2.2 MATERIALS AND METHODS

DENV isolate: Cells were infected with the prototypical laboratory strain of serotype-2 DENV, New Guinea C (NGC). Virus propagation was performed in BHK-21 cell for 6 days, resulting in a titer of $5x10^6$ PFUs/ml as measured by plaque assay in BHK-21 cells (propagation and plaque assay performed by Randy Stalter). Uninfected BHK supernatants were collected for use as negative infection controls.

DENV specific antibodies: Pan-DENV antibody D3-2H2-9-21 (2H2) was propagated in a mouse hybridoma from mice immunized with all four DENV serotypes (ATCC); it binds the viral protein prM in the cytoplasmic region of productively infected cells and on the surface of mature virions. Anti-DENV antibody 15F3-1 (15F3) was propagated in a mouse hybridoma from animals infected with only DENV serotype-1 antigen; it binds the viral NS1 protein from DENV

serotype-1 only (ATCC) and was used as a negative control. (Antibody propagation and purification performed by Xiangdong Liu).

Infection of BHK-21 cells: BHK-21 cells were cultured in a T-25 flask in DMEM (2% FBS+ Pen/Strep) at 37^0C (5% CO_2) until confluent. At such time the media was aspirated from the flask and 100 µl of undiluted virus stock or BHK supernatant (negative control) was added. Inoculums were distributed across the cell monolayer by gently hand rocking the flask. Following 2 hour incubation at 37^0, the virus was removed and the cells were washed thoroughly with pre-warmed DMEM (2%FBS+ Pen/Strep). Fresh media was added and the cells were incubated at 37^0C (5% (CO_2)) for 48 hours. Samples were run on an LSRII flow cytometer and analyzed using FlowJo software.

Staining BHK-21 cells for flow: Media was aspirated and discarded; adherent cells were trypsinized and washed in fresh media. Alexa647-fluorophore-conjugated anti-mouse Fab fragments were used to label the Fc portion of the pan-DENV antibody 2H2 or the DENV-1 serotype specific antibody 15F3 (Zenon-Labeling Kit: Invitrogen). These were used for an intracellular stain following fixation and permeabilization of cells (BDCytoFix/Perm Kit). Additionally, a time course of BHK-21 infection was performed with data collected at 0, 6, 18, 30, and 48 hours p.i. Samples were run on an LSRII flow cytometer and analyzed using FACSDiva software (BD Bioscience).

Fluorescent microscopy of BHK-21 cells: BHK-21 cells (DMEM+2%FBS+Pen/Strep) were incubated (37^0C (5% CO_2)) in 64 well plates containing sterile cover slips until cells were confluent and adherent on slips (40 hours). Media was replaced with 200 µl of fresh media or 1:10 dilutions of day 6 BHK supernatant or DENV. Following a 2hr incubation (37^0C (5% CO_2)) inoculants were aspirated and cover slips were washed and cultured in fresh medium for 24 hours. Cells were fixed and permeabilized (BD CytoFix/Perm) and stained with the unconjugated primary antibodies IgG2a (BD Pharmingen) and 2H2 (ATCC) followed by a goat-anti-mouse Alexa488 secondary antibody (Invitrogen). Hoechst stain, a membrane permeable stain which binds DNA at A-T regions allowing for visualization of nuclei, was applied. Cover slips were inverted and adhered onto microscope slides with gelvatol. Slides were viewed on an Olympus Provis microscope with a fluorescent light source (University of Pittsburgh, Centers for Biological Imaging).

Deriving DCs from CD14+ monocytes: DCs were propagated from purified monocytes as previously described (53). Briefly, human monocytes, previously isolated *via* $CD14^+$ microbead selection from healthy human blood donors, were cultured in DC media supplemented with GM-CSF and IL-4. Cells were cultured in 6 well plates at a density of 3×10^5 cells per ml and 3 ml per well (37^0C (5% CO_2)). On day two and four of the incubation fresh cytokine supplemented media was added to each well. Immature mdDCs were infected on day five.

DENV infection of mdDCs: Undiluted NGC virus (titer 5×10^6 PFU/ml) or day 6 BHK-21 supernatant was added to each well (3ml) to obtain a virus dilution of 1:10 and an infectious dose of approximately 1 PFU/cell. Inoculants were distributed by gently swirling culture plate. At no point during the incubation (37^0C (5% CO_2)) were inoculants removed; cells were harvested 48 hours post infection using gentle agitation with FACS buffer to remove adherent DCs. DENV susceptibility was assesses by flow cytometry using intracellular staining with Alexa647-Zenon-conjugated 2H2 or 15F3.

Maturation assay: The phenotype of mdDCs following 48 hours of DENV or mock infection was assessed by flow cytometry using extracellular antibodies: HLA-DR (PerCp-Cy-5),CD80 (FITC), CD86 (PerPc), CD83 (PE) or appropriate isotypes. Cells were fixed and permeabilized prior to incubation with Alexa647-Zenon-conjugated anti-DENV 2H2 or 15F3. Subsequent experiments assessed the capacity of DENV infected mdDCs to mature in response to external stimuli. A cytokine cocktail containing: polyI:C (20µg/ml), rhIFN-γ (1,000u/ml), rhesusTNF-α (50ng/ml), and rhesus IL-1β (25ng/ml) was added to wells 2 hours p.i. Following 48 hours of stimulation/infection cells were stained with surface-labeling antibodies as above and fixed and permeabilized prior to staining with Alexa647-Zenon-labeled DENV antibodies. Analyses were performed using an LSRII flow cytometer and FACSDiva software (BD Bioscience). Antibody information is available in Table 2.

Apoptosis assay: To detect apoptosis in mock and DENV infected mdDCs, cells were stained for flow cytometry with a U/V dye (that is excluded by intact cell membranes) to differentiate live cells from dead and CD14 (PE) or isotype control. Following fixation and permeabilization (BDCytoFix/Perm) the cells were stained with anti-active-caspase-3 (FITC) or isotype, and anti-Dengue 2H2 (Alexa647-Zenon-conjugated). Samples were run on an LSRII flow cytometer and analyzed using FACSDiva software (BD Bioscience). Antibody information is available in Table 2.

2.3 RESULTS AND DISCUSSION

2.3.1 Productive DENV infection of BHK-21 cells

After 48 hours of infection approximately 45% of the infected BHKs are positive for prM, detected with the pan-DENV antibody 2H2, while no staining is detected with the serotype-1 specific antibody (15F3) or in mock infected cells (Figure 1A). Additional staining using a primary/secondary antibody protocol was performed to confirm that the Zenon-labeled antibody stained with similar efficiency (data not shown).

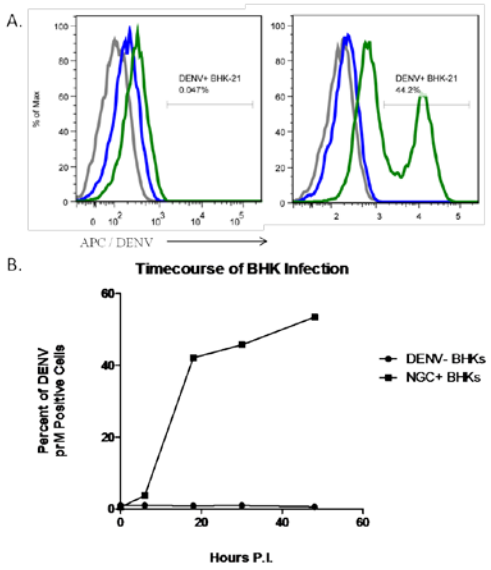

Figure 1: BHK-21 cells are susceptible to DENV infection:

A) Mock infected BHK-21 cells (left) and DENV-2 infected BHK-21 cells (right). Histogram of fluorescent intensity of unstained cells (gray line), Alexa647-Zenon-conjugated DENV-1 antibody 15F3 (blue line), or Alexa647-conjugated pan-DENV antibody 2H2 (green line) (Data representative of >3 replicates). B) The percentage of DENV positive BHK-21 cells following infection as detected by Alexa647-Zenon-conjugated pan-DENV 2H2 antibody.

A time course of BHK-21 infection was performed to confirm the observation made at 48 hours p.i., since the 2H2 antibody is not necessarily indicative of a productive viral infection

(Figure 1B). The minimal amount of prM detected at the six hour time point is likely the staining of extracellular virus particles; the continuous growth following this early time point is characteristic of a productive infection. Additionally, a sample of DENV infected BHKs was taken at the 48 hour time point and stained without permeabilization; there was a similar level of prM detection with this method as at the 6 hour time point (approximately 3-5%) (data not shown). Taken together, these data suggest that our isolate of NGC is infectious and that the 2H2 and 15F3 antibodies are specific and sensitive for virus detection. Subsequent infections of mdDCs and skin explants were accompanied by a BHK infection as a control for viral infectivity.

2.3.2 Fluorescent microscopy of DENV infected BHK-21 cells

Fluorescent microscopy was performed to confirm the results seen by flow cytometry. Based on the infectious life cycle of DENV discussed in section 1.1.1, we expect to see staining for viral prM protein in the cytoplasmic region of productively infected cells, as depicted in Figure 2. These images confirm the infection detected by flow cytometry in Figure 1.

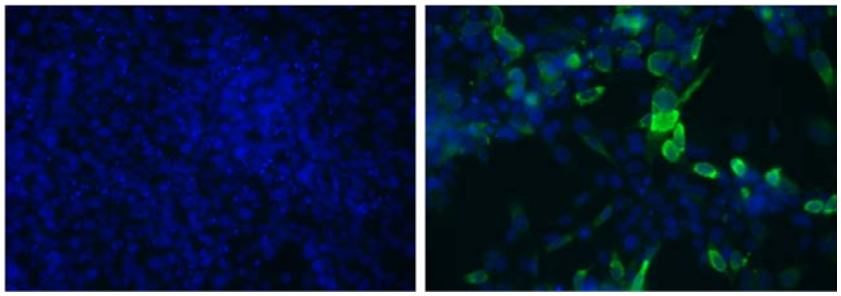

Figure 2: DENV infection of BHK-21 cells visualized using fluorescent microscopy

BHK-21cells stained with the unconjugated pan-DENV 2H2 (1^0) and goat-anti-mouse Alexa488 (2^0) antibody (green). Hoechst nuclear stain (blue). Left: mock infected cells. Right: NGC infected cells. Images are representative of two replicates.

2.3.3 Immature mdDCs are susceptible to DENV infection

Immature human mdDCs were used as an *in vitro* model for DENV infection of dDCs. The expression of MR and CD1a on these cells makes them an appropriate surrogate to investigate the susceptibility of dDCs to DENV. The mdDCs were infected on day 5 of culture; at this time >90% of the cells had differentiated into CD14- cells but remained immature based on expression of MHC-II, CD80, CD86, and CD83 (data not shown). Based on trypan blue exclusion, the survival of cells in the presence or absence of virus was similar at 48 hours p.i., with viable cell counts equal to 1.5×10^6 and 1.3×10^6 respectively. Figure 3A shows typical results seen in DENV infected populations of immature mdDC. Approximately 65% of cells up regulate expression of MHC-II and 15-30% of cells are DENV positive. In order to confirm that the level of prM positive cells seen at 48 hours is indicative of a productive DENV infection, a time course was performed (Figure 3B).

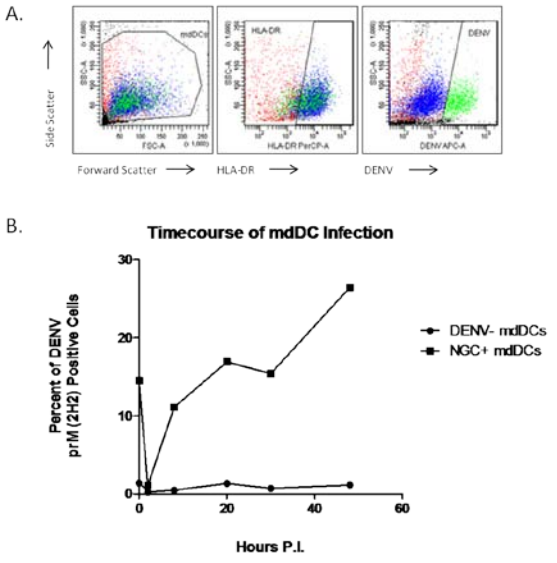

Figure 3: mdDCs become productively infected with DENV

A) DENV infected mdDCs assessed for forward and side scatter (left) HLA-DR (PerCP) (middle) and Alexa647-conjugated pan-DENV antibody 2H2 (right). Data are representative of >3 independent replicates. B) The percentage of DENV positive mdDCs following infection as detected by Alexa647-Zenon-conjugated pan-DENV 2H2 antibody.

2.3.4 DENV infection inhibits maturation of mdDCs

In the presence of appropriate stimulation, DCs become mature and activate and home to the lymph node to activate T- and B-cells in the germinal center. This mature/active phenotype can be assessed by the expression of certain cell surface markers that are required for DC-mediated T- and B-cell activation and proliferation. Amongst these are the immune synapse co-stimulatory molecules CD80 and CD86 (or B7.1 and B7.2 respectively) (25, 54), MHC-II for efficient antigen presentation, and CD83 to reduce the threshold of T- and B-cell activation (55). At 48 hours p.i. mock infected populations of mdDCs were generally 50-60%MHC^+, 20-30%$CD80^+$, 20-40%$CD86^+$, and 0-5%$CD83^+$.The presence of DENV results in 15-30% infection but no up-regulation of maturation markers, generally: 50-60%MHC^+, 20-40%$CD80^+$, 20-40%$CD86^+$, and 0-5%$CD83^+$. Interestingly none of the DENV positive cells become CD83 positive and only a portion (approximately 30-50%) of the DENV positive cells express CD80 and CD86 (Figure 4). The previously documented "bystander effect", where DENV infected cells fail to mature but bystander cells acquire a mature phenotype (44, 49), was not evident in these data. If the lack of maturation in DENV infected DCs is mirrored *in vivo* it would result in a diminished capacity of infected DCs to clear virus and initiate appropriate adaptive immune responses.

To assess whether soluble external stimuli can restore DENV infected mdDC maturation, cells were exposed to a cytokine cocktail two hours p.i. The cocktail used contained: (1) the synthetic dsRNA analog poly I:C which induces DC maturation *via* TLR3 signaling, and (2) three pro-inflammatory cytokines, rhIFN-γ, rhesusTNF-α, and rhesusIL-1β. It was confirmed that the rhesus cytokines were >95% identical to human by both amino acid and protein sequence (data not shown). Representative data of MHC-II, CD86, and CD83 expression are displayed in Figure 4. Cytokine stimulation results in maturation of bystander cells and the partial restoration of infected mdDC maturation. MHC-II and CD86 are up-regulated on $DENV^+$ cells in the presence of the cytokine cocktail, while CD83 remains absent from all $DENV^+$ cells. The reduction in overall DENV infection makes it difficult to fully assess maturation. Both poly I:C and IFN-γ have potent anti-viral effects and likely resulted in the inhibition of secondary (and higher) rounds of infection (49). Often the use of other maturation stimuli are employed when working with mdDC, such as TLR 7/8 agonists, LPS, and CD40L (49, 52), however, these stimulants failed to up-regulate CD83 in control experiments and thus were not considered

effective (data not shown). Taken together these data suggest that DENV infection does not result in the maturation and activation of DCs; however, in the presence of pro-inflammatory cytokines the immature phenotype can be partially reversed.

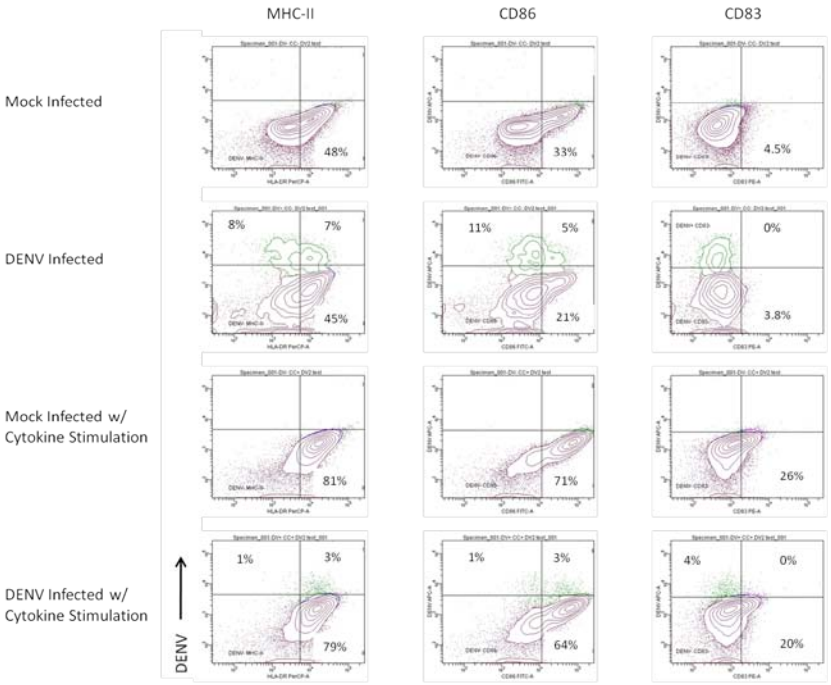

Figure 4: DENV partially inhibits mdDC maturation

Expression of MHC-II, CD86, and CD83 (X-axes) vs. Alexa647-conjugated pan-DENV antibody 2H2 (Y-axis) in mock infected (top row) and DENV infected (second row) mdDCs in the absence of cytokine cocktail maturation stimuli and after stimulation with cytokine cocktail (bottom rows). Representative data of two experimental replicates.

2.3.5 DENV infection induces apoptosis in mdDCs

During viral infection it is critical to mount an appropriate adaptive immune response. DCs play a critical role here, in which they become active, mature, and migrate to the lymph node to activate T- and B-cell mediated responses. Therefore, survival of infected DCs is vital in DENV clearance. To establish whether mdDCs survive during DENV infection, the level of active-caspase-3 was measured in DENV and mock infected cells. Caspase-3 is an intracellular cysteine protease activated during early apoptosis downstream of both the intrinsic and extrinsic cell

death pathways (*via* caspase-8 or -9). Caspase-3 becomes cleaved and can be recognized in this active form using an anti-active-caspase-3 antibody (BD). The gating scheme is pictured in Figure 5; dead cells and cells expressing CD14 were excluded, the overall percentage of active-caspase-3 positive cells was analyzed, and finally the percentage of active-caspase-3 positive cells in a defined number of DENV infected and bystander cells was assessed.

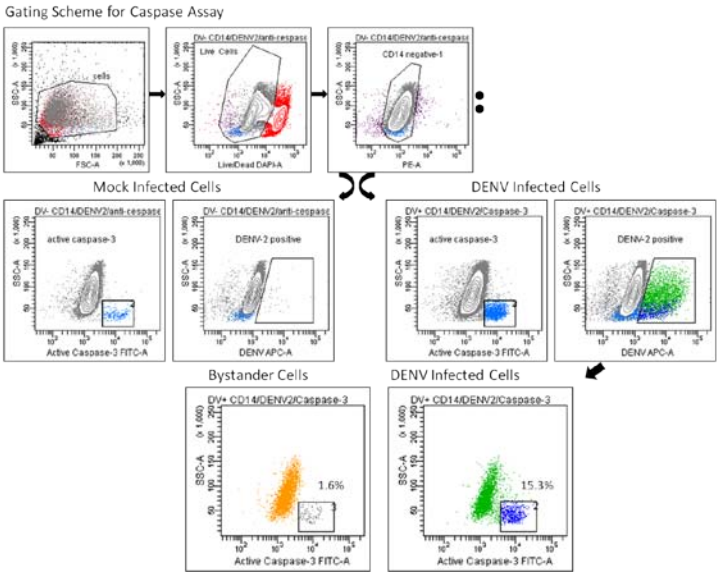

Figure 5: Enhanced apoptosis of DENV infected mdDCs compared to bystander cells

Viable mock and DENV infected mdDCs selected by forward and side scatter and exclusion of live/dead dye. CD14 (PE) negative cells assessed for expression of active-caspase-3 (FITC) and Alexa647-conjugated pan-DENV antibody 2H2. Equal numbers of DENV+ and bystander cells are 15.3% and 1.6% active-caspase-3 positive, respectively.

Following 48 hours of treatment with virus or negative control the overall survival based on trypan blue exclusion does not differ between populations (discussed in 2.3.3); however this method of survival analysis is dependent on the late stage characteristic of membrane fractionation. Analyzing earlier stages of cell death (activation of caspase-3) reveals that 9-15% of DENV infected cells are apoptotic, while less than 2% of bystander and mock infected cells are undergoing apoptosis. DENV infected cells are significantly more apoptotic than uninfected

or bystander cells (p=0.0061 and 0.0076 respectively) (Figure 6). Taken together these results indicate that DENV infection has deleterious pro-apoptotic effects on DCs.

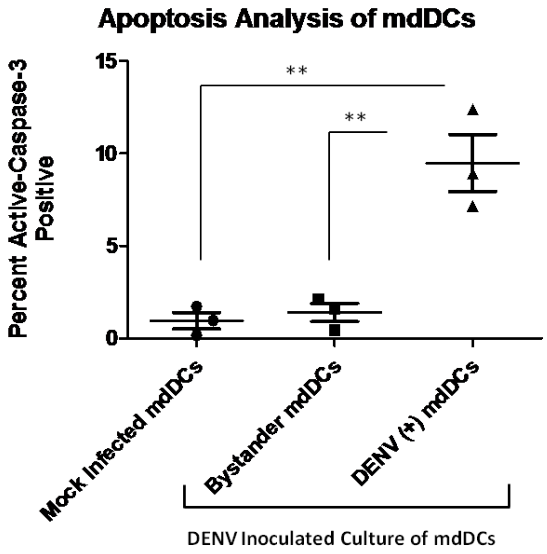

Figure 6: DENV infected mdDCs significantly more apoptotic than bystander and mock infected cells
Percent active-caspase-3 positive mock infected mdDCs (left), bystander mdDCs (middle) and DENV(+) mdDCs (right). Unpaired T-test of the differences of means between: mock infected and bystander cells p=0.5385, mock infected and DENV(+) cells p=0.0061, bystander and DENV(+) cells p=0.0076.

These studies clarify the effects of DENV infection on mdDCs in terms of maturation and survival. Our results indicate that populations of mdDCs exposed to DENV fail to mature beyond the level that is observed in mock infected cells. We found that the immature phenotype of DENV infected mdDCs can be partially reversed in the presence of soluble factors, namely three pro-inflammatory cytokines, IFN-γ, TNF-α, IL-1β, and a synthetic TLR3 ligand (poly I:C). However, the presence of these stimuli fails to restore the expression of CD83 on DENV infected DCs. This could be indicative of a specific down-modulation of CD83 induced by a yet unidentified viral factor. These data are contradictory to work that suggesting that the immature phenotype of $DENV^{+}$ DCs is only reversed in the presence of activated T-cells (51, 52). Additionally, our work shows that DENV infected DCs become apoptotic at an MOI of

1PFU/cell. This contradicts findings that DC apoptosis occurs as a result of viral burden and is only induced at MOIs greater than five (44).

The current hypothesis regarding the introduction and dissemination of DENV involves immune cells of the skin. Mosquitoes deposit virus into the epidermis and dermis in the course of a blood-meal. Here, DENV infects cutaneous antigen-presenting immune cells. These cells are thought to mature and migrate to the cutaneous draining lymph node resulting in infection of MØs and MOs. The amplification and dissemination of virus then continues *via* the lymphatics (17). However, the results presented here indicate that DENV infected DCs may not contribute to the dissemination of virus or the induction of adaptive immunity due to their failure to acquire a mature/active phenotype. DENV may evade the immune response by inhibiting DC migration to the lymph node and by inducing DC apoptosis. Further studies are needed to understand the mechanisms of how DENV induces these deleterious changes in DCs.

3.0 EFFECTS OF DENV ON CUTANEOUS IMMUNE CELLS

3.1 INTRODUCTION AND BACKGROUND

The skin is densely populated with mononuclear phagocytes that are responsible for monitoring the environment for pathogens and initiating appropriate immune responses. Immune cells of the skin include dermal macrophages (dMØ), dermal dendritic cells (dDCs) and epidermal Langerhans cells (LCs). Because of the vector borne nature of DENV it is important to understand the role that these cells play in infection. Currently it is believed that they are targets of infection and that they contribute to the spread of virus *via* the lymphatics (17). However, there is a limited body of evidence supporting this hypothesis. Previous studies involving inoculation of healthy and cadaveric human skin explants, as well a biopsy of one symptomatic recipient of a tetravalent DENV vaccine, suggest that cutaneous cells with dendritic morphology become infected with DENV (43, 45). However, a comprehensive phenotype of infected skin cells has not been established and it is therefore unclear whether dMØ, dDCs, and/or LCs are DENV targets. Developments in the field of cutaneous immunology have resulted in a panel of antibodies that can be used to characterize resident and emigrant cell subsets using flow cytometry and *in situ* analyses. The phenotypes of skin immune cells are discussed in section 1.2.2 and summarized in Table 1. Methods outlined in previous DENV studies, as well as skin immunology studies, can be used to clarify the degree to which cutaneous immune cells become infected with DENV-2, what phenotype they portray, and how they contribute to disease pathogenesis.

Table 1: Skin immune cell phenotypes

Cell Marker / Cell Type	CD1a	CD1c	LANGERIN/CD207	MR/CD206	DCSIGN/CD209	CD163
Langerhans' Cells:	YES	YES/NO	YES	NO	NO	NO
Dermal DCs:						
Subset 1	HIGH	YES	YES/NO	YES	NO	NO
Subset 2	DIM	YES	NO	YES	NO	NO
Subset 3	NO	YES	NO	YES	NO	NO
Dermal MØ:	NO	NO	NO	YES	YES	YES

3.2 MATERIALS AND METHODS

Skin explants: Methods were adapted from skin studies in the simian model (unpublished data). Briefly, healthy human skin explants from subjects undergoing elective surgery were received and cultured within four hours of removal. Samples were processed in two ways: (1) subdural fat and connective tissue were trimmed from the dermal side and skin was cultured full-thickness or (2) a hand-held dermatome was used to render split-thickness skin with a final thickness of ≈0.37µm. Skin was cut into 1-2 inch squares, rinsed with 1xPBS followed by DC media, and placed dermal side down onto 50 mesh grids in 60x15mm culture dishes.

Inoculation and culture conditions of skin explants: Split-thickness skin was inoculated *via* scarification (30). Varied concentrations of DENV or negative control were placed on the epidermis of sections; a 27 gauge needle was used to create superficial scratches through the inoculums. Full-thickness skin was inoculated by injection. DENV or negative control were injected with a 29 gauge needle; injections were as superficial as possible and resulted in a visible bubble of inoculant≈(100µl) trapped under the epidermis. Scarification/injection was performed without media in culture dishes to ensure that infection of emigrant cells was not due to virus spilling into the media. Following a two hour incubation (37^0C (CO_2 5%)) pre-warmed DC media was added underneath grids; incubation continued at 37^0C for 48 hours.

Quantification of migratory cells: Migratory cells were harvested by agitation with FACS buffer, passed through a 70µm cell strainer and washed 1x with 10ml of FACS buffer. Red blood cells were lysed with 1xACK. The number of live migratory cells was assessed by trypan blue exclusion. Skin was removed from mesh grids, rinsed with 1xPBS, and scanned or traced to

obtain exact size measurements. Open source NIH software (ImageJ) was used to quantify the number of migratory cells per unit area of skin.

Staining migratory cells for flow cytometry: Extracellular antibody stains included: HLA-DR (APC-Cy7), CD1a (PE-Cy5), CD1c (Pacific Blue), CD163 (FITC), and langerin (CD207) (PE). Cells were fixed and permeabilized (BD CytoFix/Perm) prior to intracellular staining with 1µg of Zenon-conjugated Alexa-647 2H2 or 15F3. Samples were run on an LSRII flow cytometer and analyzed using FlowJo or FACSDiva software. Additional antibody information is available in Table 3.

Processing skin samples for *in situ* analyses: Mock and DENV infected skin samples were sucrose infused (30% sucrose, overnight, 4^0C) and snap-frozen with or without 2%PFA fixation. Samples for immunolabeling were embedded in optimum cutting temperature (OCT) compound and sectioned to 6µm thickness on a MicroCryotome (University of Pittsburgh, Centers for Biological Imaging).

***In situ* staining for cutaneous immune cells and DENV**: Skin cryosections were maintained at -20^0C until immunolabeling was performed (within two weeks of sectioning). Sections obtained without fixation were post-fixed in ice cold acetone for 5 minutes. Prior to applying primary antibodies slides were thawed (10 minutes at room temp), rehydrated with 1xPBS, and blocked with 2%BSA or 20% goat serum. Unconjugated primary antibodies including: mouse anti-HLA-DR (1:100), mouse anti-CD1a (1:100), mouse anti-CD1c (1:200), mouse anti-CD163 (1:200), mouse anti-DC-SIGN/CD209 (1:250), and mouse anti-MMR/CD206 (1:200) were diluted in either 0.5%BSA or 2% goat serum corresponding to the blocking method. Primary antibodies were applied for 1 hour. Isotype specific secondary antibodies (Invitrogen) goat-anti-mouse Alexa 549 (IgG1) or Alexa 647 (IgG2a and IgG2b) or Alexa 546 Fab fragments were applied for 30 minutes. Staining for DENV was performed with unconjugated or biotinylated 2H2 overnight at 4^0C prior to cell-surface marker staining. Secondary antibodies used for virus detection were Alexa 488 or streptavidin Alexa 488 or 647. Hoechst stain was applied for nuclear visualization. Cover slips were adhered with gelvatol. Slides were viewed on an Olympus Fluoview confocal microscope (University of Pittsburgh, Centers for Biological Imaging). Antibody information is available in Table 4.

3.3 RESULTS AND DISCUSSION

3.3.1 Emigrant cutaneous immune cells from split- and full-thickness skin

Emigrant cells from split-thickness skin were collected and stained as described in section 3.2; the antibody panel is detailed in Table 3. Viable cells were gated by side and forward scatter and approximately 30-40% of the migratory population was viable based on this criterion (Figure 7). Expression of MHC-II was used as the first inclusion criteria for defining migratory DCs and MØs. Averages of 65-80% of viable cells were positive for MHC-II across three replicates; this number equates to 12-25% of the total migratory cells being classified as immune cells. CD1a and CD1c were used to define dDC populations; three populations were apparent based on CD1a: $CD1a^{-}$, $CD1a^{dim/medium}$, and $CD1a^{high}$. $CD1a^{-}$ and $CD1a^{dim/medium}$ populations stained brightly for CD1c. Cells of the $CD1a^{high}$ population, and to a lesser extent the $CD1a^{dim/medium}$ subgroup, expressed CD207 (langerin); about 10% of all MHC-II positive emigrant cells express CD207. The origin of these $CD207^{+}$ cells is not clear since epidermal LC and a subset of dDCs have been shown to express CD207 (32, 33). Further staining with CD103 would be necessary to differentiate $CD207^{+}$ dDCs from LCs. Alternatively, the separation of the dermis and epidermis would clarify the origin of these langerin positive cells. However, the process of separating dermis from epidermis, often *via* collagenase digestion, is likely to alter the phenotypes of migratory cells and thus this method was avoided.

Other emigrant populations remain undefined based on flow cytometry and require further investigation. Approximately 20% of cells that express CD1a do not express CD1c or CD207; this is unexpected based on the currently accepted phenotypes of cutaneous immune cells (29) (Table 1). Additionally, there is a rather large portion of MHC-II positive cells that fail to stain for any other antibodies in the panel (Figure 7). These cells express lower levels of MHC-II than cells that stain for DC markers; this phenotype is characteristic of dMØs (see 1.2.2). However, without the inclusion of CD163 (the defining cell-surface marker for dMØs) it is not possible to identify these cells as such. This MHC low population could also include keratinocytes, which up-regulate this marker in the presence of pro-inflammatory stimuli (56). It is unlikely that DENV infection is the only source of pro-inflammatory stimuli since the proportion of these

cells is equivalent in DENV and mock infected samples (data not shown). It is possible that the trauma from surgery and *ex vivo* processing up-regulates MHC-II expression on keratinocytes.

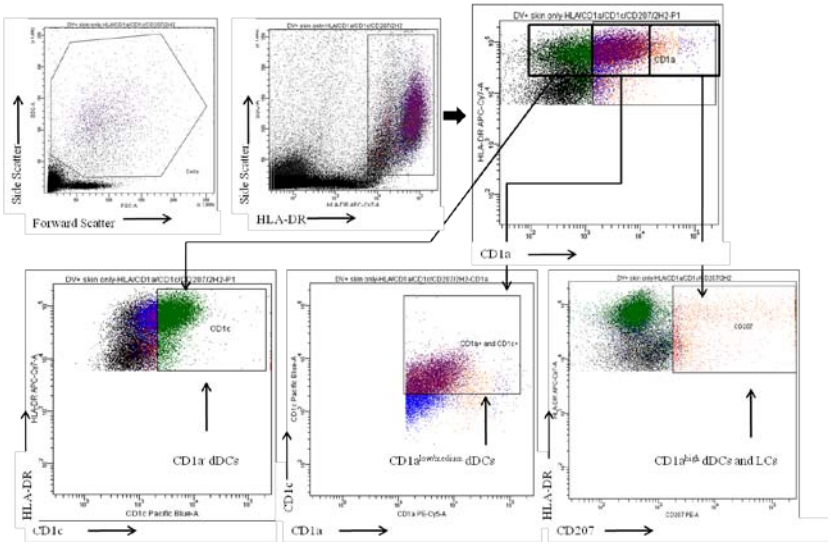

Figure 7: Emigrant cells from split-thickness skin

Emigrant cells from DENV inoculated skin after 48hrs culture: viable cells selected by forward and side scatter and HLA-DR(APC-Cy7) expression. HLA-DR^{+} cells contained three populations based on CD1a(PE-Cy5): CD1a^{-} cells and 80% of CD1a$^{dim/medium}$ cells express CD1c^{+} (PacificBlue) (left and middle arrows), CD1a^{high} cells are CD207^{+} (PE) (right arrow). Data are representative of three independent experiments performed on skin samples from three individuals.

One disadvantage of using a dermatome to create split-thickness skin sections is that it stimulates the migration of dDCs, dMØs, and potentially LCs. Therefore, we also investigated DENV infection using full-thickness skin (Figure 8). Here, we cultured full-thickness skin in the presence and absence of virus. Emigrant cell collection and staining procedures are outlined in section 3.2 and details of antibodies are summarized in Table 3. Viable cells were gated based on side and forward scatter; an average of 20% of the total migratory population was viable based on this criteria. Only cells with proportionate area and height, indicative of single events, were analyzed further (Figure 8). As before, expression of MHC-II was an inclusion criterion for defining migratory immune cells. CD1a^{-} and CD1a$^{dim/medium}$ phenotypes were evident amongst CD1c^{+} cells; the CD1a^{high} population was diminished when compared to migratory cells from

split-thickness skin (see Figure 7). The small fraction of CD1a^{high} cells co-expresses CD207/langerin. However, the total percentage of CD207 expressing cells was <3%. It is likely that cells from the epidermis failed to migrate out of full thickness skin; this would explain the reduction in MHC-II^{+} cells as well as the lack of CD207^{+} cells. Inclusion of CD163 revealed that about 10% of MHC-II^{+} cells are dMØs, which express CD163 and fail to stain for the CD1 markers and CD207.

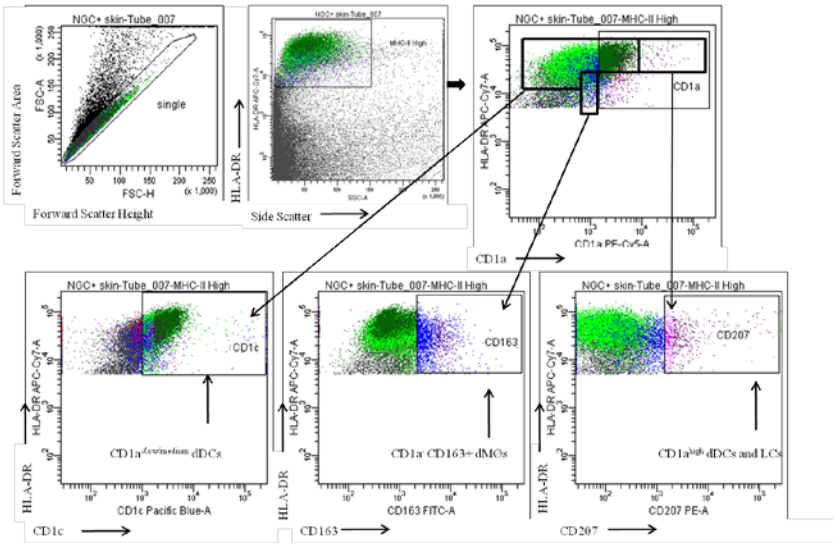

Figure 8: Migratory cells from full-thickness skin

Emigrant cells from DENV inoculated skin after 48hrs culture: viable cells selected by forward and side scatter (not shown), forward scatter height and area (single events) and HLA-DR(APC-Cy7) expression. MHC-II^{+} contained two populations based on CD1a(PE-Cy5): CD1a^{-} cells and 75% of CD1a$^{dim/medium}$ cells express CD1c^{+} (PacificBlue) (left arrow), 10% of CD1a^{-} cells are CD163^{+} (middle arrow). Minor population of CD1a^{high} cells are CD207^{+} (PE) (right arrow). Results are representative of four experimental replicates performed on skin explants obtained from four individuals.

Again, there are migratory populations that cannot be defined based on the current staining protocol. 20-30% of cells that express CD1a do not express CD1c or CD207, which does not fit the defined phenotype of any cutaneous immune cells (29) (Table 1). One possibility, in the case of full- and split-thickness emigrant cells, is that cells expressing only MHC-II and CD1a represent dDCs and LCs that have down-regulated CD207 upon migration. It has been shown

that langerin expression is lower upon arrival at the cutaneous draining lymph node (35). Additionally, studies have shown that langerin is internalized upon migration (57) and therefore may have been missed in the extracellular staining performed here (30). A small population of MHC-II positive cells does not stain for DC or MØ markers. As discussed before (page 26) it is likely that these represent keratinocytes that have up-regulated MHC-II in response to pro-inflammatory stimuli.

Emigrant cells from split- and full-thickness skin explants were stained for the presence of DENV using the Alexa647-Zenon-conjugated anti-DENV 2H2; DENV positive cells were not detected in the MHC-II^{+} cells that migrate out of skin at 48 hours p.i. (data not shown). *In vitro* data suggest that infected DCs fail to mature and thus may fail to migrate out of the skin. Alternatively, DENV infected cells could migrate from the skin but rapidly undergo apoptosis and not be detected in the emigrant population. Finally, infection rates could be at the low end of the range of detection by flow cytometry; this would inhibit additional studies aimed at identifying the phenotypes of infected cutaneous cells using the methods established here.

3.3.1.1 Quantification of skin emigrant cells

Live emigrant cells from full-thickness skin were counted, based on trypan blue exclusion, and are summarized in Figure 9. A paired t-test comparing the difference of the means reveals that the number of emigrant cells from mock and DENV infected skin is not significantly different (p=0.1492).

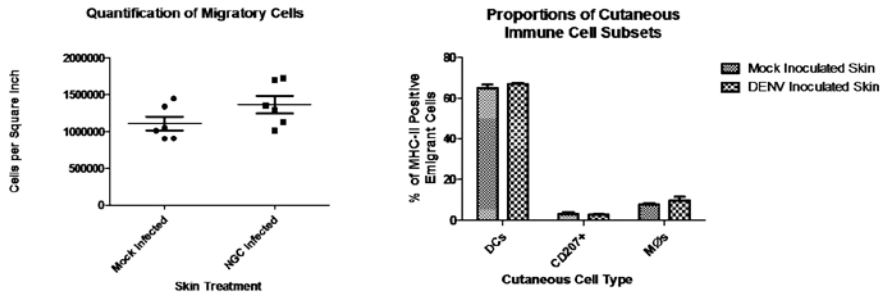

Figure 9: Quantification of migratory cells from full thickness skin

Live emigrant cells per square inch of full-thickness mock and DENV inoculated skin. Paired T-test comparing the difference of the means p=0.1492 (left) (n=5). Proportions of cutaneous immune cell subsets as a percentage of the total MHC-II positive emigrant cells (right) (representative of four individual replicates).

Typically, 50% of viable cells from full-thickness skin were positive for MHC-II; this equates to 7-13% of emigrant cells being classified as cutaneous immune cells. This percentage remained the same between DENV infected and uninfected skin, as did the proportions of subpopulations of immune cells (Figure 9). On average; 70% of emigrant cells were DCs defined by expression of CD1c, approximately 3% of cells express CD207/langerin, and 10-15% of MHC-II^{+} cells were dMØs.

3.3.2 *In situ* cell characteristics

Cryosections of full- or split-thickness skin were cut in cross-section and stained as described in section 3.2. Briefly, 6µm sections were cut from either PFA fixed or unfixed skin samples; sections from unfixed skin samples were post-fixed in acetone. Following blocking, primary antibodies to cell-surface markers were applied. These markers included: mouse anti-HLA-DR, CD1a, CD1c, CD163, DC-SIGN/CD209, and MR/CD206. Polyclonal secondary antibodies used included goat-anti-mouse polyclonal Alexa488 or isotype specific Alexa-647 or -549. Initially, cell-surface staining was performed to assess the specificity of antibodies and the distribution of their epitopes. These experiments confirmed that MHC-II is expressed in both the dermis and epidermis while expression of CD163, CD209, and CD206 is restricted to the dermis (Figure 10). While not evident in Figure 10, additional staining revealed that langerin (CD207), CD1a, and CD1c were expressed throughout the skin (evident in Figure 11 and Figure 13). This is expected based on the phenotypes of LCs and dDCs discussed in section 1.2.2.

In order to clarify the role of cutaneous immune cells in DENV pathogenesis, it is important to identify the subpopulations that become infected. It is currently accepted that three populations of dDCs are present in the skin during steady-state; these populations are CD1c^{+}CD1a^{high}CD14^{-}, CD1c^{+} CD1a^{dim}CD14^{-}, and CD1c^{+}CD1a^{-}CD14^{+} (27-30). Expression of langerin has been documented on dDCs that express high levels of CD1a (Figure 7) (30, 32, 33). dMØs fail to express the DC markers CD1a and CD1c, but are defined by expression of CD163 and dermal residence (27). Both dDCs and dMØs express MR, however the expression of DC-SIGN on these two cell types remains unclear (27, 28). In order to clarify the phenotypes of cutaneous immune cells dual staining was performed.

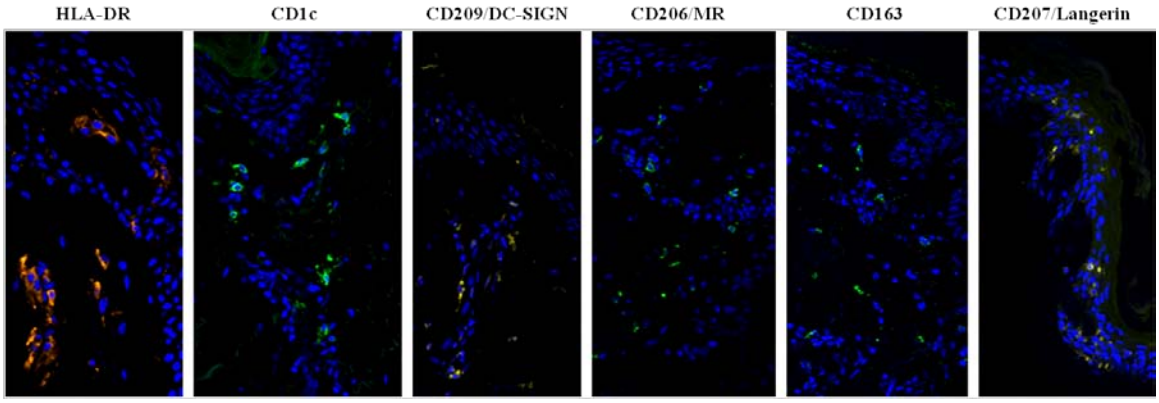

Figure 10: *In situ* staining for the distribution of immune cell markers and receptors

Mock inoculated skin sections (6µm) stained for (left to right): m α HLA-DR+g α m IgG2a specific Alexa647 (orange), m α CD1c+polyclonal g α m Alexa488 (green), m α CD209+g α m IgG2b specific Alexa647 (yellow), m α CD206+polyclonal g α m Alexa488 (green), m α CD163+g α m polyclonal Alexa488 (green), and m α CD207+g α m IgG1 specific Alexa549 (orange). Hoechst nuclear stain (blue).

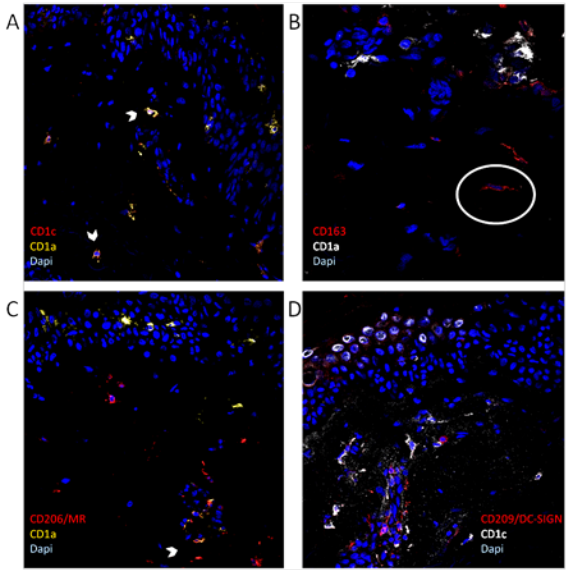

Figure 11: Cutaneous immune cell populations identified by *in situ* staining

Mock inoculated skin sections (6µm) stained for: A) m α CD1c+g α m IgG1 specific Alexa549 (red) and m α CD1a+g α m IgG2a specific Alexa647 (yellow). Co-localization indicated by arrow heads. B) m α CD163+g α m IgG1 specific Alexa549 (red) and m α CD1a+g α m IgG2a specific Alexa647 (white). Circled is a dMØ. C) m α CD206+g α m IgG1 specific Alexa549 (red) and m α CD1a+g α m IgG2a specific Alexa647 (yellow). Arrow head indicates co-localization. D) m α CD209+g α m IgG2b specific Alexa647 (red) and m α CD1c+g α m IgG1 specific Alexa549 (white). Hoechst nuclear stain (blue). Panels A and C depict representative staining from three independent replicates; panels B and D depict representative staining from two independent replicates.

Figure 11-A, shows co-localization of CD1a (yellow) and CD1c (red) in the dermis. This is the phenotype expected of dDCs (29). Bright staining for CD1a (yellow) is apparent in the epidermis; this indicates the presence of epidermal LCs (29). To confirm that CD1a expressing dermal cells are dDCs, it is necessary to establish that they do not express the scavenger receptor CD163 (27). Figure 11, panel B, image of reticular dermis cells, confirms that cells expressing CD1a (white) do not express the dMØ marker CD163 (red). It also highlights the fact that morphology cannot be used to differentiate dDCs from dMØs; the circled cell is a dMØ based on expression of CD163 but has an elongated, spindled, dendritic like appearance.

The receptor profile of dDCs remains poorly defined, yet it is hypothesized that expression of DC-SIGN and MR makes dDCs targets of DENV infection (28). In order to clarify the issue, sections were stained with CD1a or CD1c and either CD206 (MR) or CD209 (DC-SIGN) (Figure 11C and D). As expected, CD1a (white) is expressed in both the epidermis and the dermis. In the epidermis it does not co-localize with CD206/MR (red) which confirms that LCs do not express MR (Figure 11C). In the dermis some cells express both CD1a and CD206 while some have only CD206; these cells are likely dDCs and dMØs respectively. Unlike CD206/MR, CD209/DC-SIGN is not co-expressed on cells with dDC markers. This is evident in Figure 11D, where CD1c (white) does not co-localize with CD209/DC-SIGN (red). Furthermore, since LCs do not express DC-SIGN (29), the CD209 positive cells in the dermis must be dMØ.

These experiments clarified the phenotypes of immune cells in the skin. LCs can be identified in the epidermis based on expression of CD1a or CD207. The dermis contains dMØs defined by the expression of CD163. Additionally, dMØs can be identified by expression of DC-SIGN since we have confirmed that this receptor is not expressed on DCs in the dermis. Dermal cells expressing CD1a, CD1c, and/or CD207 are dDCs or migratory LCs; additional staining would be necessary to differentiate these populations.

3.3.3 DENV detection in skin

Having validated the assay for phenotyping cutaneous immune cells, the subsequent studies were aimed at determining the degree of DENV infection in skin and specifying the cell types infected. The methods for DENV detection are detailed in section 3.2. Briefly, cryosections were stained with unconjugated or biotinylated primary antibodies (IgG, 2H2, or 15F3) overnight.

Polyclonal goat-anti-mouse antibodies used for virus detection were Alexa 488 or streptavidin Alexa-488 or -647. Staining in pre-fixed skin using unconjugated 2H2 is depicted in Figure 12A; background levels of fluorescence were established in mock infected skin. Staining above background is apparent in the cytoplasm of dermal resident cells (yellow). However, it is unclear whether these cells represent dMØs, dDCs, or migrating LCs. Additionally, the epidermis, particularly the stratum basale and stratum corneum, fluoresce uniformly in $DENV^{+}$ skin to a greater degree than in mock infected skin. This is likely the detection of DENV infection in keratinocytes, however the use of a keratinocyte specific antibody would be required to confirm this hypothesis. Additionally, it is possible that prM protein in virus inoculum adheres to the dead keratinocytes of the stratum corneum and is detected in this most superficial layer of the epidermis. Similar results were seen staining with biotinylated 2H2 primary (on $DENV^{+}$ and $DENV^{-}$ skin) followed by streptavidin Alexa488 (Figure 12B).

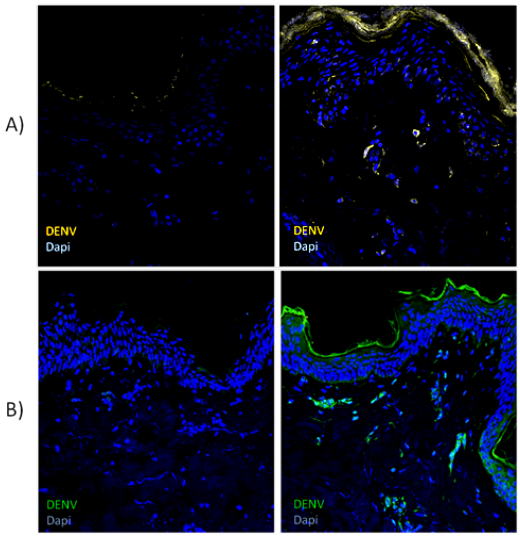

Figure 12: DENV antigen is detectable in dermal and epidermal cells of inoculated skin

A) PFA fixed skin sections (6µm) mock infected (left) or DENV infected (right) stained with unconjugated pan-DV antibody 2H2 + polyclonal g α m Alexa488 (yellow). B) PFA fixed skin sections (6µm) mock infected (left) or DENV infected (right) stained with biotinylated pan-DV antibody 2H2 + streptavidin g α m Alexa488 (green). Hoechst nuclear stain (blue). Images are representative of results from three independent replicates using skin explants from two individuals; skin inoculated *via* scarification.

These data suggest that DENV infection does occur in both dermal and epidermal populations of cutaneous cells; however it does not clarify the specific subpopulations of cutaneous immune cells that become infected with DENV. Multiple methods of dual staining, for DENV protein and cell-surface markers, failed to reveal appropriate staining patterns. Nonspecific binding of primary and secondary antibodies often resulted in apparent co-localization of virus and cell marker in both DENV and mock infected skin sections. Additionally, the limited DENV antigen in the skin prohibited the additional blocking steps required to inhibit nonspecific co-localization. However, preliminary data suggest that these problems have been resolved with the following staining method: (1) blocking sections with goat serum (2) biotinylated mouse-anti-DENV-2H2 (3) streptavidin-goat-anti-mouse secondary (4) monoclonal mouse-anti-cell marker primary (5) goat-anti-mouse single Fab fragment secondary. This method has been used to assess the degree of infection in dDCs, dMØs, and LCs (Figure 13).

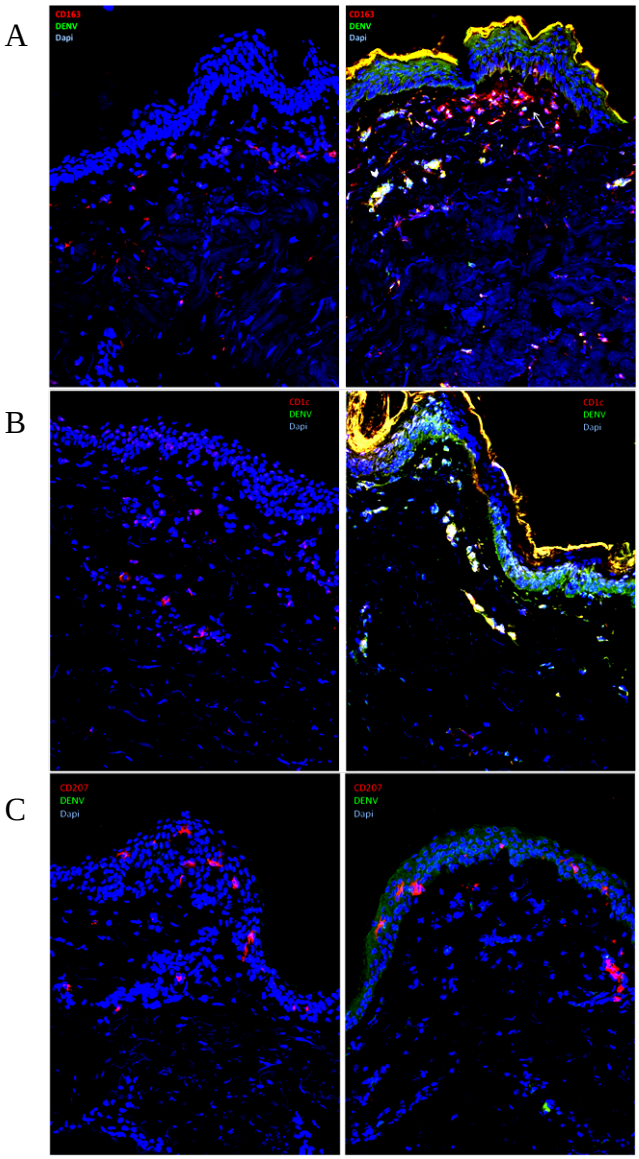

Figure 13: Dermal resident MØs and DCs are targets of DENV infection

A) PFA fixed skin sections (6µm) mock infected (left) or DENV infected (right) stained with biotinylated pan-DENV antibody 2H2 + streptavidin g α m Alexa488 (green) and m α CD163 + single Fab fragment g α m Alexa 546. Arrow head indicates co-localization, arrow indicates uninfected dMØ. B) PFA fixed skin sections (6µm) mock infected (left) or DENV infected (right) stained with biotinylated pan-DENV antibody 2H2 + streptavidin g α m Alexa488 (green) and m α CD1c + single Fab fragment g α m Alexa 546. C) Acetone fixed skin sections (6µm) mock infected (left) or DENV infected (right) stained with biotinylated pan-DV antibody 2H2 + streptavidin g α m Alexa488 (green) and m α CD207 + single Fab fragment g α m Alexa 546. Hoechst nuclear stain (blue).

DENV can be detected in CD163 expressing cells, suggesting that dMØs are targets of DENV infection (Figure 13A). In DENV inoculated skin explants, infected MØs reside in the lower papillary or upper reticular portion of the dermis, while uninfected dMØs are concentrated directly beneath the epidermis. This could indicate selective recruitment of uninfected MØs to the infected keratinocytes in the basal layer of the epidermis. It is likely that this reorganization of dMØs in DENV inoculated skin occurs to promote the clearance of virus and apoptotic keratinocytes.

DCs located in the dermis are targets for DENV infection based on the co-localization of DENV antigen and CD1c in DENV inoculated skin (Figure 13B). It is unclear whether these cells represent dDCs or migratory LCs since CD1c is expressed on both of these cell types (32, 33). DENV was not detected in epidermal LCs (Figure 13C); however, this does not indicate that LCs are unsusceptible to DENV infection. The frequency of cell specific DENV detection in the stratum spinosum and granulosum of the epidermis is consistently low relative to that in the dermis (see Figure 12), this could explain the failure to detect DENV in epidermal LCs. Alternatively, the overall lack of DENV detected in this staining could indicate that this section was far from the site of inoculation and is representative of bystander cutaneous cells.

It is evident that DENV is capable of infecting both dMØs and some populations of dermal or epidermal DCs. Further studies will be required to confirm this finding and to clarify the effects of DENV infection. Upon pathogen recognition dMØs are capable of producing inflammatory cytokines that aid in the recruitment of additional immune cells from the blood (28). This could aid in the clearance of virus and the activation of adaptive immunity. However, it is unclear whether this occurs during DENV infection. In response to maturation stimuli, dDCs quickly mature up regulate expression of CCR7 and migrate to the cutaneous draining lymph node (31). However, *in vitro* studies suggest that DENV infection of dDCs does not stimulate maturation. This could render infected dDCs incapable of viral clearance and diminished in their capacity to activate adaptive immunity. It is difficult to speculate the effects that DENV infection would have on LCs, as the immunological role of LCs remains unclear (39-41).

4.0 DISCUSSION

Overall, the pathogenesis of DENV is poorly understood. It is suspected that cutaneous immune cells are primary targets of infection and that they contribute to viral dissemination and adaptive immune activation; however there is limited evidence to support this hypothesis (17). The lack of an appropriate animal model for DENV and the limited access to patient samples has made biologically relevant studies of DENV difficult to perform. Here, we used healthy human skin explants, in conjunction with human mdDCs, to study the degree of cutaneous infection and the potential role of cutaneous immune cells in DENV pathogenesis.

It is currently hypothesized that initial DENV infection occurs in cutaneous immune cells and that subsequent migration of infected cells to the lymph node results in dissemination of virus (17). However, *in vitro* data presented here supports findings that viral infection does not stimulate the maturation of DCs (44). It is probable that insufficient maturation of DENV infected dDCs and LCs would render these immune sentinels incapable of migration out of the skin; this would limit the degree to which dDCs and LCs contribute to virus dissemination *via* the cutaneous draining lymph node. This is supported by the lack of DENV positive cells observed in emigrant skin cell populations. Additionally, our data confirm that dDCs do not express DC-SIGN (28) and that LCs lack defined DENV receptors (10, 11). It is possible that this indicates a reduced susceptibility of dDCs and LCs to DENV infection compared to dMØs. The expression of DC-SIGN and MR on dMØs suggests that they would be susceptible to DENV infection (10, 11, 29); a hypothesis confirmed here by immunolabeling of CD163 and DENV in *ex vivo* inoculated skin samples. In response to invading pathogen, the role of dMØs is primarily one of cytokine production and pathogen clearance; these cells have limited migratory and immunostimulatory capacities (27, 28). Also, infection of dMØs likely results in trafficking of DENV into poorly acidified phagosomes, thereby limiting viral replication (46). These facts indicate that dMØs do not contribute greatly to the amplification and dissemination of virus.

In addition to limiting the amount of viral replication and producing immune modulating cytokines, dMØs may contribute to DENV clearance. DENV inoculation of skin explants results in the infection of dMØs as well as an altered distribution of uninfected dMØs compared to mock infected skin. Dense populations of uninfected dMØs are evident in the papillary layer of the dermis, just deep to the epidermis. DENV infection of keratinocytes in the basal layer of the epidermis likely alters the immune environment such that these uninfected cells are recruited to this site. Here, dMØs may aid in the clearance of virus as well as apoptotic cell bodies. Further investigation of dMØs, as well as keratinocytes, is necessary to understand their dynamic roles in cutaneous DENV infection.

The minimal detection of DENV infected cells in the dermis of skin sections suggests that the spread of virus within the skin is limited. Previous *in vitro* studies indicate that, while DENV infected DCs do not acquire a mature phenotype (44), they do produce TNF-α and IFN-α (49-51, 54). IFN-α is likely produced following the recognition of dsRNA, the DENV replication intermediate (9), by TLR3. TLR3 is a potent inducer of type-I interferons (IFN-α and -β), which have antiviral and immunostimulatory effect on neighboring cells (58). Production of pro-inflammatory and anti-viral cytokines by DENV infected cells may stimulate the maturation of bystander cells and the down-regulation of DC-SIGN (44). This would render bystander cells less susceptible to DENV infection. This supports the hypothesis that the spread of DENV within the skin is limited.

Previous work has suggested that LCs are primary targets for DENV infection. Studies have identified DENV positive epidermal cells as LCs based solely on their epidermal location (43) or on their location and co-expression of CD1a (45). This work identified high levels of DENV prM protein in epidermal cells of inoculated human skin explants. However, this detection was primarily in the stratum basale and stratum corneum of the epidermis where LCs do not reside. Combined with the lack of detection in langerin^{+} cells of the dermis and epidermis, these data suggest that, while LCs may become infected, keratinocytes are the primary epidermal target of DENV. Additionally, it remains unclear how DENV infected LCs may contribute to DENV immunopathogenesis. Further, the immunological role of LCs is controversial. *In vitro* studies suggest that the primary function of LCs is the initiation of immune responses to epidermal threats (39). Contradictory *in vivo* data suggest that LCs are not necessary for the induction of

immune responses and may play an immunoregulatory role (39, 41). These inconsistencies further complicate our understanding of the role of LCs during DENV infection.

The induction of adaptive immune responses is vital to the clearance of pathogens, especially intracellular pathogens such as DENV. DCs play a critical role here, in which they become active, mature, and migrate to the lymph node to activate T- and B-cell mediated responses. The results presented here indicate that DENV infected dDCs, and potentially LCs, may fail to contribute to the induction of adaptive immunity. Insufficient expression of the immune synapse molecules MHC-II, CD86 and CD83 on DENV infected mdDCs supports this hypothesis. While expression of MHC-II and CD86 are up-regulated on DENV infected DCs stimulated with soluble maturation stimuli, the expression of CD83 on these cells remains absent. It is likely that a yet unidentified viral factor is responsible for the specific down-modulation of CD83 expression. This could represent an immune evasion technique of DENV. CD83 is known to reduce the threshold necessary for antigen specific B-cells activation (55), and it is probable that DENV down-modulates this molecule in an effort to inhibit the production of virus neutralizing antibodies. Additionally, a diminished capacity to migrate and present antigen to naïve T-cells allows DENV to avoid recognition and destruction by cell-mediated immunity. Further studies are needed to understand the mechanisms of how DENV causes these deleterious changes in DCs.

In addition, we confirm that DCs become apoptotic upon DENV infection (44). This pro-apoptotic effect of DENV was observed at MOIs as low as 1viral PFU/cell. We speculate that infected DCs that are incapable of maturation undergo programmed cell death. Further studies are needed to assess apoptotic effects of DENV infection in cutaneous immune cells.

The work presented here has established an *ex vivo* model for further investigation of DENV infection in subsets of cutaneous immune cells, as well as to establish their consequent phenotype. Elaborating on these methods, cell-surface staining for maturation markers, including CD86, CD83 and CCR7, can be used to confirm whether $DENV^{+}$ cutaneous immune cells acquire a mature phenotype. Additionally, this method can be used to confirm the pro-apoptotic effects of DENV infection that are observed in mdDCs. Furthermore, the use of this *ex vivo* skin model could be extended to studies of other arboviruses, such as West Nile virus (WNV). Similar to DENV, WNV is a reemerging infectious agent that causes life-threatening illness and is not preventable by vaccination (2). Elucidating the role of skin immune cells could aid in the

understanding of WNV pathogenesis and the development of a safe and effective vaccine. Also, this system could be used to study variability of virus infectivity in mosquito vs. vaccine infections. Limited studies suggest that the infectivity of DENV, and potentially other arboviruses, is affected by mosquito saliva (59). This warrants investigation as it could impact the efficacy of purified virus vaccines if cutaneous infection is a primary route of virus introduction.

The incidence and severity of DENV cases have increased dramatically in recent years (4). Our current understanding of DENV pathogenesis is limited; this fact contributes to difficulties in developing effective vaccines and treatments. Due to the vector-borne nature of the virus, a primary target of infection is thought to be the skin immune system. Therefore, elucidating the effects of infection on cutaneous immune cells will be vital in our efforts to understand DENV pathogenesis and develop safe and effective therapeutic and preventative treatments.

APPENDIX

ANTIBODY INFORMATION

Table 2: Maturation and apoptosis antibodies

ANTIGEN	CONJUGATE	ISOTYPE	CLONE	SPECIES	COMPANY
HLA-DR	APC-Cy7	IgG2a	L243 (G46-6)	Mouse	BD #335796
CD80	PE	IgG1	L307.4	Mouse	BD#557227
CD86	FITC	IgG1	2331(FUN-1)	Mouse	BD#555657
CD83	PE	IgG2b	HB15A	Mouse	Immunotec#PNI M2218
CD14	PE	IgG2b	MφP9	Mouse	BD#347497
Caspase-3	FITC	IgG	C92-605	Rabbit	BD#559341

Table 3: Emigrant skin cell antibodies

ANTIGEN	CONJUGATE	ISOTYPE	CLONE	SPECIES	COMPANY
HLA-DR	APC-Cy7	IgG2a	L243(G46-6)	Mouse	BD#335796
CD1a	PE-Cy5	IgG1(κ)	HI149	Mouse	BD #555808
CD1c	Pacific Blue	IgG1(κ)	L161	Mouse	BioLegend # 331507
CD207	PE	IgG1	DCGM4	Mouse	BeckmanCoulter#IM3577
CD163	FITC	IgG1	5C6-FAT	Mouse	BMA Biomedicals #T-1062

Table 4: *In situ* staining antibodies

ANTIGEN	CONJUGATE	ISOTYPE	CLONE	SPECIES	COMPANY
HLA-DR	Pure	IgG2	L243(G46-6)	Mouse	BD#347360
CD1a	Pure	IgG2a	NA1/34HL	Mouse	AbD serotec #MCA80T
CD1c	Pure	IgG1	L161	Mouse	AbD serotec #MCA694T
CD207	Pure	IgG1	DCGM4	Mouse	Beckman Coulter #IM3449
CD163	Pure	IgG1	5C6-FAT	Mouse	Novus Biologicals #BM4041
CD206 (MR)	Pure	IgG1	15-2	Mouse	AbD serotec #MCA2155T
CD209 (DCSIGN)	Pure	IgG2b	120507	Mouse	R&D Systems #MAB161
CCR7	Pure	IgG2a	150503	Mouse	R&D Systems #MAB197
CD83	Pure	IgG1	HB15	Mouse	AbD serotec #MCA1582T

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