DEFINING DENGUE VIRUS INFECTION IN HUMAN SKIN

by

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ABSTRACT

The skin is the primary site of dengue virus (DENV) replication following the bite of an infected mosquito, but the factors that contribute to productive infection in human skin and virus spread out of skin are not understood. We defined the dynamics of DENV infection in human skin explants using quantitative in situ imaging. A transient interferon-α response occurred prior to detectable virus replication, which initially established in cells in the epidermis. DENV infected a wide variety of cell types including Langerhans cells (LC), dermal macrophages (Mϕ), dermal dendritic cells (DC), fibroblasts, mast cells, and lymphatic endothelium, but keratinocytes were the earliest and quantitatively most important target of DENV infection, contributing to 60% of overall infected skin cell over time. DENV infection led to the recruitment and infection of LC, dermal DC, and dermal Mϕ. These immune cells emigrated out of the skin in increased number as a result of infection, presumably leading to dissemination of virus. Infection of keratinocytes led to the abundant production of inflammatory mediators, most significantly IL-1β. Blocking keratinocyte-derived IL-1β reduced the infection of LC, dermal DC, and dermal Mϕ by 75-90% and decreased the total number of infected cells in epidermis and dermis by 33% and 65%, respectively. In the first demonstration of antibody-dependent enhancement of DENV infection in human skin, we showed that the presence of heterotypic DENV-immune serum enhanced the recruitment and infection of dermal Mϕ by 50-70%, and increased emigration of myeloid cells

out of skin. *Aedes aegypti* mosquito salivary gland extract did not impact dermal Mϕ recruitment or infection with DENV, with or without immune serum. Blocking FcγRIa and FcγRIIa inhibited antibody-mediated infection of dermal Mϕ, and decreased the number of cell emigrants, resulting in reduction of the overall number of infected cells in the dermis by 70%, without notable changes in the epidermis. Ethnic differences in skin immune responses to DENV were observed for the first time in our study. In comparison with skin from Caucasians donors, skin from African American donors maintained robust antiviral IFN-α responses for at least 48 hours. This was observed in association with less DENV replication, a reduced production of IL-1β in the epidermis, less recruitment and infection of LC and dermal Mϕ, and less cell emigration out of the skin. These findings suggest that innate immune responses in skin control DENV replication and spread, and equates with epidemiologic data that African ancestry protects against severe dengue. Our findings highlight the importance of skin and the complex interplay between resident and immune skin cell populations in DENV infection and dengue pathogenesis. Defining DENV infection in human skin therefore has considerable public health significance because these data will provide a rationale for exploration of therapeutic strategies through targeting the mechanism DENV exploits skin microenvironment and preventing the risk of systemic infection as well as severe dengue.

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1.0 CHAPTER ONE: INTRODUCTION

1.1 HISTORY AND CURRENT GLOBAL STATUS OF DENGUE

1.1.1 Evolution of dengue virus (DENV)

Dengue fever is an old disease caused by infection with any of four serotypes of dengue virus (DENV), DENV-1, 2, 3 and 4. The origin of DENV is unclear but most likely it originated as a mosquito-specific virus (1). DENV were maintained by vertical transmission among canopy-dwelling mosquito species responsible for sylvatic (forest) cycles, which periodically adapted to involve lower primates, and later humans (2). DENV capable of infecting humans likely evolved in the forest cycle and moved into rural areas after urbanization had become common. The geographic and evolutionary origin of the virus remain under discussion to recent days. It is unclear whether DENV originated in Asia, in Africa, or in both areas.

The Asian-origin hypothesis suggested that dengue fever in Asia was predominantly transmitted by *Aedes albopictus*, an Asian mosquito species (3). The earliest record of an epidemic case with dengue-like illness, characterized by rash, fever, pain in eyes, muscles, joints, and bleeding in various organs (4), was documented in a Chinese medical encyclopedia, first published during AD 265-420. Nevertheless, this raises a question whether the true etiology was DENV, as the

manifestation of arthralgia could be compatible with a disease caused by Chikungunya virus (CHIKV). All four serotypes of DENV are transmitted in sylvatic cycles in Malay Peninsula, whereas only DENV-2 has been documented in a forest cycle in Africa (5). Serological surveys in the 1950s confirmed that the people and canopy-dwelling animals living in diverse ecologies in Malaysia had a similar prevalence rate of DENV-1 neutralizing antibody (Ab), increasing with age, suggesting that dengue endemics were common in the area (6). Phylogenetic analysis placed the Asian sylvatic strains in a deep position in the phylogenetic tree (7). This provides further support for the Asian-origin of the virus.

Alternative theories supporting an African origin speculated that dengue was transmitted by *Aedes aegypti*, a species of mosquito that has the ancestral type as well as domestic populations in sub-Saharan Africa (3). Nowadays, it is a primary vector of DENV and the related Zika virus, and inhabits much of the tropical and subtropical region. DNA sequencing analysis of DENV and other related flaviviruses indicated a progenitor originating from Africa (8). Genetic studies proposed a phylogenetic tree of *Aedes aegypti* showing genetic similarities between mosquito populations in the Americas and those in Asia-Pacific regions, indicating the notion of global westward development of dengue (9). Thereby, it is believed that dengue spread from West Africa through the import of African slaves to non-immune indigenous populations since the 17th Century, and from there to the Asia-Pacific region as the global shipping industry expanded in the 18th and 19th Centuries. Earliest epidemics of dengue-like illness in the New World were reported in French West Indies (1635) and Panama (1699), in one of the Atlantic ports where enslaved Africans entered, supporting a disease widespread from Africa following the slave trade

routes. Because the *Aedes* African mosquito was a highly efficient vector, it rapidly infested when introduced into port cities, moved to inland cities, and caused urban epidemics (10).

1.1.2 Overview of dengue history and its emergence as a global public health threat

1.1.2.1 Dengue in the past (17th Century to mid-20th Century)

Prior to an arrival of *Aedes aegypti* in Asia, dengue outbreaks were limited, short-lived and infrequent as dengue urban epidemics were unknown. Many observations suggested an introduction of the mosquito species in Asia and Australia in the last half of the 19th Century (3). Dengue epidemics became common in the region and increased in frequency, as *Aedes aegypti* distribution expanded.

The global pandemic of dengue began during the Second World War (1930s-1940s) (4, 11). The war activities provided an ideal larval habitat for *Aedes aegypti*, with water storage and constant movement of new susceptible individuals as well as their eggs and viruses, resulting in a greatly expanded geographic distribution and increased densities of the mosquito vector, and increased dengue epidemics. Also, it has created the perfect conditions for initiating the 20th Century dengue pandemic worldwide.

Following the end of the Second World War, many countries experienced strong economic growth which was a driving force of unprecedented, rapid urban growth that continues today. Millions of people migrated from rural areas to urban cities for work. A rapid societal expansion resulted in inadequate management of infrastructures, supplies and wastes. As effective mosquito control had not been fully developed, DENV transmission progressively increased accordingly,

with increasing frequency of epidemics occurring in migrant workers and children. Together, fast-paced population growth and uncontrolled global trade and travel, combined with a large population of mosquitoes living in intimate association with crowded human populations, were key factors that inevitably led to hyperendemic conditions where the multiple DENV serotypes began to co-circulate (12, 13). It was in this setting that the emergence of dengue hemorrhagic fever (DHF) was observed in Southeast Asia. DHF is a severe and fatal hemorrhagic disease associated with DENV infection in a small minority of infected individuals. The first epidemic was recorded in Manila, Philippines in 1953-1954, followed by another in 1956, and a subsequent epidemic in Bangkok, Thailand in 1958 (14, 15). It is believed that many DHF cases in such regions went unclassified throughout the 1950s. Since then, it has changed the core belief of dengue that was once thought to have limited clinical significance.

Throughout the 17th to the early 20th Centuries, vector-borne diseases including malaria, yellow fever, dengue and plague were responsible for more human illness and death than all other causes combined (16). Yellow fever received much attention after the vector role of *Aedes aegypti* was first demonstrated by Walter Reed. Later, dengue was shown to have a similar transmission cycle. That knowledge pushed forward new possibilities of eradicating the diseases. Shortly, the large-scale prevention and control program was established by the Pan American Health Organization (PAHO) in the Americas in 20th century (1947-1970). The program that was implemented emphasized elimination of mosquito breeding sites through environmental hygiene along with limited use of chemical insecticides, and succeeded in 23 out of 27 countries, mainly in Central and South Americas. Field operations indicated that sampling sites were either free of infestations or that the mosquito was present in exceedingly small numbers at yellow-fever

receptive areas in the US. Correspondingly, the threat of urban yellow fever as well as dengue was almost eliminated. The control of urban yellow fever in the Americas through the eradication of *Aedes aegypti* was marked as a great achievement. On the other hand, the eradication plan or effective vector control was never achieved in Asia.

1.1.2.2 Emergence/Resurgence of dengue (1970 to present day)

The *Aedes aegypti* eradication campaign was disbanded in the early 1970s because yellow fever and dengue were no longer a critical problem (16), resulting in proliferation and invasion of this mosquito in nearly all countries that had once achieved eradication (17). The period of failed public health support on arboviruses happened to coincide with a globally expanding urbanization as well as increased movement of DENV both into and within the Americans (18), leading to a dramatic increase in dengue that reached staggering levels in only a decade (1970-1980). Nevertheless, the clinical presentation reported was self-limiting dengue fever. Dengue during that time was a hypoendemic disease as either DENV-2 or DENV-3 viruses were present at one time. Epidemiologic evidence suggested that DENV-1 as well as DENV-4 were introduced to the Americas from Asia, rapidly spreading from islands in the Caribbean to inland countries.

In 1981, Cuba was the first country in the region that suffered a serious DHF outbreak (19, 20). There were 344,203 cases of dengue-like illness, 10,312 cases of DHF reported, and 158 deaths. It was following by more DHF cases in other American countries, which in total accounted for about 10,000 DHF cases and 165 deaths during 1981-1992. DENV-2 appeared to be a serotype that was most associated with fatal cases. These epidemics of DHF, particularly the one that occurred in Cuba, were thought to be caused by an introduction of a strain of DENV-2 from

Southeast Asia. The sequence of events associated with the changing epidemiology of dengue indicated that the Americas were likely facing a similar situation as Southeast Asia's in the 1950s and 1960s.

The transmission of dengue in Asia has been persistently maintained. Failure to control the mosquito vector coincided with a period of economic expansion, which is a key driver of intense urban growth, globalization, and growing industries since the 1960s (13). This setting has a profound impact on dengue, especially in major cities as they provide an ideal, sustainable environment for DENV to fully adapt to humans, without the need to replicate in a sylvatic cycle. Unfortunately, this continues to the present time where a region experiences repeated epidemics, with dengue outbreaks getting progressively larger (21). Singapore, a country that once had dengue in controllable levels, experienced a drastic resurgence of dengue epidemics that still lasts to this day (22). As DHF has been endemic in this region, the frequency and magnitude of epidemic DHF increased, with major epidemics occurred every 3-5 years. It is a leading cause of hospitalization and death among children in many countries in Asia (23).

The introduction of air transportation during 1960s-1970s has changed the epidemiology of dengue. It provides a much larger landscape and faster routes to rapidly import dengue from endemic areas to non-endemic areas. Many dengue-endemic countries are popular tourist destinations. Like migrant workers, travelers are at significant risk of acquiring the disease caused by strains of virus capable of causing epidemics, and contribute to its spread throughout the country, region, and globally. With most countries in the Americas changing from non-

endemic or hypoendemic conditions to hyperendemic in less than 30 years since the 1970s (23), there has been rapid amplification of the disease driven by all possible factors combined.

While yellow fever has ceased to be a global threat because of the vector eradication program and mass yellow fever vaccination campaigns in the mid-20th Century, dengue has emerged as a major public health problem. Although dengue research has been making advances, a safe and effective vaccine has yet to be found because of the underlying complex pathogenic mechanisms. Further research into dengue disease pathogenesis will hopefully help solve this global health threat of the 21st Century.

1.1.3 Current global distribution

Currently, the distribution of dengue has been reported in more than 125 countries globally. The World Health Organization (WHO) estimates that 2.5 billion individuals are at risk of exposure to dengue, with approximately 50-100 million infections, 500,000 severe cases and 12,500 deaths, mainly in children at an age younger than 15, occurring annually (23). A recent epidemiological survey suggests 3.6 billion people are at risk, producing 390 million DENV infections annually, resulting in approximately 96 million cases with apparent clinical manifestations and 15,000 deaths (21). These recent estimates have dramatically raised the profile of the disease. According to recent United Nations world estimates, the current world population is 7.6 billion (24). Therefore, using the estimates above, approximately 50% of the global human population is at risk of contracting DENV. As globalization, tourism and commerce are progressively increasing in our modern world, coupled with poor surveillance systems in many inaccessible and underdeveloped parts of the globe, this projection is likely an

underestimate. WHO has currently labeled dengue as one of the fastest-growing viral threats worldwide.

1.1.4 Current approaches for dengue prevention and control

Currently, the cornerstone of dengue prevention remains *Aedes aegypti* mosquito vector control and avoidance of mosquito bites, due to lack of specific prophylactic or antiviral treatment options and effective dengue vaccines (23). Environment management is a classical but still up-to-date vector control approach that aims at preventing or minimizing vector propagation and reducing human-vector-pathogen contact through changing a natural environment to suppress stages in mosquito life cycle and using mosquito repellents. Insecticides are restricted for use as an emergency vector control method during outbreaks. Unfortunately, vector control approaches are limited in number and not as safe or efficacious as needed (25). Misuse of insecticides can mediate resistance in mosquitoes and environmental toxicity (26). Biological control measures offer more tools for vector control without chemical contamination of the environment. These controls constitute anti-DENV interventions based on the introduction of living organisms that will prey upon, parasitize, compete with, or otherwise reduce the abundance of the mosquito. Previously, the copepod strategy, using water fleas to prey on newly hatched larvae, has been successfully implemented in Vietnam (27, 28). However, the application comes with limitations as it requires a persistence copepod population in known breeding sites and is only employed against an immature stage of the mosquito life cycle. An effect is thus delayed as reduction of the mosquito larval population takes time to have a significant impact on adult mosquitoes. Also, the results do not always correspond to reduction in disease transmission, making it an unsuitable method especially during dengue outbreaks.

Recently, attempts have been made to develop a new set of biocontrols that could be a solution for a global control of dengue, including *Wolbachia*-based technology (29). *Wolbachia pipientis* are maternally inherited, intracellular bacteria that are known to live in and manipulate the reproduction of many insect species in order to enhance their own, resulting in a shorter lifespan of their hosts. This life-shortening phenotype has led to an idea that the bacteria can be used to invade *Aedes* mosquito populations to suppress DENV transmission. Under controlled conditions, a dramatic 3-log reduction in virus replication, measured as the DENV genome copy, was observed in *Aedes aegypti* infected with *W. pipientis* when compared to uninfected controls, indicating that the impact of bacterial infection is not only limited to host longevity, but also vector competence (30, 31). Small-scale field tests suggest that *W. pipientis* was able to invade the wild mosquito with close to a 100% maternal transmission rate (31, 32). Presumably, such rapid bacterial invasion among mosquitoes would greatly decrease the transmission rate of DENV in human populations. While this genetically-modifying mosquito strategy is promising, it remains largely experimental and requires answers to many questions regarding implementation in the field.

Whereas genetically engineering mosquitoes appears encouraging to reduce infectious mosquito populations, dengue vaccine development efforts are facing disturbing news from an approved dengue vaccine. In 2015, Sanofi Pasteur's Dengvaxia® became the first dengue vaccine to be licensed for use in individuals aged between 9-45 years old in dengue endemic countries, mostly in Asia and Latin America (33). The Philippines was the first country to grant the use of the vaccine which was introduced in public school-based immunization program in April 2016, targeting more than 730,000 school children. The vaccine has also been used in other countries,

including Brazil. Shortly thereafter, Sanofi Pasteur made a public announcement in November 2017 about new analysis from the vaccine trial and attempts to update product label. The findings suggested that the vaccine provided a long-term safety and efficacy only to those who had previously been infected with DENV. DENV-naïve vaccine recipients, however, were at risk for severe dengue disease after vaccination upon a subsequent dengue infection. Despite these safety data, the company claimed that there have been no reported deaths that were related to dengue vaccination. To date, over one million individuals have received at least one dose of vaccine. The vaccine program in the Philippines is now entirely suspended and the FDA has halted the sale of the vaccine. In Brazil, the dengue vaccination effort is still ongoing with the usage restriction to provide vaccine only to those previously infected with DENV. The WHO has supported the decision of the Philippine government to suspend the vaccine program and has recommended a restriction based on the company's data.

In fact, the possibility of these safety issues whereby the dengue vaccine sets up dengue-naïve recipients for severe illness has been shown in a clinical trial of CYD-TDV (referring to Dengvaxia®), a live attenuated tetravalent vaccine based on a recombinant virus constructed from yellow fever. It was able to show an efficacy of over 90% in preventing severe dengue in children 9 years and older who were hospitalized in year 3 of a phase III clinical trial conducted in over 35,000 schoolchildren aged 2-16 years in 10 endemic countries (34, 35). However, the hospitalization rate for severe dengue among seronegative children ages 5 years or younger was five-fold higher than placebo control. The hospitalized risk increased 1.6-fold in vaccinated children younger than 9 years old (36). It was this reason that Dengvaxia® was recommended to recipients 9-45 years old. Recent studies have suggested that the overall efficacy of Dengvaxia®

is dictated by immune status, not age (37). Given recent observations, many dengue experts voiced concerns that CYD vaccination induced low levels of DENV-specific antibodies in seronegative individuals, resulting in development of severe disease upon primary DENV infection through Ab-mediated enhancement (ADE), and argued for further explicit investigations on this issue (38-40).

As dengue will continue to be a global threat in the oncoming years, it is clear that there are no effective dengue vaccines available for therapeutic options. Nevertheless, a number of dengue vaccines are in various stages of development and some have shown promising results. For example, it has been shown in the recent 18-month phase II randomized, controlled trial that Takeda's live attenuated dengue vaccine candidate (TAK-003) provided a complete protection against all four DENV strains regardless of prior virus exposure and immunization schedule (41, 42). It is now being tested in a large-scale phase III clinical trial which is expected to be completed in 2018.

1.2 DENGUE VIRUS

1.2.1 Virus classification

DENV is a non-segmented, positive-sense, single-stranded RNA virus in the genus flavivirus, family Flaviviridae (43). DENV is related to other mosquito-borne flaviviruses such as yellow fever virus, Zika virus (ZIKV), Japanese encephalitis virus, West Nile virus (WNV) and St. Louis encephalitis virus. There are four genetically distinct serotypes; designated as DENV-1,

DENV-2, DENV-3, and DENV-4, which can be distinguished by serological and molecular methods. Molecular studies on the nucleotide sequences of DENV genomes allow identification of the agent into genotypes (44).

1.2.2 Virus structure

Dengue viruses have a spherical shape and are approximately 45-50 nm in size (45). The basic structural organization of virus particles can be divided into four components (from the inner core to the outer shell): DENV genome, nucleocapsid, envelope and membrane glycoproteins.

1.2.2.1 DENV genome

The DENV RNA genome is a linear, single strand which spans approximately 11-kb (46). The RNA bears a type I cap structure, m7GpppAmp, at its 5' end and lacks a 3'-polyadenylated tail. The uninterrupted, long open reading frame encoding a large viral polyprotein is flanked in 5' and 3' by untranslated regions (UTRs). Both ends carry a number of cis-acting signals such as stem loops and conserved sequences required for viral replication and translation. There are complementary sequences in UTRs that induce the cyclization of the genome (47). Genome cyclization is the formation of specific secondary RNA structures formed by the interactions between two ends of the complimentary cyclization sequences of the viral genomic RNA that regulate different viral processes together with host and viral factors (48). It is a potential conserved mechanism among Flaviviruses to ensure that the viral replication is confined to only undamaged, full-length genomes.

1.2.2.2 Nucleocapsid

The inner core contains an icosahedral nucleocapsid about 30 nm in diameter. It is a single molecule of the dengue RNA genome formed in complex with multiple copies of the capsid (C) protein. Capsid proteins act as multifunctional RNA chaperones as they promote correct folding of RNA molecules by either preventing or resolving their misfolding. Efficient genome packaging by capsids also protects the RNA from contact with host immune sensors, or any environmental hazards in the infected cell. The capsid-RNA interaction in DENV has been shown to have high affinity but low specificity (49), suggesting that the nucleocapsid assembly in the endoplasmic reticulum (ER) is likely non-specific and mainly driven by electrostatic forces. The capsid protein has been shown to bind to membranes (49), suggesting that DENV capsids may translocate the genome into host cells through a receptor-independent interaction with the viral envelope, which enhances the infection process.

1.2.2.3 Envelope and membrane glycoproteins

The nucleocapsid is sheathed in a membrane called the envelope which is a lipid bilayer acquired from the host. The viral envelope is embedded by envelope (E) and membrane (M) glycoproteins. These proteins form a protective layer that control the viral entry into host cells. The E protein is the dominant surface protein of the mature virus. The E protein has two N-linked glycosylation sites and structurally can be divided into three domains: The N-terminal central domain I, the elongated dimerization domain II, and the Ig-like domain III. The E protein functions as a fusion molecule because of the fusion peptide located at the distal end of domain II. Domain III serves as the receptor attachment domain. Due to its high proportion on the virus particle, the E protein contributes to most of the antigenic sites and is also the target for

therapeutic monoclonal antibodies (50, 51). The M protein is a product from the precursor prM processing during virus maturation.

DENV goes through substantial conformational and translational movements of viral structural proteins during the virus life cycle, suggesting that the particles have substantial dynamic capabilities (52). The immature virus is converted into an infectious mature form by the cleavage and release of the prM protein. Prior to cleavage, prM on the immature virion is arranged with the E protein in a 1:1 manner. The prM moiety covers the hydrophobic fusion peptide at the end of domain II. This trimeric prM-E configuration is thus believed to be inefficient in infection as fusion of virion particles to host membranes is blocked. It also makes immature virus particles appear with spikes. On the other hand, the surface of the mature virion is relatively smooth as the E protein lays parallel to the viral membrane. In this conformation, domain III protrudes from the surface of the E protein and thus many of the Ab neutralizing epitopes are exposed. These epitopes permit the recognition of virions by Ab. Dynamic properties of E protein play an important role in the immune control of virus infection (45).

1.2.3 Virus life cycle: entry, replication and secretion

1.2.3.1 Entry

Infection begins with attachment of DENV particles to putative host molecules such as heparan sulfate (53, 54). Envelope protein is mainly responsible for interactions with receptors for virus attachment and entry. After binding to the receptor, the virus can be invaginated into the cytoplasm using cellular components called clathrin-coated vesicles, or it directly enters the cytoplasm via the fusion of the viral and host membrane. Following internalization, a low-pH

dependent fusion of the envelope of DENV and the host endosomal membranes leads to the release of the nucleocapsid into the cytoplasm of the infected cell. The viral genome is then released from the nucleocapsid by an unknown mechanism. A study showed that this step involved the ubiquitination-dependent degradation of capsid proteins (55). Viral proteins released following the uncoating step have been found to play a role in modulating host innate immune response (56).

1.2.3.2 Replication

In positive-stranded viruses, the genomic RNA serves as a template for translation and replication (46). When the genome is released into the cytoplasm it is presented to the ER where translation and processing immediately occur. The genome encodes a polyprotein in the following order: (N-terminal)-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-(C-terminal). This polyprotein is processed into ten individual polypeptides of varying length by proteases present in the ER lumen of host cells, called signalase and furin, as well as nonstructural proteins (NS3/NS2B). There are three structural proteins: capsid, and two transmembrane linked glycoproteins, precursor membrane protein (prM), and envelope protein (E). The seven nonstructural (NS) proteins: NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 have a variety role in viral genome replication and subversion of the host cell response for facilitating the production of virus progeny (57).

It is generally accepted that the cytoplasm is where RNA replication and nucleocapsid formation occurs. However, the replication stages are complex and do not occur freely in the cytoplasmic region (58). The process requires extensive reorganization and morphological changes of the cellular membrane environment induced by DENV (44). DENV genome replication is carried

out by a viral replication complex (RC) which is assembled on the cytoplasmic side of the invaginated perinuclear ER membrane, called the vesicle packets (VP). Although the exact component of the RC remains unclear, it has been shown that it is a highly organized complex associated with all types of viral NS proteins, viral genomic RNA, and host-derived proteins and lipids (44), resulting in bulging membranes into the lumen (VP). VP represent replication niches for DENV in the cytoplasm. The RC associated with the virus-induced VP is a great architecture for efficient virus production for many reasons. Host-derived membranes sequestrate the viral factor and genome from the immune detection. This modified structure facilitates viral replication by accumulating essential components in place and allowing rapid transport of viral proteins or host factors between interconnected compartments. Besides VP, the virus induces the formation of other intracellular structures that are continuous with the ER, known as convoluted membranes (CM), which appear to be the site of translation and polyprotein processing (59).

A prerequisite for DENV genome replication is the negative sense RNA template because an open reading frame in the positive viral genome is amplified through a negative strand (47). The synthesis of the negative strands is initiated when viral polymerase (NS5 RdRp) within the RC binds to the 5’ end at the stem loop A, next to the 5’ cap, and the RC aggregates on the 3’ end of the positive sense template. During replication, the negative sense remains bound to the genomic RNA, forming a double stranded replicative form (RF). These RF then serve as the template to produce new positive-sense RNA. In each round of synthesis, a single nascent positive strand is copied from the negative strand within the RF. The replication occurs through an asymmetrical semiconservative mechanism, resulting in multiple copies of positive strand RNA being synthesized. While the new positive strand is synthesized, it remains bound to the negative strand

and displaces the pre-existing positive strand, creating the replicative intermediate (RI). These displaced positive RNA progenies are either used as templates for further synthesis of negative strands and generation of RI recycling, or as mRNA for translation of viral proteins, or as replication products ready to be capped and methylated by NS3 and NS5, preparing the positive strand genomic RNA for packaging into virus particles.

1.2.3.3 Secretion

Following RNA replication and protein translation, the newly synthesized genomic RNA is encapsidated by virus-encoded capsid protein, creating nucleocapsid formation. The virus particles assemble on the surface of the ER adjacent to the sites of replication as nucleocapsids bud through the ER lumen to obtain an envelope membrane. The recruitment of E and prM proteins deposited on the opposite side of the ER lumen completes an assembly of immature virions. Nascent virions in the ER need to be transported from the ER to Golgi apparatus, and then transported to the plasma membrane before they are released outside the cells.

During the traffic through the Golgi and trans-Golgi network, prM protein is cleaved by cellular furin protease due to acidification, creating pr and M (52). M protein remains on the particle to form a heterodimer with E protein, whereas soluble pr protein is released from the mature virion when the virus egresses. A prM region provides resistance to acidic environments and prevents the premature fusion of E protein. Following prM cleavage, E protein undergoes a major conformational change which allows the 180 envelope copies in the virion to form 90 head to tail dimers which are arranged in an icosahedral symmetry, resulting in a smooth appearance of the mature virion. Theoretically, virus particles exit the infected cells in a mature and infectious form. However, cleavage of prM is often incomplete, resulting in the release of a variety of

intermediate virus forms. Levels of prM cleavage indicate an acquisition of infectivity because the pr region mechanically prevents the receptor-binding E glycoprotein from undergoing membrane fusion. Thus, the conformation of virions secreted from infected cells ranges from fully cleaved (infectious, mature particles) to partially cleaved prM (intermediate particle with somewhat infectious), to non-cleaved prM (non-infectious, immature particles). prM-containing immature particles account for 30% of virions secreted from cells infected with DENV (60).

1.2.4 Transmission

DENV is transmitted by *Aedes* species of mosquito. While the virus cannot spread directly from person to person, a viremic individual can infect *Aedes aegypti* species (61). DENV can also be transmitted by *Aedes albopictus*. The virus is maintained in two ecologically and evolutionary distinct transmission cycles: a sylvatic cycle and a domestic cycle (62).

1.2.4.1 The main vector: *Aedes aegypti* mosquito

Aedes aegypti is a mosquito species of the subgenus Stegomyia. It is believed to have an origin in Africa, where both sylvatic and domestic forms occur (63). Because this species transmitted urban yellow fever for centuries, it is, thus far, commonly known as the “yellow fever mosquito”. *Aedes aegypti* is distributed in tropical and subtropical regions around the globe (64). The invasions occur most during the warm weather season and the populations do not survive the winter. *Aedes aegypti* is adapted to live in close proximity with human dwellings. It is essentially an urban mosquito. This characteristic extensively complicates its control.

1.2.4.2 Transmission through a blood meal

The successful transmission of DENV relies mainly on three factors: host, mosquito, and virus. A susceptible mosquito needs to feast on any DENV-infected individual with a sufficiently high viremia. A study showed that the titer of virus in human blood influences the infection in mosquitoes (65). A higher titer of virus takes a shorter incubation period of about 12 days in a mosquito, whereas it takes mosquitoes up to 3 weeks to transmit the virus when feeding on individuals with a lower virus titer. People with asymptomatic infections have been shown to have a lower viremia level than those who are in a period prior to an onset of symptoms or with symptomatic infections. Viremia levels vary throughout the course of infection. In those who develop symptoms, it has been observed that the titer of virus is highest during day 1 to 4 of illness.

In mosquitoes acquiring a DENV infection, it is critical for DENV to infect the mosquito salivary gland to be delivered together with saliva into host skin (66). After a blood meal is taken, viremic blood first arrives into the mosquito midgut. The virus must successfully infect the midgut epithelium to replicate to a higher levels and spread into a hemocoel, which is a body cavity between the organs in which blood or hemolymph flows and bathes the tissue and organs. This function of the cavity inadvertently helps disseminate the virus to several mosquito tissues including the salivary glands. Sufficient virus replication in the salivary gland ensures infectious levels of virus that will likely be transmitted to a new vertebrate host for the next feeding time. The titer of virus transmitted by an infected mosquitoes transmit is highly variable, ranging from 10^3 to 10^7 plaque forming units (PFU) (67). The number of virions needed to productively infect humans is unknown.

Only female mosquitoes, which need a rich source of pre-digested blood to nurture their eggs, have the modified physiology necessary for taking blood from warm-blooded creatures, particularly humans in a case of *Aedes aegypti*. The proboscis is the female's mouth part that is adapted for pain-free penetration of the skin of hosts, and is used for probing and cannulating a blood vessel, and for aspiring blood. The proboscis is a long six-needlelike system encased in a sheath called a labium which makes the needles look like a single tube (68). In fact, a proboscis includes two sharp maxilla for anchoring mouthparts while drilling the skin, two mandibles for holding the skin apart, a bendable labrum for finding and piercing a blood vessel and sucking blood, and a hypopharynx for injecting saliva and sometimes incidentally delivering virus particles (69). Skin is thus the first organ that encounters DENV in the human host.

1.3 DENGUE DISEASE AND PATHOGENESIS

1.3.1 Dengue case classification and levels of severity

DENV infection results in a broad range of clinical features from asymptomatic infection to dengue fever (DF) to a severe form of disease called dengue hemorrhagic fever (DHF) that can progress to a life-threatening dengue shock syndrome (DSS). The latest classification system views dengue as one disease entity with different clinical presentations, often with unpredictable clinical evolution and outcome (70). Patients are classified depending on their overall levels of disease severity into two groups, as having dengue (with or without warning signs) or severe dengue. The basic dengue case definition involves patients with fever and two of the following criteria: nausea/vomiting, rash, aches and pains, leukopenia, and tourniquet test positive. Most

cases present with a self-limiting non-severe illness followed by recovery without complications. However, approximately 10% of reported cases progress to severe dengue, mostly characterized by vascular leakage with or without hemorrhage, and shock. A major concern has been that individuals progressing to severe disease are difficult to define when notable features present together.

1.3.2 The course of dengue illness: febrile – critical – recovery phases

Following bites from an infected mosquito, there is an incubation period that lasts 4-7 days (minimum 3 days, maximum 14 days), and the individual may abruptly develop symptoms afterwards. The illness usually follows three phases: febrile, critical, and recovery (70, 71).

1.3.2.1 Febrile phase

Patients typically experiences sudden onset of high-grade fever (39-40◦C). This phase commonly persists for 3-7 days and terminates abruptly; most patients recover without serious complications after the temperature settles. These patients are classified as having non-severe dengue. Mild hemorrhagic manifestations such as skin petechiae or bleeding from any organs are uncommon, but sometimes present. Enlarged liver is often noted. After the febrile phase, patients with a reduction of leukocytes (leukopenia), but no increase in capillary permeability, will mostly improve, whereas those showing signs of plasma leakage tend to progress to worse conditions.

1.3.2.2 Critical phase (leakage phase)

A number of systemic problems may develop around day 3-6 of dengue illness when the temperature decreases to 37.5-38◦C or less. The most serious manifestation is an increase in capillary permeability which can occur in any age group, but it is predominantly recognized in children and young adults. Once this occurs, it defines the timing of onset of the critical phase lasting for 24-72 hours, and results in profound loss of plasma volume, increasing hematocrit levels, and subsequent potentially fatal hypovolemic shock that is the hallmark of DSS. The increased capillary permeability often occurs in conjunction with hemorrhagic manifestations and abnormal hemostasis. Skin petechiae or easy bruising are the most frequent hemorrhagic manifestations during this phase. Changes in total white blood cell count are consistent with early suppression of platelet production in bone marrow as well as leukopenia, all reaching a nadir during the critical phase before gradually increasing to normal levels over a few days. Evidence suggests that the severity of hematologic abnormalities has a strong correlation with the severity of the plasma leakage (72).

1.3.2.3 Recovery phase

The plasma leakage and abnormal blood counts during the critical phase are usually transient. On day 6-8 of illness, a gradual reabsorption of extravascular compartment fluid begins in the 48-72 hours following the onset of recovery phase. The clinical outcome in patients receiving prior careful management is commonly good in a short-term period. Most patients develop skin rash during the transition from critical to recovery phases. The rashes vary ranging from a mild maculopapular rash to a new skin rash called “islands of white in the sea of red” (73, 74).

1.3.3 Risk factors responsible for the severity of dengue

Dengue pathogenesis remains poorly understood, as the nature of dengue fever and severe dengue is complex and varied, with the involvement of multiple organs and intricate virus-host interactions. The multifaceted clinical presentation and diagnostic challenges of dengue clearly indicate that no single variable should be considered as a sole mechanistic cause of the clinical outcome. Alternatively, many factors are concomitantly involved in the expression of dengue phenotypes. These risk factors are virus, host, vector and others such as environment, socio-economic status and race (75). Understanding how factors determine the dynamics of dengue is important for discovering, developing, and implementing methods to prevent infection and improve patient outcomes.

1.3.3.1 Viral factors

The risk of developing severe disease after infection by one of the four DENV serotypes may differ depending on the serotype of the infecting virus. All four DENV serotypes have the potential to cause severe and fatal disease in humans (76). Studies in the Kingdom of Tonga and Thailand have shown DENV-1 and DENV-3 to cause more severe outcomes in the absence of previous DENV infection than DENV-2 or DENV-4 (77). While primary DENV-2 infection is rarely linked to severe disease, many epidemiologic studies in various regions including Thailand, Nicaragua, Cuba, Colombia, and Burma, have demonstrated that secondary DENV-2 infections are most frequently associated with DHF and shock as compared to the other serotypes (78-81). However, exceptions have been documented in different locations; DENV-3 infection in Brazil were more likely to show an association with shock, abdominal pain and exanthema as compared to DENV-2 and DENV-1 (82).

It is difficult to draw solid conclusions for serotype-specific differences in disease severity because most previous studies did not take differences among genotypes and strains within each serotype into account. It has been suggested that some genotypes of DENV-2 are more virulent due to their within-host fitness. It is believed that the virus with genetic variants showing faster replication and higher viral loads tend to produce more severe disease. Studies have shown that the Southeast Asian genotype of DENV-2 associated with dengue disease severity is able to replicate more efficiently in vitro than the American genotype of a similar serotype (83). This high fitness feature provides the Asian strain the potential to displace strains with lower relative replicative ability when co-circulation occurs as it has been observed in the America prior to 1981 (84) (refer to 'History' section). Only a few cases of severe manifestations associated with DENV-2 were documented in Latin America and the Caribbean during the time when the American genotype of DENV-2 circulated. The introduction of the virulent Asian DENV-2 stain into the region corresponded with the first DHF/DSS epidemic in Cuba (20). The replacement of the Native American DENV-2 with an invading DENV-2 genotype of Asian origin resulted in more severe cases observed. This epidemiologic event also support a positive relationship between within-host fitness and virulence in dengue virus (85).

1.3.3.2 Human host factors

Individual susceptibility seems to influence the occurrence of severe dengue. Studies have shown that severe dengue was found more frequently among children than in adults (86). With an estimation of half a million cases of severe dengue occurring each year, more than 90% of those cases required hospitalization and were children under the age of 15. In a study in Vietnam, it was reported that children between age 1-5 years had four times higher odds of dying from dengue than children between age 11-15 years, even though the incidence of DSS was found to

be highest in a group between the ages of 6 and 10 (87). These findings are consistent with physiological observations that younger children inherently have more penetrable blood endothelium and are more likely to suffer from plasma leak, shock and poor clinical outcome (87). The risk of developing severe dengue is also high in the infants (0-12 month), especially those who are born to DENV-immune mothers (88). It has been hypothesized that maternal Ab may play a role in symptomatic or severe dengue in infants (88).

The association between host genetic background and dengue disease severity was indirectly suggested in 1906 by Agramonte's statement that "black people seem to have a remarkable degree of resistance to dengue disease" (89). In support of the observation, epidemiological studies in multiracial areas such as Cuba and Haiti suggest that individuals of African descent have a lower risk of developing severe disease, as compared to those of European descent (19, 90). One explanation for these differences in dengue has been shown to be variation in T-cell response among White and Black Cuban population (91). More recently, a genome-wide association study identified genetic polymorphisms that protect people with an African ancestry from dengue hemorrhagic manifestations. These candidate genes such as OSBPL10 and RXRA are known to play a role in virus replication and immune function (92). However a limited number of studies, mainly the epidemiological observations, examining dengue across ethnic groups have been conducted. Further studies are certainly required to understand the biology behind this variation. Addressing these knowledge gaps is important because it would help provide a basis to move towards specialized and effective therapeutic strategies. Other host genetic determinants such as HLA alleles and variants in cytokine genes have also been suggested to influence disease outcomes (93).

1.3.3.3 Immune pathogenesis

The temporal mismatch between the viral loads and the course of dengue illness indicates that the pathologic symptoms of dengue are not exclusively caused by the virus (70). During the critical phase, the characteristic capillary permeability of severe dengue manifests relatively slowly when the viral burden is in sharp decline or is no longer detectable in blood. This suggests that virus-host interactions occur early and trigger protective antiviral responses to control and eliminate the virus, which may inevitably aggravate dengue pathogenesis through highly activated responses even after rapid clearance of virus. Evidence suggests that the host immune response plays an important role in causing many clinical complications associated with severe disease (94, 95). Malnourished children have been shown to have a lower risk of developing DHF/DSS than obese children due to their suppressed immune response (96). Although DENV infection infrequently leads to death, any patients have a potential to develop to severe dengue and die of progressive shock and multi-organ impairment (70). There is a correlation between the progress into shock and the switch from a Th1 to a Th2 response, together with an increase in Th2-type cytokines (97), supporting that an abnormal and exaggerated host immune response modulates dengue progression.

The immune pathogenesis hypothesis is supported by the observation that many severe dengue complications occur in serologically confirmed patients with previous history of dengue (19, 78, 98). It is suggested that, during the secondary challenge of a different (heterotypic) virus serotype, the immune system preferentially activates the cross-reactive response from memory to the primary challenge, rather than constructing a new response from naïve cells. This process is termed original antigenic sin which refers to the predominance of the original antigen, cross-

reactive antibodies or T-cell responses to the prior DENV during the secondary infection (99). Halstead has proposed that previously infected individuals, children or adults, have an immune risk factor, DENV-reactive IgG antibodies (Ab) which can enhance sequential DENV infections in Fcγ-receptors (FcR) bearing cells when the presence of Ab is at sub-neutralization concentrations. This phenomenon is called Ab-dependent enhancement or ADE (100). Generally, Ab responses defend the body from viral infection in a number of ways including the binding of Ab to virions that may interfere with virion adhesion to cellular receptors, block fusion with host membranes, cause aggregation of virus, or direct the lysis of viruses. However, Ab can opsonize virus and the Fc portion of Ab crosslink the virus to membranes of Fc receptor-expressing effector cells such as phagocytes, activating the phagocytosis and intracellular disposal. This Fc-mediated uptake process appears to be a double-edged sword in the case of dengue as it also serves as an underlying mechanism of ADE (101).

A cohort study using dengue patients' blood samples has demonstrated that cross-reactive Ab have a 100-1000-fold lower avidity for the subsequently infecting virus (102). At the stage of DENV transmission, pre-existing Ab generated to the primary infecting virus will not have a sufficient avidity or titer to neutralize the secondary infecting virus, impairing virus clearance. Instead, low avidity Ab opsonize the virus, form immune complexes and lead to targeting of Fcγ-receptor expressing cells, resulting in increased internalization and viral replication. More severe clinical manifestations are associated with greater virus burden in the body (103, 104).

The ADE phenomenon provides a rational explanation to the severe dengue in infants born to DENV-immune mothers (105). In this case, the mother passively transfers Ab, including IgG

specific for her previous DENV serotype, to her unborn baby through placenta during the last trimester of pregnancy. Maternal IgG is thus implicated as a risk factor for developing disease in infants who later become infected with DENV for the first time. Severe dengue occurs during primary infection typically in babies between 4 and 11 months, a period when levels of maternally acquired Ab to DENV wane, supposedly to a point where Ab are no longer sufficient to neutralize infection. That, in turn, exacerbates the primary infection by ADE, resulting in severe dengue (106). The exact mechanism of ADE of DENV infection in infants is not well understood, whether it resembles or differs from those occurring in older children and adults. Nevertheless, it has been speculated that ADE in infants is facilitated similarly to in vitro observations as the neonatal and adult monocytes display similar expression of FcγRIa or FcγRIIa (101).

Another hypothesis in support of the immune pathogenesis of severe dengue is original antigenic sin of T cell responses (107). It has been believed that cross-reactive T cells constructed against the original infecting serotype during secondary infection may also play a role in progression to severe dengue. The amino acid variations among four DENV serotypes can result in many variant peptide sequences with slight differences of amino acid. As T cell epitope recognition requires only short peptide fragments derived from replicating dengue for being T cell determinants, it is likely that the different infecting serotypes may lead to a complexity of T cell response to variant peptides that potentially offers cross-reactivity during secondary infection (108).

Analysis of T cell lines and clones often shows highly variable levels of cytokine production and cytotoxicity when stimulated with titrations of different peptides (109). Importantly, the phenotype and magnitude of both CD4 and CD8 T cell responses has been shown to strongly correlate with the severity of dengue disease; T cells in DHF produced more cytokines and showed lower levels of degranulation while those observed in DF produced much higher degranulation in an absence of cytokines (110). Paradoxically, studies have demonstrated that many T cells showed a high affinity for previously infecting serotypes, but had a relatively low affinity for secondary infecting serotype (109, 111), which was also reflected by comparatively poor T cell responses to secondary infection. These data suggest a potential of original antigenic sin of T cell responses in which a cross-reactive response can be rapidly raised from memory T cells, but appears to be pathogenic with low affinity to current infections (112). Activated T cells have been shown to produce a plethora of inflammatory cytokines such as TNF-α, IFN-γ, IL-1, IL-6, IL-8, IL-18, MCP-1, and CXCL chemokines. Many of these factors are detected in the blood of individuals undergoing the onset of severe dengue (113). However, further investigations are needed to show the direct role of the T cell response in development of severe dengue, especially because this hypothesis remains in conflict with the protective role of T cells that has been widely demonstrated in animal and human studies (114, 115). Furthermore, the cross-reactive T cell response cannot explain the ADE of infection in infants because T cells do not directly pass through the placenta, suggesting that requirements for promoting ADE in adults may also not include T cell responses.

In all proposed hypotheses, the core mechanistic concept of immune-pathogenesis has been the production of pro-inflammatory cytokines during DENV infection that is thought to cause

detrimental effects on endothelium and tissues, leading to tissue damage and life-threatening clinical outcome. This process of a cytokine storm mediating capillary permeability is compelling because it helps consolidate the higher virus burden from ADE of infection with the magnitude of immune activation in affecting innate immune cells as well as T cells.

1.4 SKIN

A wide variety of dengue clinical manifestations ranging from mild illness to fatal disease suggest that there are many factors influencing clinical outcome, and the virus has the potential to affect all human organ systems. That, in turn, results in varying degrees of disease in individual patients. The purpose of the dissertation is to understand an early event of DENV infection in human skin. This section focuses on the integumentary system and its importance in the context of dengue.

1.4.1 Dengue symptoms referable in skin

Studies have shown that skin irregularities are the most common clinical manifestation of laboratory-confirmed dengue cases, affecting approximately 65% of all patients (116). The confirmation rate of dengue is up to 100% in patients presenting with concomitant skin rash, itching, and petechiae (117). This suggests that recognition of skin rash helps with an early diagnosis for dengue fever. The link between the presence of dengue rash and the development of severe dengue disease is inconclusive based on a limited number of studies. A meta-analysis

study has suggested skin rash as one of the predictive signs associated with the progression of severe dengue (118).

Dengue rash can be categorized into two types by the onset and appearance linked to a particular phase during the course of illness. The initial dengue rash occurs within the first 24-48 hours, coinciding with or shortly after the onset of fever and lasting for several days (119). The rash involves a prominent flushing erythema of the skin on the face, neck, and chest that may be seen in around 20% of dengue patients (120). It is believed to be the result of capillary dilation. A diffuse maculopapular rash, first appearing on the trunk and later spreading to the face and extremities, has also been described in about 30% of patients in the first few days of illness (121-123).

The second type of dengue rash develops during convalescence when fever is subsiding (defervescence). It thus can be called recovery rash. As noted earlier, this rash has been described as “white islands in the sea of red”. It is characterized by a generalized eruption of intense red erythema and dense petechiae scattered with multiple small round of normal skin (117). Typically, it is marked by pruritus and prominently presents on the lower limbs, although the arms and trunk may be affected in some adults. This characteristic commonly persists in dengue recovery patients for over 1-2 weeks before fading gradually with skin peeling (desquamation). It is likely that patients make a full skin recovery after the improvement in other symptoms. With its distinct presentation, this recovery rash is one of the specific clinical signs associated with DENV infection. The mechanism for dengue rash remains ill-defined, but is thought to represent damage in small blood vessels under the skin. However, it is not known

whether the damage is due to the direct effect from virus, or the inflammatory response of blood vessels which is induced by the interaction between virus and skin cells.

1.4.2 Biology of human skin

The skin is the central organ in the integumentary system, which also includes other accessory structures such as hair, nails and certain glands. The term “integumentary” originates from a Latin word ‘tegere’ that means ‘to cover’. As the terminology suggests, the skin continuously covers the entire external surface of the body, and is continuous with the mucous membranes lining various body openings. It accounts for about 12-15% of total adult body weight, with a surface area of 1-2 meters. Indisputably, the skin is the largest organ of the human body and provides a natural barrier. Alterations in the skin invariably affect the overall wellbeing of an individual.

The skin not only provides a mechanical covering for internal structures, it is in fact a specialized complex organ consisting of several distinct layers: the epidermis and dermis, which are the main structural layers, and a closely associated underlying layer of subcutaneous tissue (the hypodermis). Each layer is composed of its own structures and components, but all layers work together as a single structure to perform unique and critical functions including self-repair when breached, protection against invading agents, thermoregulation, excretion, homeostasis and sensory perception.

The epidermis is the outermost layer of the skin and is highly cellular, consisting primarily of keratinized, stratified squamous epithelium. The epidermis houses a number of skin cell

populations including keratinocytes, Langerhans cells, Merkel cells, and melanocytes. Among these cell types, keratinocytes comprise the majority of the cells in the epidermis. They produce and store the protein keratin, which is an intracellular fibrous protein that gives skin the water-resistant property. The epidermis is a continuously self-renewing epithelium layer and can be sub-categorized into multiple layers or strata according to the morphology and position of keratinocytes as they differentiate and move upward. The average epidermal thickness of is 0.1 mm.

The dermis comprises a bulk of the skin with thickness ranging between 1-3 mm in different parts of the body and according to sex and age. The dermis is relatively acellular, and the major component of dermis is collagen which represents 70% of the skin's dry weight. Collagen fibers provide structure and tensile strength and are capable of retaining water to keep the skin hydrated. Elastic fibers provide elasticity and resilience to the skin, enabling movement, but have little role in stress-resistance. The dermis is largely supplied with a vascular system involving arteries, veins and lymphatics. These microcirculatory vessels are smaller branches from underlying large vessels of the muscles that ascend the fat tissue, enter the deep dermis and vertically penetrate to the upper dermis, but do not extend into the epidermis. Given the fact that only the dermis and hypodermis are vascularized and the epidermis contains no blood vessels, another function of the dermis is to regulate normal body temperature and to support and nourish the epidermis with vital nutrients and oxygen.

1.4.3 Immune function of the skin/ skin response to infection (an integrated view)

Skin is a versatile organ that fulfils many functions for a particular purpose to secure the integrity of the body. One of its important functions is to elicit a robust immune reaction against an endless variety of external dangers ranging from allergy, fungal growth, bacterial, viral and parasitic infections; these may come from agents which reach the skin from outside such as insect bites or injury, from the blood, or from an inherent instability of the skin cells present since birth or acquired later in life. In the meantime, the skin has to finely tune an appropriate balance of such reactions between protection from pathogens and prevention against innocuous substances including self-antigens or commensal microbiota. Dysfunction of the immunologic barrier leads to infection, skin hypersensitivity, skin cancer, inflammatory skin conditions and allergy, which could plausibly result in harmful consequences in other organ systems and death.

Skin can be defined as a dynamic, responsive immune organ (124). The skin immunologic barrier is built on a network of structural, cellular and molecular components (125). Intact skin provides physical and chemical barriers including structural integrity, slightly acidic pH, secretion of antimicrobial chemicals and water-resistant lipids, making skin relatively resistant to most extraneous matters. Also, the epidermal cell shedding reduces the chance of organisms colonizing or infecting the epidermis. Typically, microorganisms must breach the skin to gain entry into the body. As an interface organ, the skin contains a large population of resident immune cells that are key for tissue homeostasis and immune surveillance (126). Immune responses originating in the skin are mounted and carried out by these resident or infiltrated cells in the skin. Such cell populations are main players in either innate or adaptive immune systems. Innate immunity is commonly immediate, non-specific, diverse response that protects against a

wide range of invading pathogens, but does not exhibit memory (127). On the other hand, the adaptive response has a high degree of specificity as well as memory, but develops slower than the innate response. In favorable circumstances, innate and adaptive responses effectively cooperate and influence each other to mount a robust, specific immune response against insults. Collectively, the complexity and interactions of cells and molecules in skin constitute a first line of defense on the interface between the internal milieu and the external world.

1.4.3.1 Innate immune response in the skin

Human skin is home to a diverse population of cells including keratinocytes and Langerhans cells (LC) in the epidermis as well as dermal dendritic cells (DC) and skin-resident macrophages (Mϕ) in the dermis. Keratinocytes are the most abundant cells of the skin and are important for skin immune responses (128). They are likely the first cells that any skin-invading pathogens will encounter as they penetrate the epithelial barrier. Keratinocytes are the primary cells that express, store and release constitutive and inducible antimicrobial peptides (AMP), forming an innate chemical shield in the epithelial layers. AMP are evolutionarily conserved, predominantly small cationic proteins that directly bind and form pores on anionic cell walls and membranes of many bacteria, fungi and viral envelopes, resulting in microbial killing (129). The most important keratinocyte-derived AMP in human skin are human β-defensins1, 2, and 3, RNase 7 and the cathelicidin LL-37 (130). In addition to the direct antimicrobial activity, some AMP function as early warning signals to trigger a host response. LL-37 has chemotactic effects on mast cells, neutrophils and T cells, and stimulates endothelial cell proliferation (131). LL-37 was observed to activate G-protein-coupled receptor P2X7, inducing IL-1β processing and release from lipopolysaccharide-primed monocytes (132). Human β-defensins recruit immature DC and memory T cells through chemokine receptor 6 (133). This suggests that the presence of AMP in

the skin offers many non-specific functions to modify the local inflammatory responses and activate innate immune mechanisms of host cells in response to infection.

An essential function of the innate immune system is sensing invading microorganisms (134). The human skin is equipped with a variety of germ line-encoded pattern recognition receptors (PRR) which are expressed by both keratinocytes and resident immune cells in the skin. These receptors are responsible for recognizing specific pathogen components, known as pathogen-associated molecular patterns (PAMP). PAMP are highly conserved molecules that are present and widely shared by many pathogens, but are not expressed in host cells. They are indispensable for the survival or infectivity of the pathogen and are thus not subjected to selective pressure. There are four different subsets of PRR including Toll-like receptors (TLR), Retinoic acid-inducible gene (RIG)-I-like receptors (RLR), NOD-like receptors, and C-type lectin receptors. Recognition of PAMP with multiple families of PRR provides the host multiple mechanisms to sense and rapidly respond to a diverse range of infectious agents. This sensing strategy of the innate immune system also helps activate the specific part of the adaptive immune system, which sequentially mounting downstream cascades of immune response commensurate with the microbial invasion. The host innate immune response detects and responds to microbial stimuli mainly through recognition of the TLR family. A number of TLR recognize PAMP molecules on the cell surface such as lipopolysaccharides, flagellin, and yeast mannans (135). Multiple TLR including TLR-3, 7, 8, play a role in sensing intracellular viral nucleic acids and have been implicated in DENV invasion (136). TLR-3 recognizes dsRNA which is commonly generated during the replication cycle of DENV (137).

In human skin, there are many cell types that express functional TLR including keratinocytes, Langerhans cells, monocytes/macrophages, DC and lymphocytes (138). Each of these cell populations has distinct expression patterns of TLR. For example, keratinocytes constitutively express most TLR (139); basal keratinocytes express TLR-2/4 mRNA and suprabasal keratinocytes express TLR 1-5, TLR-7, and TLR-10 mRNA, suggesting that keratinocytes in different layer of epidermis express different type of TLR. The TLR family is responsible for the recognition of pathogens in the extracellular and endosomal compartments. The RLR family including RIG-I and MDA-5 monitors the cytoplasm for the presence of RNA viruses. It is a critical element of the anti-viral defense status in many cell types including keratinocytes, fibroblasts, and DC. Recognition of DENV occurs through a combination of both RIG-I and MDA-5 (140). Engagement of PRR with PAMP triggers a series of signaling pathways that activate transcription factors such as NF-kB and AP-1, resulting in the production of AMP, proinflammatory cytokines such as tumor necrosis factor α (TNFα), Interleukin-1 (IL-1) and IL-6, chemokines, type I interferons (IFN) and induction of immune responses necessary to eliminate the pathogens.

In response to virus infection, viral genetic elements (viral PAMP) trigger the activation of type I interferon cascade, leading to the production and secretion of type 1 IFN, including IFN-α and IFN-β, by infected cells (141). Secreted type 1 IFNs are potent antiviral cytokines that can bind to the IFN-α/β receptors in the same cell or adjacent cells as an alert. This triggers a signaling cascade via Jak/STAT phosphorylation and subsequent induction of IFN-stimulated genes containing IFN-stimulated response elements that exert antiviral functions. The expression of multiple IFN-stimulated genes mediates the restriction of viral infection in various pathways. For

example, protein kinase R suppresses the proliferation of virus-infected cells and 2′5′-oligoadenylate synthase activates RNase L, which inhibit virus replication by cleaving viral RNA. The oligoadenylate-RNase L pathway has been shown to reduce DENV infection in human cells. DENV has developed a variety of mechanisms to counteract the type I IFN system whether it inhibits type I IFN production or antagonizes type I IFN signaling, therefore affecting the induction of functional IFN-stimulated genes (136). The ability of DENV to interfere with STAT2 is one of the most striking immune evasion strategies observed in DENV infection (142). As STAT2 is essential for controlling the transcription of IFN-stimulated genes, DENV NS5 forms a complex with host STAT2, resulting in STAT2 targeted to the host proteasome machinery for degradation (143). This is one of the main mechanisms that allows DENV to establish infection in humans and potentially affects the induction of effective adaptive immune responses.

1.4.3.2 Adaptive immune response in the skin

Adaptive immunity consists of cell-mediated and humoral responses, elicited by T cells and B cells (134). These T- and B- lymphocytes employ a diverse repertoire of antigen specific receptors that are not encoded in germ line but are generated de novo corresponding to each stimulation, providing vertebrate hosts with a highly flexible, broad range of specific responses to pathogens. They also have an ability to generate and retain memory of past immunologic challenges, which can persist for decades and produce more rapid and strong responses to successive exposure to the re-challenged antigen. Besides keratinocytes and other innate immune cells, skin is also populated by resident lymphocytes. Normal skin contains about 20 billion T cells, which is twice as many T cells as the whole blood volume, indicating that the immune defense at the skin interface is a high priority (144). These resident cells from both innate and

adaptive immunity act as the immune-surveillance system in the skin under normal conditions. This setting would help overcome the logistic problems when the skin comes in contact with pathogens as it readily allows the access of a number of T cells to antigen-presenting cells, and subsequently expedites the initiation and activation of adaptive immune response.

Innate mechanisms determine the effective development of adaptive immune responses. Innate immunity defines the type and the strength of responses to facilitate T cell development and entry into tissues. Any inappropriate induction of local responses could modulate T-cell and innate immune effector cells entering the tissues without actual stimuli or existing pathogens in the system. These could turn the effective immune clearance of pathogens into the associations for skin inflammatory diseases such as dengue.

1.4.4 Skin cells and their role in early DENV infection

1.4.4.1 Keratinocytes

Keratinocytes represents the major cell population in skin as they are present throughout the epidermis from the outermost skin layer to the deeper epidermis. Keratinocytes not only provide the keratin structure of the skin but also function as innate immune sentinels with the detection and control of pathogens via the expression of PRR (145). In the steady state, inflammatory mediators are rarely detectable in keratinocytes, whereas upon stimulation, keratinocytes increase the production of pro-inflammatory and immunomodulatory cytokines, including IL-1α, IL-1β, IL-6,-10,-12,-17, and IL-18, TNF, and chemokines (CC-chemokine ligand (CCL) and CXC-chemokine ligand (CXCL) (128, 146). This activation can lead to multiple consequences. For example, the expression of CCL20 in activated keratinocytes activates and recruits

Langerhans cells and attracts T-cells to the skin (147, 148). The fact that keratinocytes express sensing receptors and abundantly produce mediators clearly demonstrates that keratinocytes have an active role in initiating and regulating host defense. In the context of dengue, these cells are likely to be the first to encounter the virus-containing saliva being inoculated into the skin (149). However, little has been explored about the role of keratinocytes during DENV infection. A study has reported that DENV infection of primary keratinocytes stimulates the transcriptional activation of intracellular RNA virus sensor, type I IFN genes and antimicrobial proteins, indicating the initiation of antiviral innate immunity against DENV (150).

1.4.4.2 Langerhans cells

Langerhans cells (LC) are a distinct subset of DC present in the mid-epidermis (151). The highly specialized antigen-presenting LC are equipped with PRR to detect, ingest, and process antigens present in the skin. After taking up pathogens and becoming activated, LC increase their expression of MHC class II and co-stimulatory molecules and migrate out of skin to T cell areas in draining lymph nodes, where they secrete chemokines that allow the attraction of naïve T cells and induce the proliferation and differentiation of antigen-specific T cells. Whether migratory LC carry antigens to skin-resident DC in skin or blood-derived DC in draining lymph nodes is questionable. The migration of LC to draining lymph nodes also occurs at a much lower rate in steady state. Although the homeostasis of LC to repopulate cells in the epidermis remains elusive, studies have shown that CCR2-expressing monocytes are direct LC precursors (152). In addition to their classic dendritic appearance, the unique feature of LC is the presence of tennis racket-shaped Birbeck granules, a part of the endosomal recycling compartment (153). LC express the c-type lectin receptor langerin (CD207), the lipid-presenting molecule CD1a and Fc receptors (154). While langerin has recently been observed in dermal CD103+ DC and lymph

node resident CD8+ DC (151, 155), it remains a useful and reliable marker for identifying LC in tissues. Because of their role in T cell stimulation, it is believed that LC plays a role in eliciting immune responses to protect the host following pathogen uptake (156). In support of this notion, studies have reported that langerin functions as an antiviral receptor in HIV infection by binding and leading to degradation of this virus. However, the protective role of LC has been challenged by studies of certain skin-invading pathogens; LC in skin-draining lymph nodes do not present antigen derived from herpes simplex virus-1 (157, 158). During Leishmania infection, LC induces regulatory T cells which leads to reduced effector T cell responses (159). The study of LC in DENV infection is limited. Epidermal LC have been indicated as the first target cells of DENV infection based on their infection in a skin biopsy obtained from the recipient of a live attenuated tetravalent DENV vaccine (160). A study using isolated cells from human skin explants has reported that LC did not support ADE of DENV infection, while DC and Mϕ did in the same study (50). The contribution of LC to DENV infection remains largely unknown.

1.4.4.3 Dermal dendritic cells

Dermal DC that reside in the dermis are myeloid or conventional DC. These cells are the key antigen presenting cells linking innate and adaptive immune responses in humans and other mammals. In the steady state, DC typically have an immature phenotype that display high levels of phagocytic activity; these cells sample their skin surroundings, picking up antigens from damaged cells, pathogens, or a commensal microorganism. Upon pathogen recognition and PRR engagement, DC become activated, produce inflammatory cytokines and chemokines, and switch chemokine receptor expression, facilitating their migration out of skin and into lymphatics which take them to the draining lymph nodes. This activation is generally termed DC maturation. DC are a heterogeneous group of cells that can express moderate to high basal levels of MHC class

II, which is increased during maturation. This facilitates a switch from antigen capture by immature DC in skin, to antigen presentation to lymphocytes in lymph nodes by mature DC. There are several distinct DC subsets in the epidermis and dermis but the defined myeloid DC population encompasses the CD141+ DC and CD1c+ DC (161). Dermal DC can be best identified in situ by expression of CD1c because CD1c+ DC are the major population of human myeloid DC in blood and tissues, whereas the number of CD141+ DC is relatively small, approximately 10% of human myeloid DC (162), and expression of CD141 is observed on other cell types such as endothelial cells. Both DC types are good at migrating and presenting antigen to T cells in the draining lymph node. While CD141+ DC have a superior capacity in cross-presenting exogenous antigens to CD8 T cells (163), which are abundant in the epidermis, CD1c+ DC are better at stimulating naïve CD4 T helper cells in the dermis. The CD1c+ DC can produce a broad range of cytokines that fine-tune the T cell immune response in the skin (162). The dengue field has widely recognized the importance DC in DENV pathogenesis. DC-SIGN molecules were shown to mediate DENV attachment to DC cell surface (164). DC are among the first cells to be infected with DENV and can support robust DENV replication, resulting in high level production of virions and of inflammatory mediators (165-167). The infiltration of monocytes generates DC which serve as additional targets for DENV infection (168). The migration of DENV-infected DC into draining lymph nodes leads to systemic dissemination of DENV and infection of blood monocytes, which can become the dominant cell type infected by DENV. Although the direct contribution of DC on the outcome of ADE remains unclear, studies indicated that DC are one of the main cell types supporting immune complex infection (169). The effect of mosquito saliva during ADE infection involves an increase in migration of DC to skin-draining lymph nodes (170).

1.4.4.4 Dermal macrophages

Macrophages (Mϕ) are present throughout the dermis as resident cells or monocyte derived cells that infiltrate into the skin in response to inflammation (171). Mϕ are long-lived, biosynthetically active cells with potent endocytic and phagocytic functions, which play a role in maintaining tissue homeostasis and resolution of inflammation through the clearance of apoptotic cells and cell debris (172). Mϕ are endowed with a variety of PRR, lectins, and scavenger receptors, suggesting the role of immune surveillance to protect the host through innate immunity. In addition to surface receptors, a number of evolutionary conserved reactions including the release of calcium and hydrogen peroxide by damaged cells lead to an immediate activation of Mϕ. Mϕ are able to modulate their properties upon contact with different insults, different cell types and extracellular matrix. During inflammation, activated Mϕ are a critical local source of chemokines, matrix metalloproteinase (MMPs), cytokines such as IL-1β and TNF-α, inducible nitric oxide synthase and other free radicals, and other secreted factors that produce and coordinate cascades of the inflammatory response. Despite low expression of MHC class II, these cells can exert antigen-presenting activity and direct T and B-cell differentiation, thus influencing adaptive immune responses. These versatile abilities allow Mϕ to rapidly recognize and respond to pathogens that successfully invade the skin (173). CD163 expression can be used to identify dermal Mϕ. CD163 is a receptor for hemoglobin-haptoglobin complex-binding scavenger receptor (174). Besides CD163, dermal Mϕ phenotype includes mannose receptor (CD206) and DC-SIGN (CD209) (175, 176). Mϕ have long been recognized as the principal cells to replicate DENV (165, 177, 178). DENV binding and internalization in Mϕ have been identified to occur through mannose receptors and CLEC5A or a cooperative interaction between these two receptors, whereas DC-SIGN expression renders resistance to the infection (175, 179).

Studies have reported that DENV-infected Mϕ release high amounts of pro-inflammatory cytokines and infectious viral particles (180). However, it remains unclear whether the inflammatory function Mϕ is favoring the virus or the host.

1.4.4.5 Fibroblasts

Fibroblasts are the most prevalent cell in human dermis. These cells are traditionally recognized for their role in synthesis, remodeling and degradation of the extracellular matrix and connective tissue proteins such as collagen which play an important role in injury repair. Beyond their role in structural support, fibroblasts are able to secret and respond to PAMP, as well as to pro-inflammatory factors, chemokines, and growth factors (181). This suggests that activated fibroblasts have the ability to participate in the maintenance of induced inflammatory responses via the expression of these factors (182). Fibroblasts can also interact with other resident cell types, particularly Mϕ, as suggested by cardiovascular disease studies (183, 184). However, few studies have examined DENV infection in fibroblasts. In vitro studies have demonstrated that primary fibroblasts from human skin were highly susceptible to DENV infection, leading to the production of type I IFN, GM-CSF and IL-6 (185, 186). Whether fibroblasts play a role in facilitating viral dissemination or inducing antiviral responses is unclear.

1.4.4.6 Mast cells

Mast cells are found in body interfaces that are in close contact with the external environment such as skin. However, the number of mast cells in skin is relatively small when compared to other resident cell types abundantly present in the skin such as keratinocytes and fibroblast. The main function of mast cells is the rapid release of pre-stored immune mediators such as histamine and the proteases (tryptase and chymase) (187). These vasoactive mediators can

increase vascular permeability and are responsible for immediate-type hypersensitivity reactions in the skin. The activation of mast cells occurs through the stimulation of the high-affinity immunoglobulin (Ig) E receptors (FcεRI). Mast cells also express other Fc receptors that bind IgG and therefore can respond to opsonized pathogens. Like other immune cells of the skin, mast cells express a variety of PRR including TLR and NLR, release reactive oxygen species, and can phagocytose organisms (188). Furthermore, mast cells can produce chemotactic factors such as CXCL-8 and TNF-α (189), known to recruit natural killer cells and neutrophils (190). While mast cells in skin biopsies fed with DENV-infected mosquitoes support DENV replication, the role of mast cells in cell recruiting has been shown to protect against DENV infection (191). DENV-infected mast cell–deficient mice had an increased viral burden within draining lymph nodes due to the lack of recruitment of natural killer and T cells to the site of infection (192). The accumulation of DENV has been shown in subcellular granules of mast cells, a compartment that can be released without degranulation, and travel intact through lymph (193). This has been proposed as an alternative mechanism of DENV spread, in addition to the immune cell migration.

2.0 CHAPTER TWO: HYPOTHESIS AND SPECIFIC AIMS

The skin is the initial and main site of early DENV replication following the bite of an infected mosquito. While LC, DC and Mϕ have been implicated as the primary target cell for DENV infection, the relative contribution of individual cell types within skin to infection remains controversial. To facilitate its spread in the skin and distal organs, DENV must develop strategies to subvert or exploit skin immune responses, which could dictate if the disease outcome is a mild febrile illness or a life-threatening condition. Thus, understanding the complex dynamics of DENV infection in skin is an essential basis for developing therapeutic options. Nevertheless, much of the current knowledge about skin involvement in DENV infection is based on conclusions drawn from findings in permissive cell lines and immune-deficient mice. Whether these findings predict the nature of DENV infection in humans is unclear. In this study, I will investigate dynamics of DENV infection and host immune responses to the virus in ex vivo human skin explants. The approach of using the skin model of DENV infection, coupled with quantitative in situ imaging and dissolvable microneedle arrays (MNA), will allow the quantitative analysis of early cellular events following DENV infection with the manipulation of skin biological processes.

My overall hypothesis is that DENV infects and exploits skin cells and host immune processes to facilitate its local replication and spread in human skin. To address this hypothesis, I propose the following specific aims:

Aim 1: Define the cellular targets of DENV infection and determine the relative importance of each target cell in human skin. Using immunohistochemistry (IHC), I will determine the dynamics of DENV infection, characterized by DENV non-structural protein 3 staining, in the epidermis and the dermis in skin explants during the first 48 hours following DENV inoculation. To further characterize skin cell types, I will incorporate specific cell markers into the system to determine the target cells of DENV and quantify the contribution of each target cell to the overall infection in skin. Two strains of DENV-2: the lab-adapted 16681 and the clinical isolate K0049, will be used for this aim for the comparison of cell types infected and the generalizability of data. I will also study the expression of IFN-α during the course of productive DENV infection to evaluate the counteracting effect between the virus and the host.

Aim 2: Delineate the mechanism underlying DENV spread in human skin. Changes in the number of skin-resident cells in response to the infection will be evaluated by IHC. To predict DENV spread beyond the skin, I will count emigrant cells in culture media and identify cell types by flow cytometry analysis. Using quantitative RT-PCR, I will determine the expression of pro-inflammatory cytokines and chemokines known to influence cell recruitment and infiltration in skin samples. The up-regulation of genes will be confirmed in skin as well as the characterization of skin cell types responsible for the production of the candidate genes. MNA delivery of neutralizing Ab to candidate cytokines and/or chemokines will allow for

determination of whether the upregulation of the factors influences the infection and recruitment of target cells.

Aim 3: Determine whether skin-resident macrophages participate in the antibody-dependent enhancement of DENV and ZIKV infection, and elucidate the role of Fcγ receptors (FcγRs) in facilitating the enhanced infection. The delivery of immune serum containing monotypic Ab to DENV-3 to skin will allow the ADE investigation of DENV-2 and ZIKV infection. Focusing on Mϕ responses, I will use anti-human CD163 and DENV NS3 to characterize Mϕ infected with DENV or ZIKV. The assessment of ADE phenomenon will be measured by an increase in the number of Mϕ as well as the percentage infected of Mϕ. I will also elucidate the impact of mosquito salivary gland extraction on primary DENV infection and ADE of DENV infection. If the enhancing serum shows a significant impact on Mϕ infection, I will further investigate the involvement of FcγRI and FcγRII in facilitating ADE infection through the blockade of the receptors by neutralizing Ab.

3.0 CHAPTER THREE: INTERPLAY BETWEEN KERATINOCYTES AND MYELOID CELLS DRIVES DENGUE VIRUS SPREAD IN HUMAN SKIN

3.1 PREFACE

This chapter is adapted from a published study (Parichat Duangkhae[1,2], Geza Erdos[3], Kate D. Ryman[1,4,†], Simon C. Watkins[5,6,7], Louis D. Falo, Jr.[3,7], Ernesto T.A. Marques, Jr.[1,2,8] and Simon M. Barratt-Boyes[1,2,7]. Journal of Investigative Dermatology. 2018 Mar; 138(3):618-626.

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Work described in this chapter is in fulfilment if specific aim 1 and 2.

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3.2 ABSTRACT

The skin is the site of DENV transmission following the bite of an infected mosquito, but the contribution of individual cell types within skin to infection is unknown. We studied the dynamics of DENV infection in human skin explants using quantitative in situ imaging. DENV replicated primarily in the epidermis and induced a transient IFN-α response. DENV infected a wide range of cells, including LC, Mϕ, dermal DC, mast cells, fibroblasts and lymphatic endothelium, but keratinocytes were the earliest targets of infection and made up 60% of infected cells over time. Virus inoculation led to recruitment and infection of LC, Mϕ and dermal DC, and these cells emigrated from skin in increased numbers as a result of infection. DENV induced expression of pro-inflammatory cytokines and chemokines by infected keratinocytes. Blocking keratinocyte-derived IL-1β alone reduced infection of LC, Mϕ and dermal DC by 75-90% and reduced the overall number of infected cells in dermis by 65%. These data show that the innate response of infected keratinocytes attracts virus-permissive myeloid cells that inadvertently spread DENV infection. Our findings highlight a previously undescribed role for keratinocytes and their interplay with myeloid cells in dengue.

3.3 INTRODUCTION

Dengue is the most common mosquito-borne virus infection worldwide, with clinically apparent cases estimated to approach 100 million per year (21). DENV replicates in skin following the bite of

an infected mosquito, but the biology of DENV infection in human skin remains ill-defined. DENV uses a range of receptors to bind to and infect host cells, including heparan sulfate, mannose receptor and DC-SIGN (164, 194, 195), and accordingly a wide range of skin cell types is permissive to DENV infection. Emphasis has been placed on the importance of myeloid cells, including LC, Mϕ and dermal DC in both human and murine models of skin infection (168, 196, 197). DENV also replicates in dermal fibroblasts, and mast cells have been shown to contribute to DENV infection in skin (198-200). DENV evades the antiviral type I IFN response and suppresses type I IFN production by DC in vitro (201), but the impact of infection on IFN production in human tissue is not known.

Relatively little is understood about the role of keratinocytes, the most abundant cell in skin, in DENV infection. Keratinocytes are emerging as important immune sentinels and initiators of skin inflammation and produce cytokines and chemokines that influence the traffic of immune cells into skin (202). DENV and the closely related ZIKV replicate in isolated human keratinocytes (203, 204) and studies with human skin explants are suggestive of DENV replication in keratinocytes in the basal layer of the epidermis (205). The interplay between keratinocytes and immune cells that are also targets of DENV infection has not been studied and may be important in the biology of DENV at the point of entry.

In this study, we developed an *ex vivo* model of DENV infection in human skin explants to quantify infection in different skin cell populations and define the early events following virus inoculation. We demonstrate that at least seven different cell types contribute to DENV replication in skin, but that keratinocytes alone make up 60% of all infected cells. We show that the innate response of

infected keratinocytes leads to recruitment and infection of Langerhans cells, macrophages and dermal DC, which then emigrate out of skin in increased numbers. Our findings show that blocking keratinocyte-derived factors, particularly IL-1β, markedly reduces the overall number of virus-infected cells in skin. The data indicate that DENV exploits the innate response of keratinocytes to attract and infect virus-permissive myeloid cells, aiding in the spread of virus.

3.4 RESULTS

3.4.1 DENV replicates primarily in epidermis and induces transient IFN-α production

To establish the *ex vivo* human skin model, we obtained discarded anonymized abdominal and breast skin from healthy individuals undergoing elective surgery. DENV was inoculated into a 1-square-inch area in the center of a 4-square-inch area of full-thickness skin. This large area was used to allow recruitment of cells into sites of inoculation. A bifurcated needle was used to repeatedly puncture the skin surface through a 50-µl bubble of media containing virus; preliminary experiments showed that this method resulted in approximately 10 µl of virus suspension being delivered into the skin. We used a relatively high-dose inoculum of the prototype DENV serotype 2 (DENV-2) strain 16681 (6.4×10^6 plaque-forming units (pfu)/10 µl delivered virus) for these initial experiments. Excess virus was removed at 2 h and skin was harvested at intervals after inoculation and stained with Ab to DENV nonstructural protein NS3, which is expressed during virus replication. DENV replication was first evident in cells at the base of the epidermis at 6 hours, and by 12 hours and 48 hours, replicating virus was detected in much of the basal layer of epidermis and the sparsely cellular dermis (Figure 1a). Using

quantitative image analysis, we found that the majority of virus replication took place in the epidermis, which contained approximately six times more infected cells than dermis at 48 hours (Figure 1b). To identify the earliest targets of virus infection at 6 hours, we stained sections with Ab to NS3 and to the major cell subsets in the epidermis, using AE1 to label cytokeratin in keratinocytes and CD207 to identify Langerhans cells. Virus replication took place largely in basal keratinocytes and was rarely detected in Langerhans cells (Figure 1c). In focal areas of infection at 6 h about 10% of keratinocytes were infected relative to 1% of Langerhans cells (Figure 1d). In addition, we found that IFN-α was transiently expressed in both epidermis and dermis, peaking from 2 to 8 hours post infection and returning to near baseline levels at 12 hours (Figure 1e, 1f).

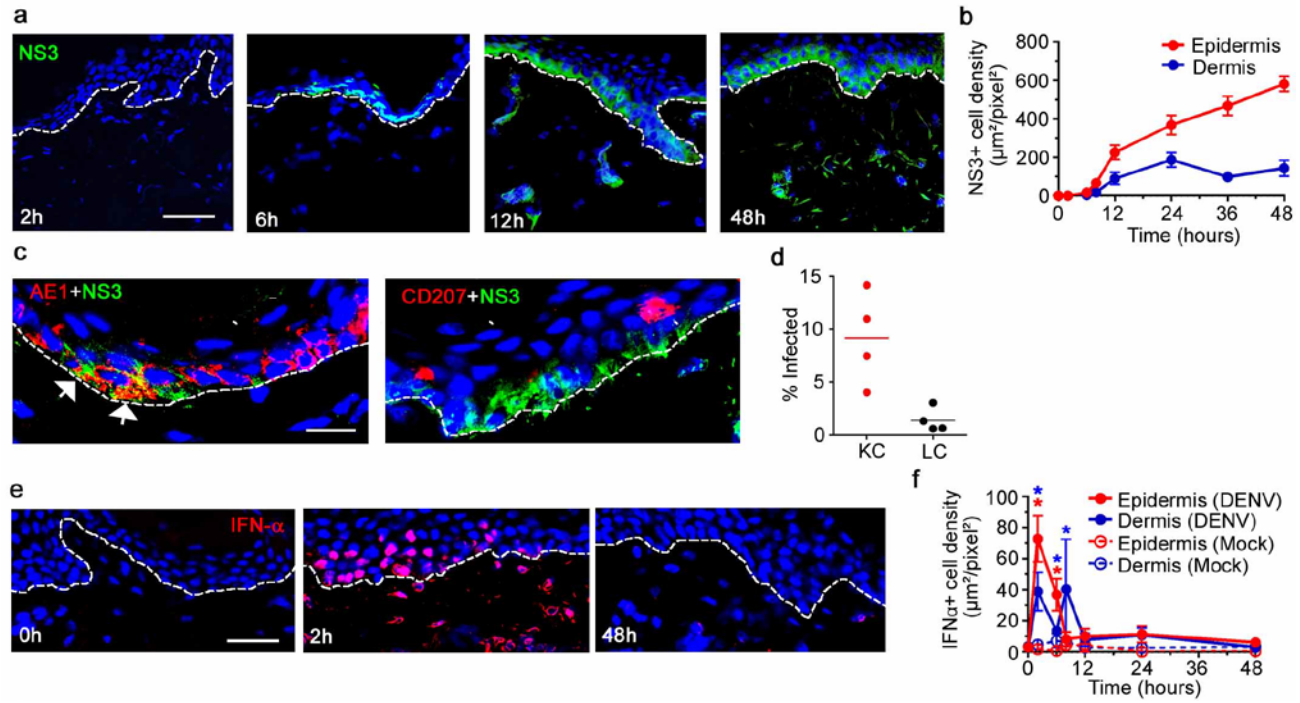

Figure 1. DENV replicates primarily in the epidermis and induces transient IFN-α expression.

(a) NS3 (green) expression in skin at intervals after DENV-2 16681 infection. Scale bar = 100 µm. **(b)** Quantification of infection over time. **(c)** Immunofluorescence with Ab to AE1 (keratinocytes [KC], red) or CD207 (Langerhans cells, red) and NS3 (green) at 6 hours. Arrows indicate infected KC. Scale bar = 25 µm. **(d)** Proportion of infected KC and Langerhans cells (LC) at 6 hours. Each symbol represents one individual and horizontal lines indicate means. **(e)** IFN-α (red) expression before and after infection. Scale bar = 100 µm. **(f)** Quantification of IFN-α-expressing cells. **(b, f)** Data expressed as mean ± standard error of mean (SEM) from four individuals. Blue staining in images represents nuclei and dotted lines indicate epidermal-dermal junction. **P* < 0.05 relative to mock infection.

3.4.2 DENV replicates widely in skin cells but infection is most abundant in keratinocytes

We next used a panel of cell-specific markers to quantify infected cell subsets in skin at intervals after inoculation. We focused on resident skin cell subsets considered to be permissive for DENV infection in vitro. We used both the 16681 prototype virus and the limited passage DENV-2 clinical isolate K0049, which was inoculated at a titer around 3 logs lower than the prototype virus (8.6×10^3 pfu/10 µl delivered virus). At 48 hours post-infection with either, isolate NS3 was readily detected in keratinocytes and Langerhans cells in epidermis (Figure 2a). In dermis at 48 hours after infection with either high-dose 16681 or low-dose K0049 strains, productive DENV infection was seen in macrophages and dermal DC, identified by expression of CD163 and CD1c, respectively (Nestle et al., 2009), as well as mast cells (defined by co-expression of CD117 and FcεRI) and fibroblasts (defined by the fibroblast-specific Ab TE7) (Figure 2b). Infection was also observed in $VEGFR3^+$ lymphatic endothelial cells, although this was less frequently observed than infection of other cell types and was not consistent between specimens (Fig. 2b). Quantitative image analysis revealed that between 30% and 75% of keratinocytes, Langerhans cells, macrophages, dermal DC, fibroblasts and mast cells were $DENV^+$ at 24 to 48 hours post infection. However, overall keratinocytes alone made up roughly 60% of all the $DENV^+$ cells from 8 to 48 hours post infection (Figure 2c).

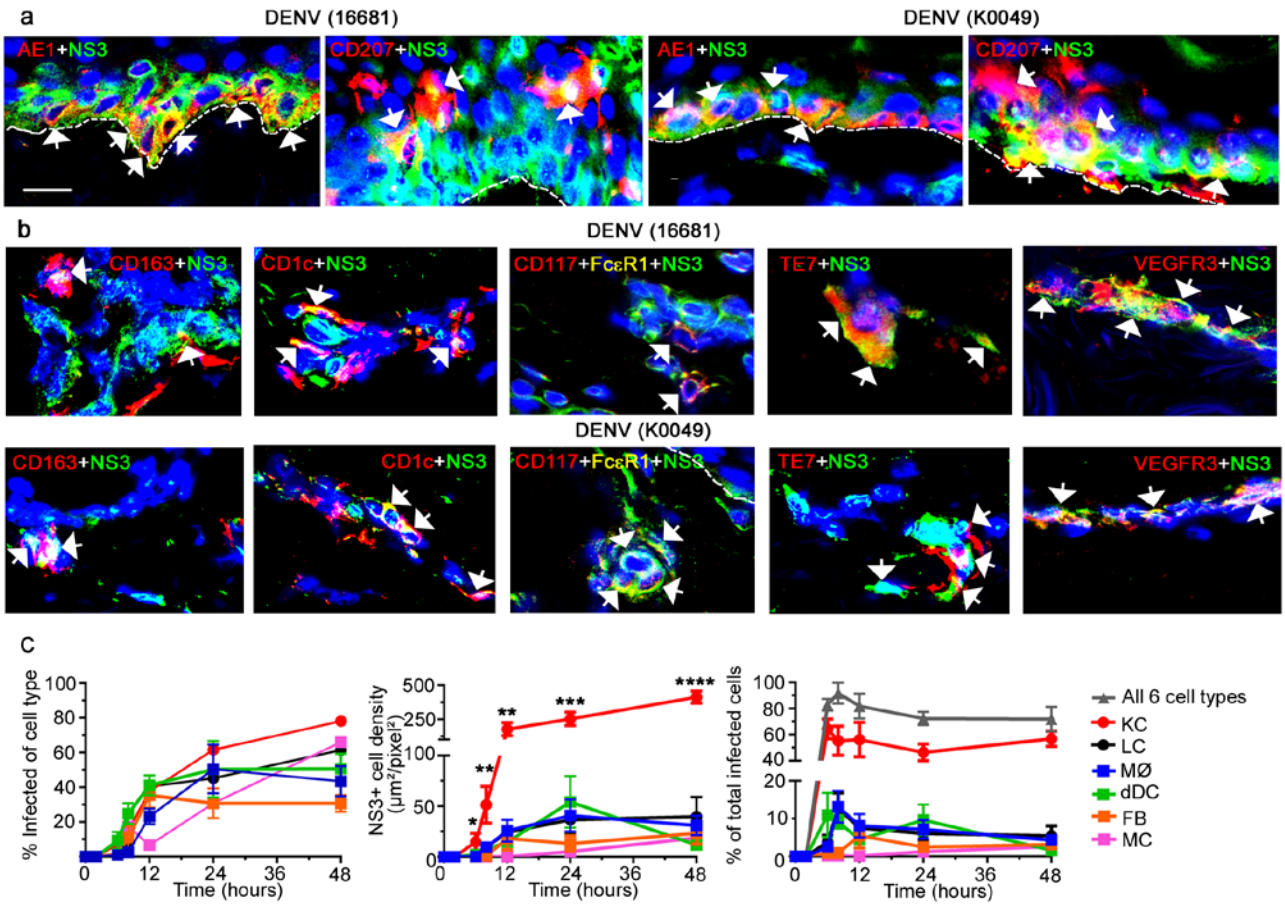

Figure 2. DENV replicates in a wide range of cells with keratinocytes, the major contributor to infection.

Staining with antibodies to specific cell markers (red) and NS3 (green) in epidermis **(a)** and dermis **(b)** at 48 h post infection with DENV-2 16681 or K0049 strains. Arrows indicate infected cells. Scale bar = 25 μm. **(c)** Proportion of each cell type infected (left), area of infection attributed to each cell type (middle), and infection as a percent of all infected cells (right). Symbols represent keratinocytes (KC), Langerhans cells (LC), macrophages (MØ), dermal DC (dDC), fibroblasts (FB) and mast cells (MC). Data expressed as mean ± standard error of mean from four individuals. **P* < 0.05, ***P* < 0.01; ****P* < .001; *****P* <. 0001 comparing KC with other cell types.

3.4.3 DENV infection drives recruitment of myeloid cells that become infected

It was apparent from these experiments that the presence of DENV resulted in greater numbers of DC and macrophages in skin, suggesting that these cells were attracted to the site of virus replication. To quantify this, we counted Langerhans cells in epidermis and macrophages, dermal DC and mast cells in dermis over time in the presence and absence of DENV. There was a 5-fold increase in the density of Langerhans cells at 12 and 24 hours post infection relative to mock-infected skin, and a similar but slightly delayed increase in the number of macrophages. Both cell subsets underwent a modest decline in number at 48 hours (Figure 3a and 3b). Dermal DC increased 10-fold at 24 hours in the presence of DENV, but returned to baseline density by 48 hours. Mast cell numbers increased in a more delayed fashion, being statistically significant at 24 and 48 hours relative to mock-infected skin (Figure 3a and b). The number of fibroblasts did not vary over time with DENV infection (data not shown). There was a strong positive relationship between the number of Langerhans cells, macrophages and dermal DC and their infection with DENV, but no such relationship with mast cells (Figure 3c). These data suggest that DENV infection results in production of factors that attract myeloid cells that in turn become infected with virus.

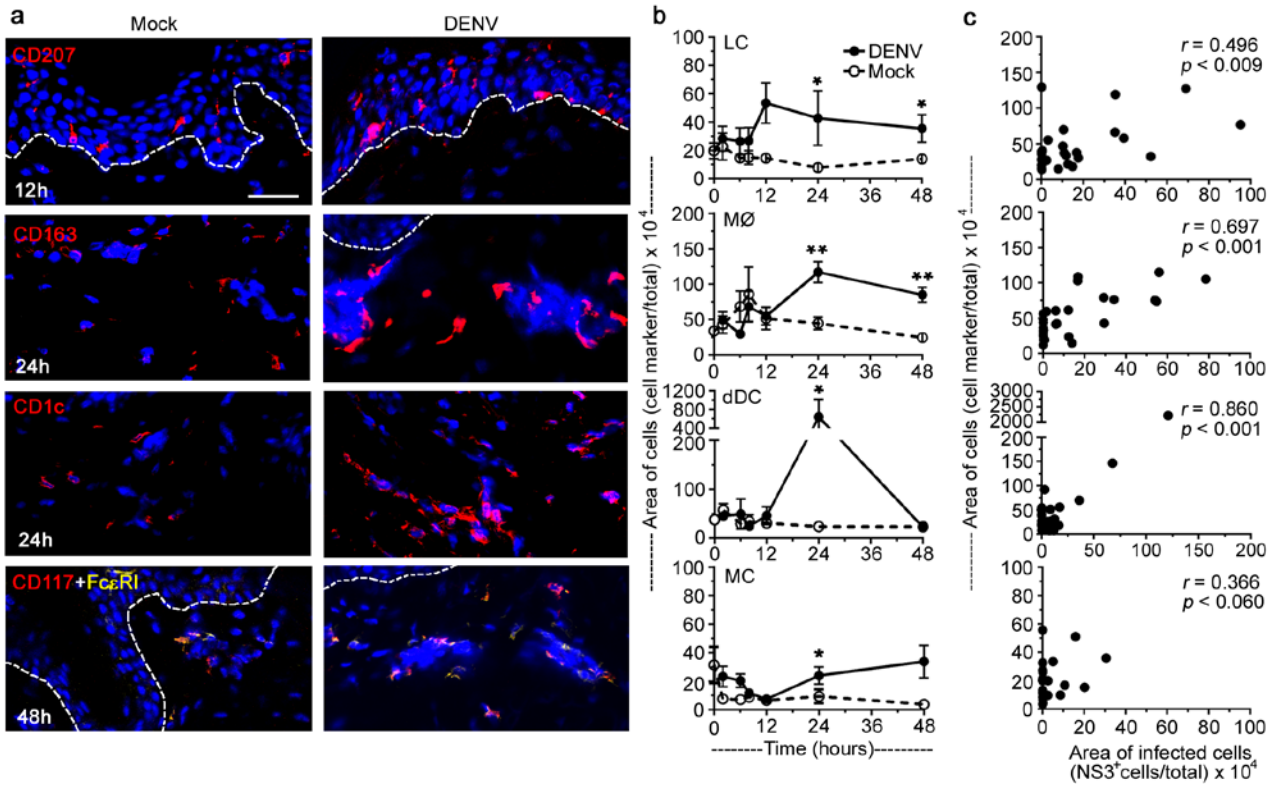

Figure 3. DENV infection in skin causes influx and infection of myeloid cells.

(a) Immunofluorescence with antibodies to individual cell markers (red) at intervals after infection with DENV-2 16681 or mock infection. Scale bar = 100 µm. **(b)** Density of Langerhans cells (LC), macrophages (MØ), dermal DC (dDC) and mast cells (MC) in mock- and DENV-infected skin. Data expressed as mean ± standard error of mean for four individuals. **P* < 0.05; ***P* < 0.01 comparing mock and infected skin. **(c)** Relationship between cell density and DENV infection for each cell type.

3.4.4 DENV infection increases emigration of myeloid cells out of skin

The decline in the density of Langerhans cells in epidermis and macrophages and dermal DC in dermis at 48 hours post infection suggests that these populations leave the skin after their recruitment to the site of infection. In support of this notion, we observed large cords of Langerhans cells present within dermis at 48 hours after DENV but not mock infection, suggesting that DENV had promoted Langerhans cell movement from epidermis to dermis (Fig. 4a). In addition, the total number of cells in media normalized to skin area increased 3-fold at 24 hours and 5-fold at 48 hours after DENV inoculation (Fig. 4b). We did flow cytometric analysis of these migrated cells, staining for CD1a; CD1c; and CD163 to identify Langerhans cells; dermal DC; and macrophages, respectively. The proportion of each of these cell types increased in media following DENV infection (Fig 4c). This was accompanied by statistically significant increases in the frequency of each of these cell subsets in media at both 24 and 48 hours after DENV infection relative to mock infection (Fig. 4d). These findings confirm that DENV infection ultimately promotes myeloid cell exodus from skin.

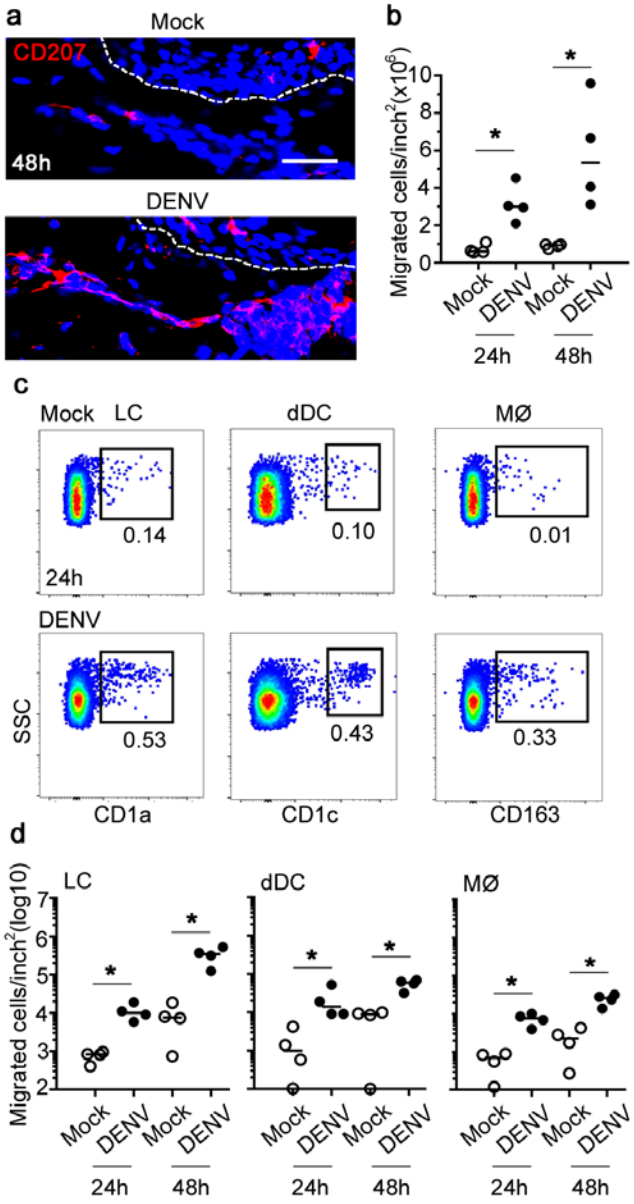

Figure 4. DENV infection promotes myeloid cell emigration from skin.

(a) Staining with antibodies to CD207 (red) to identify Langerhans cells in dermis at 48 hours post infection with DENV-2 16681 or mock infection. Scale bar = 100 µm. **(b)** Number of migrated cells collected from media per square inch of skin at 24 and 48 hours after DENV or mock infection. **(c)** Representative flow cytometry plots of migrated cells stained with indicated antibodies to identify Langerhans cells (LC), macrophages (MØ) and dermal DC (dDC) at 24 hours. SSC, side scatter. (**d**) Quantification of migrated cells. Each symbol represents a different individual. *P < 0.05 relative to mock infection.

3.4.5 DENV-infected keratinocytes express pro-inflammatory cytokines and chemokines

To begin to determine the mechanism for the observed recruitment and emigration of myeloid cells, we did real-time quantitative PCR analysis for gene expression of a panel of 10 cytokines and chemokines that are identified in skin during inflammation. Of these 10 factors, expression of IL-1α, IL-1ß, CCL20 and IL-10 was significantly increased in whole digests of skin at 48 hours as a consequence of DENV infection (Figure 5a). To determine which cells were expressing cytokines/chemokines and the relationship to DENV infection, we performed in situ immunofluorescence staining for cell markers, viral NS3 and select cytokines/chemokines. IL-1β and CCL20 were abundantly expressed primarily in the epidermis after infection with DENV strain 16681, and triple labeling indicated that the majority of each of these cytokines was expressed by infected keratinocytes. CXCL8 was also primarily expressed in the epidermis at 48 hours post infection by both virus-infected keratinocytes and uninfected cells lacking expression of AE1, likely Langerhans cells (Figure 5b). Similar findings for IL-1β and CXCL8 were noted when skin was inoculated with the lower titer of K0049 strain (Figure 5b).

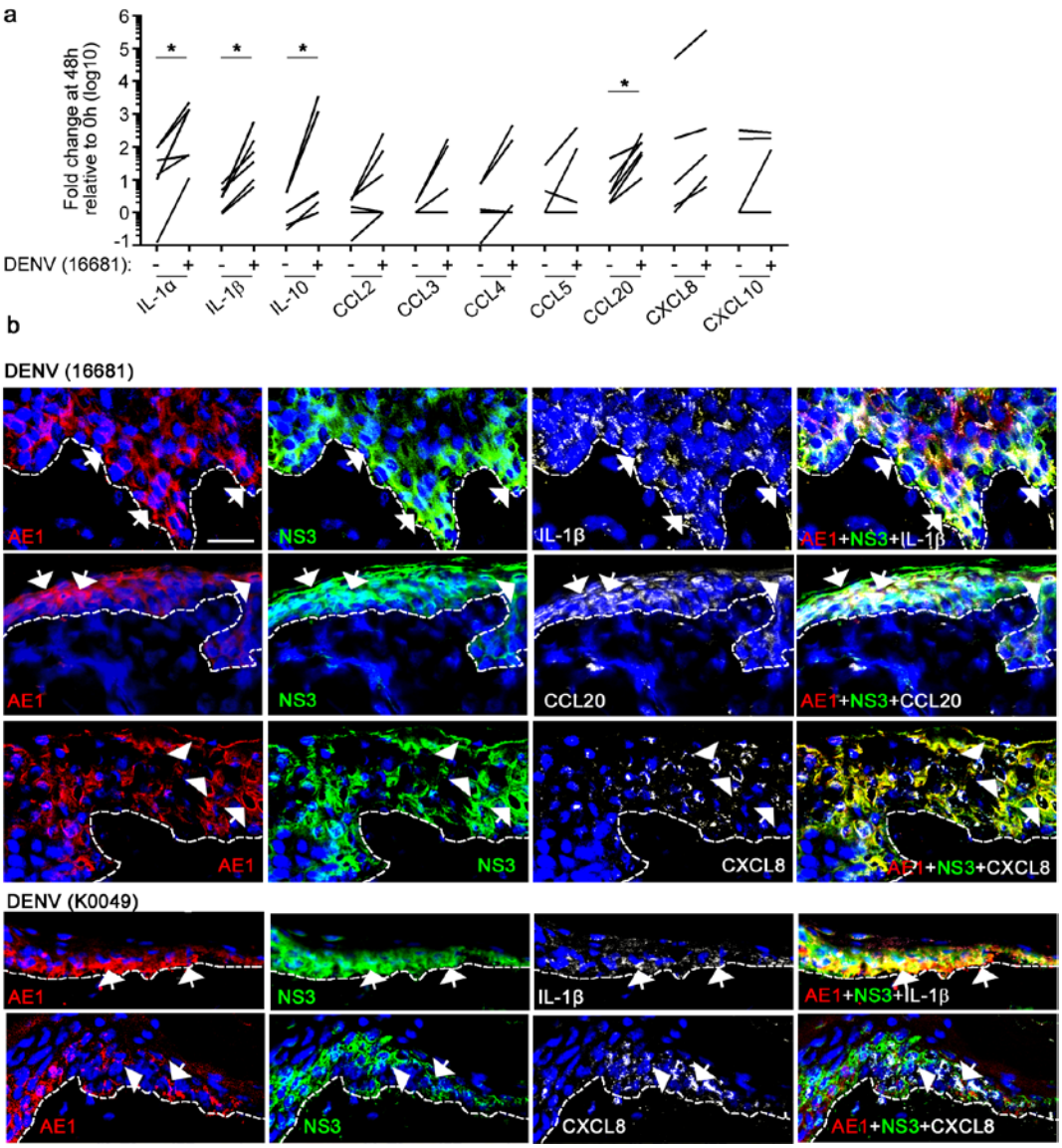

Figure 5. Expression of cytokines/chemokines by DENV-infected keratinocytes in skin.

(a) Change in expression of genes in skin in the presence or absence of DENV-2 16681 at 48 hours relative to 0 hours. Each line represents one individual. **P* < 0.05. **(b)** Immunofluorescence in skin at 48 hours post infection with DENV-2 16681 (top) or K0049 (bottom) labeled with antibodies to AE1 (keratinocytes, red), NS3 (green), and the specific cytokine or chemokine (white). Arrows indicate expression of cytokine/chemokine in infected-keratinocytes; arrowheads indicate cytokine expression in cells other than infected keratinocytes. Scale bar = 25 μm.

3.4.6 Blocking IL-1 and CCL20 markedly reduces myeloid cell recruitment and infection

To directly test the role of cytokines and chemokines produced in skin during infection to the recruitment of myeloid cells, we delivered neutralizing Ab to IL-1α, IL-1β, CCL20 and/or CXCL8 to skin 2 hours after DENV inoculation. We used Ab formulated into dissolvable microneedle arrays (MNAs) for these experiments, which penetrate the stratum corneum and deliver small volumes of Ab over a defined area of epidermis and dermis (206). MNA containing Ab to IL-1α or IL-1β or a cocktail of all four Ab significantly reduced both the total number of Langerhans cells and the number of infected Langerhans cells in the epidermis relative to isotype control Ab (Figure 6a). Within the dermis, Ab to IL-1β or CCL20 profoundly reduced both the number and infection of macrophages and dermal DC. Neutralizing Ab to CXCL8 had minimal effect on recruitment or infection of these cells (Figure 6a). In the case of IL-1β, infection of Langerhans cells, macrophages and dermal DC was reduced by 75-90%, resulting in a reduction in the total number of DENV-infected cells in the epidermis by 33% and in the dermis by 65% (Figure 6a, 6b). In situ immunofluorescence illustrated that anti-IL-1β reduced infection of Langerhans cells without impacting infection of keratinocytes in epidermis and substantially reduced the number and infection of macrophages and dermal DC (Figure 6c). These findings support the conclusion that proinflammatory cytokines and chemokines, in particular IL-1β, produced by infected keratinocytes mediate recruitment and infection of Langerhans cells, macrophages and dermal DC during DENV infection of skin.

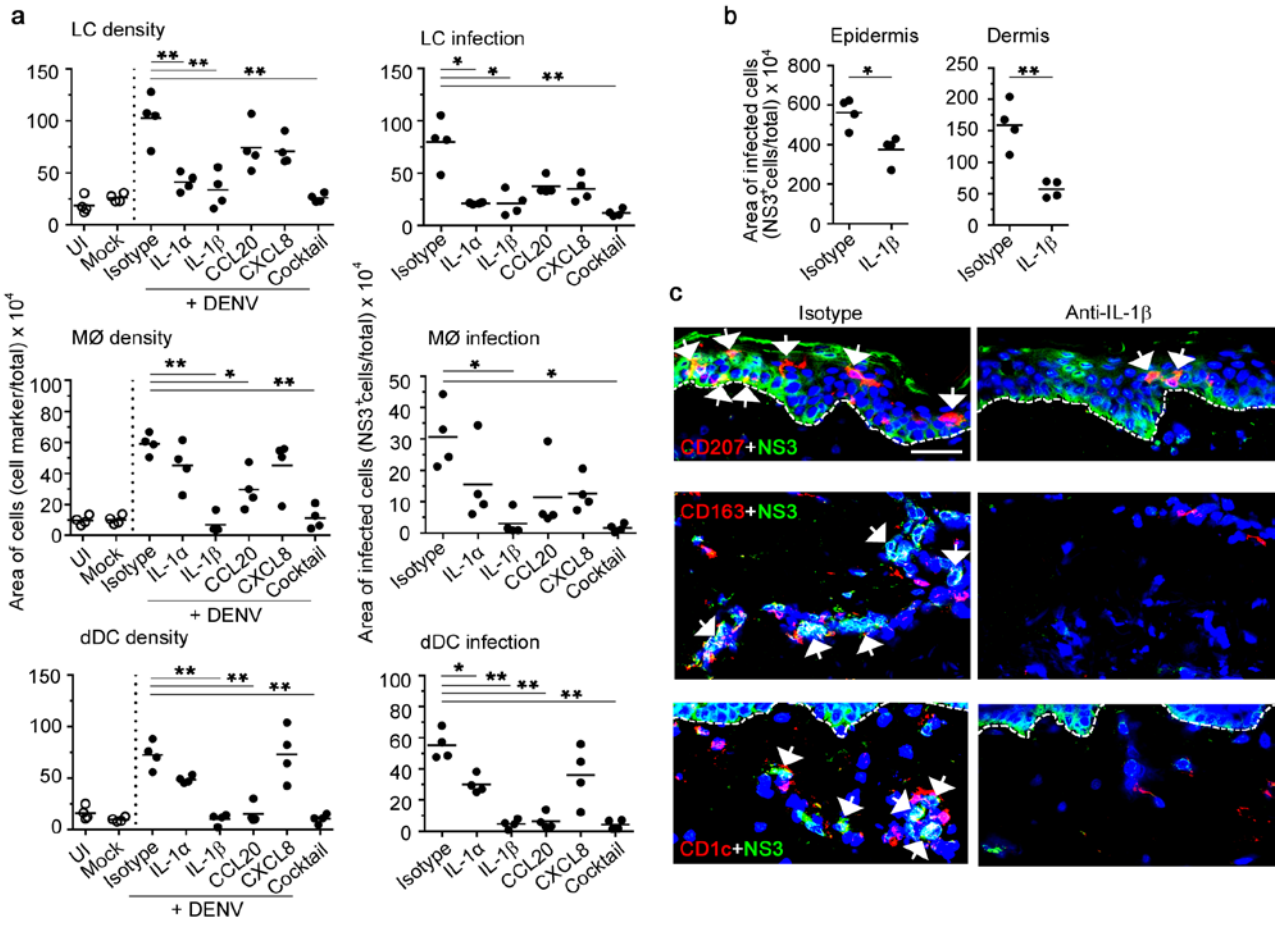

Figure 6. Blocking IL-1 or CXCL20 prevents recruitment and infection of myeloid cells in skin.

(a) (Left) Density of Langerhans cells (LC), macrophages (MØ) and dermal DC (dDC) in uninfected skin (UI) at 0 hours and mock-infected skin at 24 hours, and in dengue virus (DENV)-infected skin at 24 hours after exposure to different blocking antibodies or isotype control antibodies in dissolvable microneedle arrays. (Right) Number of DENV-infected cells of each cell type after exposure to different antibodies. **(b)** Total number of DENV-infected cells in epidermis and dermis 24 hours after exposure to isotype control antibodies or antibodies to IL-1β. Each symbol represents a different individual. **P* < 0.05; ***P* < 0.01. **(c)** Immunofluorescence with antibodies to individual cell markers and NS3 at 24 hours after DENV infection and exposure to isotype control antibodies or antibodies to IL-1β. Arrows indicate co-localization of NS3 with specific cell marker. Scale bar = 100 μm.

3.5 DISCUSSION

Both hematopoietic and non-hematopoietic skin cells support DENV replication, but the relative contribution of each to DENV infection and spread in situ is not known. We addressed this issue by inoculating virus into large-area human skin explants and quantifying the effect of DENV infection on different susceptible cell populations. Our data reveal that DENV infects at least seven different resident skin cell populations within epidermis and dermis, with efficiencies between 30 and 60% at 24 h, similar to findings in vitro (197, 198, 200, 203). However, the most abundant cell infected from a quantitative standpoint is the keratinocyte, which is the earliest target and makes up 60% of all infected cells over time. This is a function both of the high density of keratinocytes within skin and their relatively high susceptibility to infection in situ. Our findings indicate that keratinocytes play a central role in infection and spread of DENV through production of factors that draw virus-susceptible myeloid cells to the site of virus inoculation and promote their subsequent migration out of skin.

Our findings are consistent with earlier reports of keratinocyte infection in human skin explants (205) and with data from wild-type mice showing keratinocyte infection following intradermal inoculation with West Nile virus, a related flavivirus (207). A recent study showed that while 20 to 40% of Langerhans cells, macrophages and CD1c+ dermal DC isolated from collagenase-treated human skin were productively infected upon DENV inoculation, there was negligible involvement of keratinocytes in this process (196). This discrepancy could arise from the method

of cell isolation, as collagenase treatment would favor recovery of cells from dermis but not the collagen-free epidermis.

It is well described that type I IFN restricts DENV replication in human skin cells, including fibroblasts, DC and macrophages (196, 208). We found remarkably transient production of IFN-α by cells in epidermis and dermis following DENV infection, with peak expression at 2-8 h after virus inoculation. In contrast, DENV replication in isolated human keratinocytes and fibroblasts leads to type I IFN production that appears relatively sustained, out to at least 48 h (198, 203). Our data suggest that rapid blockade of type I IFN production by DENV as noted in DC in vitro (201) also occurs in infected skin cells in the context of the skin microenvironment. This blockade facilitates virus replication and spread within tissue, as well as emigration of infected cells out of skin to distal sites. Similarly, studies in IFN-α/β receptor-deficient mice showed that monocyte-derived DC are recruited to the dermis and become infected after intradermal DENV inoculation (168).

Our data show that in human skin, pro-inflammatory factors, most notably IL-1β, derived largely from infected keratinocytes, mediate recruitment of virus-susceptible myeloid cells. IL-1β is chemotactic for macrophages (209) and activates DC in skin explants, promoting T cell-stimulating function (210). CCL20 mediates recruitment of DC into inflamed skin (211) and is itself induced by IL-1β. Importantly, IL-1β in concert with TNF-α ultimately drives Langerhans cell and dermal DC exodus from skin to draining lymph nodes (212), consistent with our findings of an increase in these cell types and macrophages in media following DENV infection. Thus, myeloid cells that are brought into skin in response to keratinocytes would become

infected and then traffic to lymph nodes, initiating an adaptive immune response but also disseminating infection. Keratinocytes are an important source of IL-1 in other infections as well, but with different outcomes. In intradermal herpes simplex virus-1 infection of mice, keratinocyte-derived IL-1α mediates leukocyte recruitment to skin that serves to contain virus spread (213). In percutaneous infection of mice with Schistosome larvae, epidermal keratinocytes promote skin inflammation and wound healing, in part through release of IL-1α and IL-1β (214).

It is notable that increased circulating levels of IL-1β in dengue patients correlates with severity of disease (215). IL-1β expression by infected platelets increases vascular permeability which is a hallmark of severe forms of dengue (216). DENV antigen can be detected in the erythematous macules and papules that constitute the dengue rash seen in acute illness (197), and it is conceivable that infected keratinocytes in these lesions are an unidentified source of IL-1β and may therefore be a factor in disease pathogenesis.

A central question is how mosquito bites themselves may affect the events of DENV infection in human skin. *Aedes* mosquitoes inoculate between $10^{3.6}$ and $10^{4.7}$ pfu of West Nile virus when feeding on live mice (217), similar to the dose we used of the DENV isolate K0049 in our studies. Notably, the vast majority of West Nile virus injected by infected mosquitoes remains at the site of inoculation as opposed to being delivered directly into the circulation following cannulation of capillaries (217). Studies with other arboviruses show that mosquito bites cause edema, retention of virus in skin and recruitment of neutrophils that are a source of IL-1β. The resulting inflammatory response leads to an influx of virus-permissive myeloid cells that spread

virus infection (218). Mosquito salivary gland components and mosquito bites enhance DENV dissemination and disease in murine models (219-222). Moreover, *Aedes aegypti* saliva directly enhances DENV infection of isolated primary human keratinocytes (223, 224). These findings suggest that keratinocyte infection and subsequent recruitment and infection of myeloid cells in human skin after DENV infection may be exacerbated in the setting of mosquito bite inoculation.

In summary, we have identified a novel relationship between keratinocytes and myeloid cells that serves to promote DENV infection in human skin and is predicted to lead to greater dissemination of virus in the infected host. The data highlight a previously unidentified role for keratinocytes in DENV infection and indicate that disrupting the innate response of keratinocytes may limit DENV infection in vivo.

3.6 MATERIALS AND METHODS

3.6.1 Dengue virus

The prototype DENV-2 strain 16681 was provided by Jared Evans at the University of Pittsburgh. The low-passage DENV-2 strain K0049 was isolated in 1995 from an individual in Thailand with dengue hemorrhagic fever (83) and was obtained from BEI Resources. Viruses were propagated in C6/36 insect cells (ATCC). Tissue culture supernatant was pooled at days 5, 10 and 15 of culture and then concentrated using standard methods. Virus titers were determined by a modified focus forming unit immunoperoxidase assay using Vero cells as described previously (225).

3.6.2 Skin processing and virus inoculation

Large blocks of anonymized skin that were being discarded following elective abdominoplasty or mammoplasty at the University of Pittsburgh were used. Identifiable private information regarding skin donors was not provided and no interaction or intervention with donors was possible and as such the project did not constitute human subjects research as per the University of Pittsburgh Institutional Review Board. Residual fat was removed and skin trimmed into 25 cm^2 pieces. A volume of 50 ul virus suspension containing 3.2×10^7 pfu 16681 or 4.3×10^4 pfu K0049 was placed in a 4 cm^2 area that was demarcated in the center of the skin. A bifurcated allergy skin testing needle (Precision Medical Product Inc., Denver, PA) was used to repeatedly

puncture the skin through the liquid suspension and deliver virus into the epidermis and dermis. Inoculated explants were incubated at 37˚C (5% CO_2) for 2 h, then the skin surface was washed with PBS and wiped with sterile gauze pads to remove excess virus. By weighing skin before virus inoculation, immediately after adding virus and immediately after removal of excess virus, we determined that 10 ul virus was delivered into the tissue using this method. Tissue was placed dermis-side down on mesh grids or on filter paper in 60 x15 mm culture dishes and incubated at the liquid-air interface in complete media (RPMI 1640 media containing 10 % FBS, 100U/ml penicillin/streptomycin, 2 mM L-glutamine, 0.1 mM sodium pyruvate). Explants were collected at various intervals and the virus-inoculated central region was isolated, submerged in 30% sucrose overnight at 4˚C, and then frozen for subsequent RNA isolation and immunohistochemistry. At 24 and 48 hours, media were collected to harvest cells, which were treated with DNase, counted, and stained with Ab for flow cytometric analysis.

3.6.3 Microneedle arrays and blocking Ab

Neutralizing Ab to human IL-1α, IL-1β, CXCL8 or CXCL20, either alone or together, or isotype control Ab (R&D Systems) were formulated into tip-loaded dissolvable carboxymethyl cellulose/trehalose MNA as previously described (206). MNA contained 57.5 ng of Ab to IL-1α, IL-1β or CXCL8, or 230 ng of Ab to CXCL20, or a combination of all of these. The concentration of Ab was twice the 50% neutralization dose as provided by the manufacturer multiplied by a factor of 2.3 to account for dilution following dispersal within skin. Control MNA contained 575 ng each of IgG1 and IgG2a Ab. MNA were manually applied to explants immediately after removal of excess virus inoculum at 2 h. MNA were removed after 15 min leaving the dissolved needle tips in the skin explant.

3.6.4 Immunohistochemistry

Six µm frozen sections of skin on microscope slides were rehydrated with PBS and fixed in cold acetone for 5 min at 4˚C. Slides were incubated with blocking solution containing 5% goat and donkey serum (Thermo Fisher Scientific) overnight at 4˚C before being stained with polyclonal rabbit anti-DENV NS3 Ab (kindly provided by Sujan Shresta, La Jolla Institute for Allergy and Immunology) and mouse anti-human Ab directed against specific cell and/or cytokine markers overnight at 4˚C. Slides were washed and incubated for 45 min with donkey anti-rabbit IgG H&L Alexa Flour 488, goat-anti-mouse IgG1 Alexa Flour 546, and/or goat-anti-mouse IgG2a/2b Alexa Flour 647 (Invitrogen), depending on the combination of Ab used. Slides were stained with Hoechst dye (Thermo Fisher Scientific) for nuclear visualization. Images were viewed on an Olympus Fluoview 1000 confocal microscope. The following human primary Ab were used: keratin low molecular weight Ab-1 (AE1, Thermo Fisher Scientific), langerin/CD207 (DCGM4, Beckman Coulter), CD1a (NA1/34HL, AbD serotec), CD163 (5C6FAT, Acris Antibodies GmbH), CD1c (L161, AbD serotec), fibroblast (TE7, Chemicon), VEGFR3 (FLT4, R&D Systems), CD117 (104D2, Thermo Fisher Scientific), FcεRI (9E1, Novus Biologics), IL-1ß (6E10, Novus Biologics), CXCL20 (319F6.06, Novus Biologics) and CXCL8 (B2, Santa Cruz Biotechnology). Specificity of labeling was determined using relevant isotype-matched control Ab.

3.6.5 Quantitative image analysis

Nikon NIS elements AR 4.40 software was used to convert images into measurable data in a manner similar to that described previously (226). Slides were imaged at 40X magnification to

allow visualization of the full thickness of epidermis and approximately 500 um depth of dermis. Briefly, a region of epidermis or dermis or both was circumscribed and thresholds for red, green and blue fluorescence (representing staining of the specific cell marker or cytokine/chemokine, viral NS3 and nucleus, respectively) were established. The overlapping area of different colors specified different aspects of data, for example the intersection of red and blue thresholds showed a specific cell population, the intersection of green and blue showed infected cells, whereas the intersection of red, green and blue showed a specific cell population infected with DENV. Data for each individual skin specimen were collected from a minimum of 10 confocal images taken from 3 skin sections collected from different sites of virus-inoculated skin. Means from each individual were presented as an individual data point, and data are presented from 4 individuals.

3.6.6 Flow cytometric analysis

Antibodies to CD1a (SK9; BD Biosciences, San Jose, CA), CD1c (L161; Biolegend, San Diego, CA), and CD163 (GHI/61; Biolegend) along with matched isotype control Ab were used to stain cells collected from media prior to analysis by flow cytometry. Dead cells were excluded using a Live/Dead viability stain and singlets were defined using side scatter height and area. Samples were run on a BD LSR II flow cytometer using BD FACSDiva software (Becton, Dickinson, Franklin Lackes, NJ). Analysis was performed using FlowJo software, version 10.3 (Tree Star, Inc., Ashland, OR).

3.6.7 RNA isolation and quantitative real-time PCR

Total RNA was extracted from homogenized skin tissues and purified using RNeasy mini kit (Qiagen, Valencia, CA) and cDNA synthesis was done using standard approaches. Primers were synthesized by Integrated DNA Technologies (Caralville, IA) and are given in Table 1. The amplification of cytokine and reference genes was performed using a duplex format containing primed cDNA, each primer pair, and Platinum SYBR green qPCR Supermix-UDG (Invitrogen, Carlsbad, CA). Quantitative RT-PCR was performed using a 7900HT Fast Real-time PCR system machine (Applied Biosystems, Carlsbad, CA). Quantities of all cytokine targets were normalized to the corresponding 18S ribosomal RNA levels in the skin tissues.

3.6.8 Statistical analyses

Statistical analyses were performed using STATA, version 13 (StataCorp, College Station, TX) and SPSS software (IBM Corp, Armonk, NY). A paired *t* test or Mann-Whitney U test was used for two-group comparisons. A one-way analysis of variance followed by Bonferroni's multiple comparisons test was done for multigroup comparisons. The strength of linear associations was assessed by the Pearson's product-moment correlation. *P* values <0.05 were considered significant.

Table 1. Primer sequences used in the study

Gene	NCBI Gene ID	Primer (5'-3') Forward	Reverse	Reference
IL-1α	3553	CGC CAA TGA CTC AGA GGA AGA	AGG GCG TCA TTC AGG ATG AA	(Li et al., 2004)
IL-1ß	3553	ACA GAT GAA GTA CTC CTT CAA	GTC GGA GAT TCG TAG CTG GAT	(Li et al., 2004)
IL-10	3586	GTG ATG CCC CAA GCT GAG A	CAC GGC CTT GCT CTT GTT TT	(Overbergh et al., 2003)
CCL2	6347	GAT CTC AGT GCA GAG GCT CG	TGC TTG TCC AGG TGG TCC AT	(Dumoulin et al., 2000)
CCL3	6348	CAT CAC TTG CTG CTG ACA CG	TGT GGA ATC TGC CGG GAG	(Giribaldi et al., 2010)
CCL4	6351	ACC CTC CCA CCG CCT GCT GC	GTT GCA GGT CAT ACA CGT ACT	(Dumoulin et al., 2000)
CCL5	6352	ACC ACA CCC TGC TGC TTT GC	CCG AAC CCA TTT CTT TGC	(Dumoulin et al., 2000)
CCL20	6364	TTG GAT CCT GCT GCT ACT CCA CCT CTG	TTC TCG AGT ATA TTT CAC CCA AGT CTG TTT T	(Akahoshi et al., 2003)
CXCL8	3576	CTG GCC GTG GCT CTC TTG G	GGG TGG AAA GGT TTG GAG TAT GTC	(Giribaldi et al., 2010)
CXCL10	3627	GAA ATT ATT CCT GCA AGC CAA TTT	TCA CCC TTC TTT TTC ATG TAG CA	(Clarke et al., 2010)

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4.0 CHAPTER FOUR: ANTIBODY-DEPENDENT ENHANCEMENT OF DENGUE VIRUS INFECTION OF MACROPHAGES IN HUMAN SKIN

4.1 PREFACE

Data in this chapter fulfils aim 3 and will be incorporated into a manuscript for future publication.

4.2 ABSTRACT

Dengue is the most important mosquito-borne virus infection worldwide. During a bite or blood feeding, an *Aedes aegypti*, the primary vector mosquito, injects saliva containing any of four serotypes of DENV into the skin. Individuals who have been previously exposed to DENV infection are predisposed to severe dengue in subsequent infections with different serotypes of DENV or related flaviviruses such as ZIKV. This phenomenon is called antibody-dependent enhancement (ADE), and has been described in epidemiological and in vitro studies. Using an ex vivo skin model of DENV infection, we demonstrate in situ ADE in resident Mϕ, one of major targets of DENV infection. We show that the pre-existence of DENV-3 immune serum enhances heterotypic infection with DENV-2 and ZIKV in a dose-dependent fashion. At the peak enhancement, we observe a 6-fold increase in density of total Mϕ as well as infected Mϕ and up

to 8-fold increase in the proportion of Mϕ infected, resulting in 50-70% of total Mϕ being infected. ADE of DENV infection induced a 3-fold increase in cell emigration out of the skin, reflecting its impact beyond the skin. We also show that mosquito salivary gland extracts had no effect on DENV infection in the absence or presence of enhancing immune serum. Our results indicate that FcγRIa (CD64) and FcγRIIa (CD32) both facilitated the entry of infecting virus into cells as a blockade of these FcγRs together strikingly diminished the amount of Mϕ infected in skin and limited the spread of cell out of the skin, resulting in a reduction of the overall infection in dermis by 70%. Our data show that the Ab-mediated infection of Mϕ contributes to enhancement of infection in human skin and increased dissemination of virus.

4.3 INTRODUCTION

Dengue is the most important mosquito-borne disease caused by infections with any of four serotypes of DENV (23). The dengue serotypes (DENV-1,-2,-3,-4) are genetically related but are antigenically distinct viruses (227). Following the primary infection, a person develops a life-long immunity to the original DENV serotype, but only partial cross-protection against the other three serotypes (228). The co-circulation of multiple serotypes commonly occurs in dengue hyperendemic areas, rendering a high risk of infections with different serotypes (229). While primary DENV infections typically result in a non-lethal, self-limiting illness, secondary infections with different DENV serotypes increase the likelihood of severe dengue. The severe form of dengue commonly being referred to as dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (2). It remains debatable why only a proportion of dengue patients (estimated 500,000 people/year) progresses into life-threatening complications characterized by critical plasma leakage with hemorrhaging (227).

One widely accepted theory to explain the severity of dengue is a phenomenon called ADE, a mechanism involving the host Ab that enhance infection of host cells (230, 231). ADE is supported by epidemiologic evidence that show the strong association between severe dengue disease and primary infection of infants with waning maternal-derived dengue Ab (88, 232, 233). A variety of neutralizing Ab to DENV can drive ADE in vitro when used at sub-neutralizing concentrations (234-236). In support of the role of cross-reactive Ab in ADE occurring in adults, undiluted sera obtained from patients with secondary DENV infection have been shown to enhance the infection of cell lines with the specific DENV serotype isolated from the same patients. No enhancement of DENV was detected when sera from patients with primary DENV

infection were used (237). High viral burden is frequently present early in the course of secondary dengue illness in patients with DHF; 100- to 1000- fold higher in maximum viremia titers were observed in severe dengue patients than levels in patients with dengue fever (103, 104). These findings suggest that sub-neutralizing levels of cross-reactive Ab causing high viral loads during an early stage of infection are important in severity of dengue.

ADE is not only limited to DENV serotypes but also occurs with other closely related flaviviruses such as ZIKV. Considering that DENV and ZIKV share similar ecological distributions and a mosquito transmission mode, the emergence of ZIKV occurs in areas with high rates of DENV prevalence, which could result in DENV-ZIKV sequential infections. DENV-immune plasma collected from recovering dengue patients or monoclonal Ab to the dengue virus envelope have been shown to enhance ZIKV infection in vitro (238, 239). Studies with murine models passively transferred with human DENV immune sera demonstrated ADE of ZIKV infection in vivo (240). However, studies suggested that most DENV immunity in individuals previously exposed to DENV do not induce high-level cross-neutralizing Ab against a secondary ZIKV infection (241). It is currently not clear whether ADE caused by DENV Ab worsens clinical outcomes of ZIKV infections (242). More biological and clinical investigations are required to decipher if prior flavivirus exposure is a key factor for ADE of ZIKV infection.

The key mechanisms of ADE of DENV infection involve FcR (243). At some point, pre-existing Ab that were generated following a primary infection with DENV will wane, resulting in insufficient avidity or concentration to neutralize other infecting DENV serotypes. However, sub-neutralizing levels of these cross-reactive Ab can still bind the virus and form immune

complexes. An ability of immune complexes to increase binding and internalization predominantly occurs through engagement of surface FcγR, leading to an uptake and infection of opsonized virus (100, 244). There are three classes of FcγR in humans: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). Each receptor type can be further classified in two sub-class specificity: a and b (245). Only FcγRIa and FcγRIIa have been shown to enhance DENV infectivity in human monocyte cell lines (244, 246-248), whereas FcγRIII has low impact on ADE (249). FcγRIa requires a γ-chain subunit to initiate signaling following engagement of the Fc domain on immune complexes, whereas FcγRIIa directly transmits an activating signal through an immune receptor tyrosine-based activation motif in its cytoplasmic domain (245). The removal of γ-chain subunit in FcγRIa or the activation motif in FcγRIIa resulted in reduced infectivity of Ab-opsonized DENV (246, 247), indicating the significant role of FcγR in modulating enhancement of DENV infection.

FcγR, particularly FcγRIa and FcγRIIa, are abundantly expressed on human monocytes, Mϕ, and DC (250, 251), which have been shown to serve as major reservoir cells for DENV infection and replication (165, 252, 253). ADE in these DENV-permissive cell subsets promotes high viral loads, aberrant immune responses and tissue damage (254). Nevertheless, the ability of opsonized virus to affect early DENV infection events likely relies on a variety of FcγR-related parameters including the class of FcγR expressed, expression levels of FcγR per cell, affinity to monomeric or complexed forms of Ab, and frequency and distribution of FcγR-expressing cell types in tissue. Among these three major FcγR-expressing cell types, monocytes are mainly in circulation in a steady state, whereas tissue-residing Mϕ and DC can be found in the circulation as well as tissues. Most of the previous reports primarily used FcγR-expressing cell lines or

primary human monocytes isolated from blood to investigate the enhancement of infection (248, 255). However, once generated, Ab are also distributed and located within tissues at various Ab concentrations to protect the body from re-infections. In the case of mosquito-borne virus infection, immunoglobulins generated from the first exposure are more likely to distribute highly in skin as it is a site of transmission. A better understanding of ADE in tissues, particularly skin, will advance the dengue field and the development of vaccines.

As a portal of DENV transmission, skin encounters physical damage to the epidermis and vasculature with the simultaneous introduction of the virus and mosquito saliva. The saliva contains numerous bioactive proteins that prevent host blood coagulation, promote vasodilation, and modulate inflammatory responses to facilitate blood-feeding and virus transmission (256, 257).The immune modulatory role of mosquito saliva compromises host defenses, providing an opportunistic environment for productive DENV infections. This can be achieved in many ways such as suppression of type I IFN production, modulation of inflammatory responses, and induction of viral attachment and cell migration (258-261). As a result of these functions, mosquito saliva has been shown to enhance the replication, alter dissemination of DENV and other arboviruses, and increase disease severity in mouse models (262, 263). In addition to primary infections, a recent study has shown that mosquito salivary gland extracts (SGE) further boost the effect of ADE of DENV infection in mouse skin and result in systemically severe outcomes (221). While observations in human models have yet to be reported, these findings implicate a role of ADE and SGE in driving DENV pathogenesis in humans.

In this study, we have established an ex vivo model of DENV infection to investigate ADE of infection in human skin. We show that ADE in human skin occurs mainly in cells in the dermis. Focusing on skin-resident Mϕ, we show that DENV-3 immune serum enhances the infection of DENV-2 and ZIKV. The highest enhancing activity leads to a 6-fold increase of both total Mϕ or infected Mϕ density, and a 2- to 8- fold increase of the proportion of Mϕ infected, to the levels in which 50-70% of total Mϕ are infected. We demonstrate that the in situ ADE results in a massive DENV production from a number of cell clusters, which contain mainly Mϕ. Surprisingly, *Aedes aegypti* mosquito SGE has no effects on DENV infection in absence or presence of ADE infection. In our system, ADE occurs mainly through the FcγR pathway co-mediated by FcγRIa and FcγRIIa. Blocking these two FcγRs strikingly diminishes amounts of Mϕ infected in skin and cells spreading out of the skin, resulting in a reduction of ADE infection in cells in dermis by 70%.

4.4 RESULTS

4.4.1 Antibody-dependent enhancement of DENV-2 and ZIKV infection occurs primarily in cells in the dermis of human skin primed with DENV-3 immune serum.

To investigate ADE of DENV-2 or ZIKV infection in human skin, we introduced immune serum containing Ab to DENV-3. The DENV-3 immune serum was pooled from 7 different patients who were confirmed to have DENV-3 monotypic immunity by a plaque reduction neutralization test (PRNT) assay in a prospective cohort study in Brazil (255). The ADE assay in human skin was performed following steps depicted in Figure 1A. DENV-3 immune serum was prepared into 3 dilutions, 1/4000, 1/400, and 1/40, which were separately fabricated into MNA. The similar concentration of human AB serum was used as a negative control. To test effects of Ab to DENV-3 in enhancing viral infection, we manually applied MNA to skin before the virus inoculation. Following removal of MNAs, a low-dose inoculum (10^3 plaque-forming units/10 µl delivered virus) of 3 viruses, DENV serotype 2 strain 16681, DENV serotype 2 strain K0049, and ZIKV strain PE243 were separately inoculated into skin where the MNA were applied. Skin was harvested at 24 hours post inoculation and stained with an antibody to DENV NS3. Although skin was inoculated by a low titer of virus, it resulted in viral replication, characterized by NS3 production, detected in cells in the epidermis and the dermis (Figure 7B). A non-specific increase in the NS3+ cells in the epidermis was (Figure 7B, top). Preliminary data has shown that DENV-3 immune serum did not enhance infection of Langerhans cells in epidermis as % Langerhans cell infected ranged between 45-55% in the absence and presence of test serum (data not shown). In contrast, a significant enhancement of infection in dermis was seen with all 3 viruses, only when a 1/40 dilution was delivered. (Figure 7B, bottom).

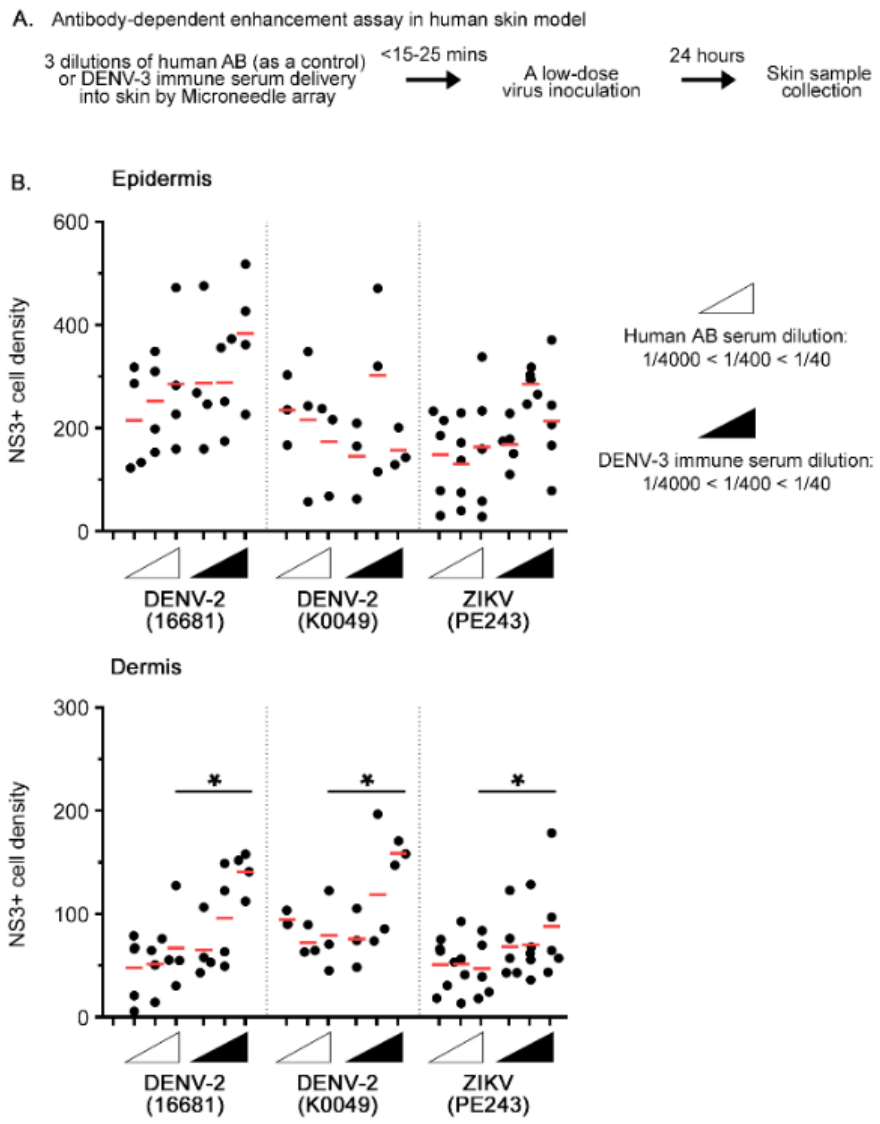

Figure 7. Antibody-dependent enhancement of DENV-2 and ZIKV infection occurs primarily in the dermis of human skin primed with DENV-3 immune serum.

(A.) A sequence of steps performing an antibody-dependent enhancement assay in human skin explants. (B.) Quantification of DENV infection in the epidermis (top) and the dermis (bottom) of human skin. Skin explants were MNA-loaded with 3 dilutions (1/4000, 1/400, and 1/40) of DENV-3 immune serum or human AB serum as a control, then were infected with a low-dose inoculum of 3 viruses: DENV-2 (strain 16681) (n=4), or DENV-2 (strain K0049) (n=3), or ZIKV (strain PE243) (n=5), for 24 hours. Skin sections were stained with pan-anti-NS3 and Hoechst dye. Each symbol represents one individual and red horizontal lines indicate means. *P < 0.05 comparing the number of NS3-expressing cells in the virus infection with DENV-3 immune serum at 1/40 dilution and the control at same dilutions.

4.4.2 DENV immune serum increases the recruitment and infection of dermal Mϕ.

As FcγR-bearing cells such as Mϕ have been suggested to facilitate ADE, we further investigated the enhancing effect of DENV-3 immune serum on Mϕ in dermis, characterized by CD163 expression. Quantitative data from in situ imaging showed that inoculation of DENV alone increased Mϕ density despite the low titer of virus used. In the presence of DENV-3 immune serum, infection of DENV-2 (strain 16681), but not the strain K0049, remarkably brought in a significant number of Mϕ close to the site of virus inoculation. These effects of the serum were observed in dose-dependent manner; the density of Mϕ was observed to increase 5- to 6- fold when the DENV-3 serum was used at 1/40 dilution. On the other hand, ZIKV infection, did not cause the recruitment of Mϕ with or without DENV-3 immune serum (Figure 8A). We previously have shown a relationship between cell recruitment and infection in which cells are recruited by the virus to serve as its additional targets (264). Corresponding to those findings, here we found that a 6-fold increase of infected Mϕ was observed following DENV-2 16681 infection with 1/40 immune serum. The highest serum dilution tested (1/4000) also induced an increase of infected cells but with a much lower magnitude (Figure 8B). Our data has shown that DENV-3 immune serum acted in a dose-dependent manner, and all 3 serum dilutions markedly increased the percent of Mϕ infected with DENV-2 (16681) (Figure 8C). The highest enhancing serum activity at 1/40 dilution led to the infection in ~50% of total Mϕ which was increased from ~20% in the infection with the presence of 1/40 control serum dilution. Similarly, ZIKV infection with DENV-3 serum was enhanced in the same manner with 1/40 dilution of test serum increasing the % Mϕ infection from 5-10% to ~25%. Unlike the 16681 strain and ZIKV, all 3 dilutions of DENV-3 serum increased similar levels of % Mϕ infected with DENV-2 (K0049), which were from 20-30% to 40%. Overall, the 1/40 diluted serum mediated-enhancement

resulted in a possible 2- to 8-, or 2, or 1.5- to 4-fold increase of the % Mϕ infected with DENV-2 (16681), DENV-2 (K0049), or ZIKV (PE243), respectively, when calculated from each individual. (Figure 8D). DENV-2 (K0049). The enhancing activity of DENV-3 serum caused a substantial number of Mϕ recruiting nearby areas of inoculation and the enhancement of Mϕ infection. These ADE phenomena in skin were frequently seen in situ as NS3 abundantly produced by infected cell clusters including infected Mϕ and other infected cell types (Figure 8E).

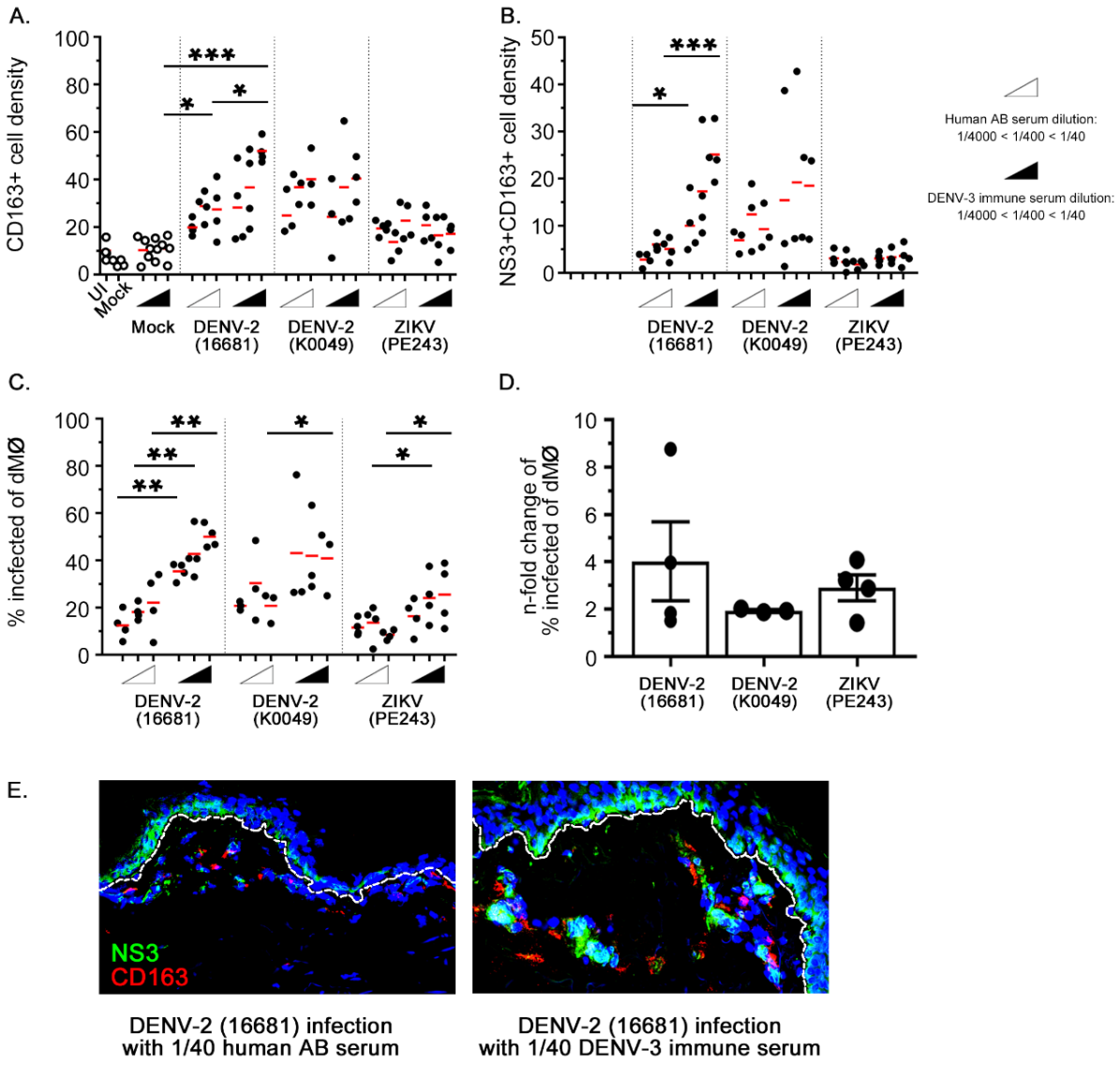

Figure 8. DENV-3 immune serum facilitates the enhancement of dermal macrophage infection.

Skin explants were MNA-loaded with 3 dilutions (1/4000, 1/400, and 1/40) of DENV-3 immune serum or human AB serum as a control, then were infected with a low-dose inoculum of 3 viruses: DENV-2 (strain 16681) (n=4), or DENV-2 (strain K0049) (n=3), or ZIKV (strain PE243) (n=5), for 24 hours. Skin sections were stained with anti-pan DENV NS3, anti-human CD163 and Hoechst dye. **(A-C)** Quantitative data of macrophage infection in dermis at 24 hours: **(A)** Density of macrophages **(B)** Density of infected macrophages (**C**) Percent of macrophages infected. Each symbol represents one individual and red horizontal lines indicate means. *P < 0.05, **P < 0.01, ***P < 0.0001 comparing the infection with DENV-3 immune serum and the control at the similar dilution. (**E.**) Representative images showing Ab-mediated enhancement of DENV-2 infection of dermal macrophages. Infection of cells in skin containing

1/40 dilution of human AB serum (left) or skin containing 1/40 dilution of DENV-3 immune serum (right). Colors represent marker staining; red indicates CD163/scavenger receptors on dermal macrophages, green stains DENV NS3, and blue represents nuclear staining. Dotted lines signify a junction between the epidermis and the dermis.

4.4.3 Female *Aedes aegypti* mosquito salivary gland extracts do not exacerbate DENV-2 infection or DENV-ADE infection of macrophages, or enhance cell migration.

The serum dilution at 1/40 clearly exhibited the highest enhancing activity on Mϕ density and infection. This serum dilution was chosen for further investigations. We sought to decipher the impact of *Aedes aegypti* mosquito SGE on DENV-2 infection as well as the ADE infection of Mϕ. To elucidate the effect of mosquito saliva on DENV infection and spread in human skin, we performed an ex vivo SGE-mediated enhancement assay on our human skin model of DENV infection following steps depicted in Figure 9A: For ADE-induced conditions, MNAs containing 1/40 dilution of DENV-3 immune serum or human AB serum were applied to the surface of skin specimen and were removed after 15 minutes of MNA application. The MNA steps were followed by an inoculation of DENV alone or mixed with SGE equivalent to one mosquito (a pair of salivary glands). For direct infection, SGE or PBS (used as a control) was mixed with a low-dose inoculum of virus (10^3 PFU), and together inoculated into skin. Following 24 hours post inoculation, skin and migrated cells in culture media were harvested. We have shown that SGE alone did not increase the recruitment of Mϕ and the number of infected Mϕ (Figure 9B-C). DENV infection at a low titer significantly induced the 2-fold recruitment of Mϕ. In the presence of DENV-3 serum in skin, the recruitment of Mϕ was strongly augmented with a 3-fold increase compared to amounts observed in DENV infection with the control serum. When SGE was

added into the system, we found a negligible increase of the number of Mϕ as well as infected Mϕ in a non-ADE condition, compared to corresponding conditions without SGE, suggesting that SGE had no effects on the recruitment of Mϕ in ADE condition (Figure 9B). A 5- to 7-fold increase of the number of infected Mϕ was observed in DENV-ADE infection. With SGE mixed with the virus, the quantity of infected Mϕ was highly increased in the ADE infection and the increased level was as comparable as those observed without SGE, indicating that these changes were mediated solely by DENV-3 immune serum effects (Figure 9C). The proportion of Mϕ infected (infected Mϕ/total Mϕ) is a direct indicator for the ADE of Mϕ infection. An approximately 20% of Mϕ became infected in DENV infection; the percent increased to ~ 30% with SGE added and is substantially enhanced to ~ 60% when DENV-3 serum alone was in the system. DENV-ADE infection with SGE led to ~ 55% of Mϕ infected which was comparable to the ADE infection without SGE, suggesting non-significant effects caused by the SGE (Figure 9D). In addition to the enhancement of DENV infection in skin, we observed that the dissemination of DENV or ZIKAV, characterized by cell emigration out of the skin, was markedly augmented by DENV-3 serum, but not the SGE (Figure 9E).

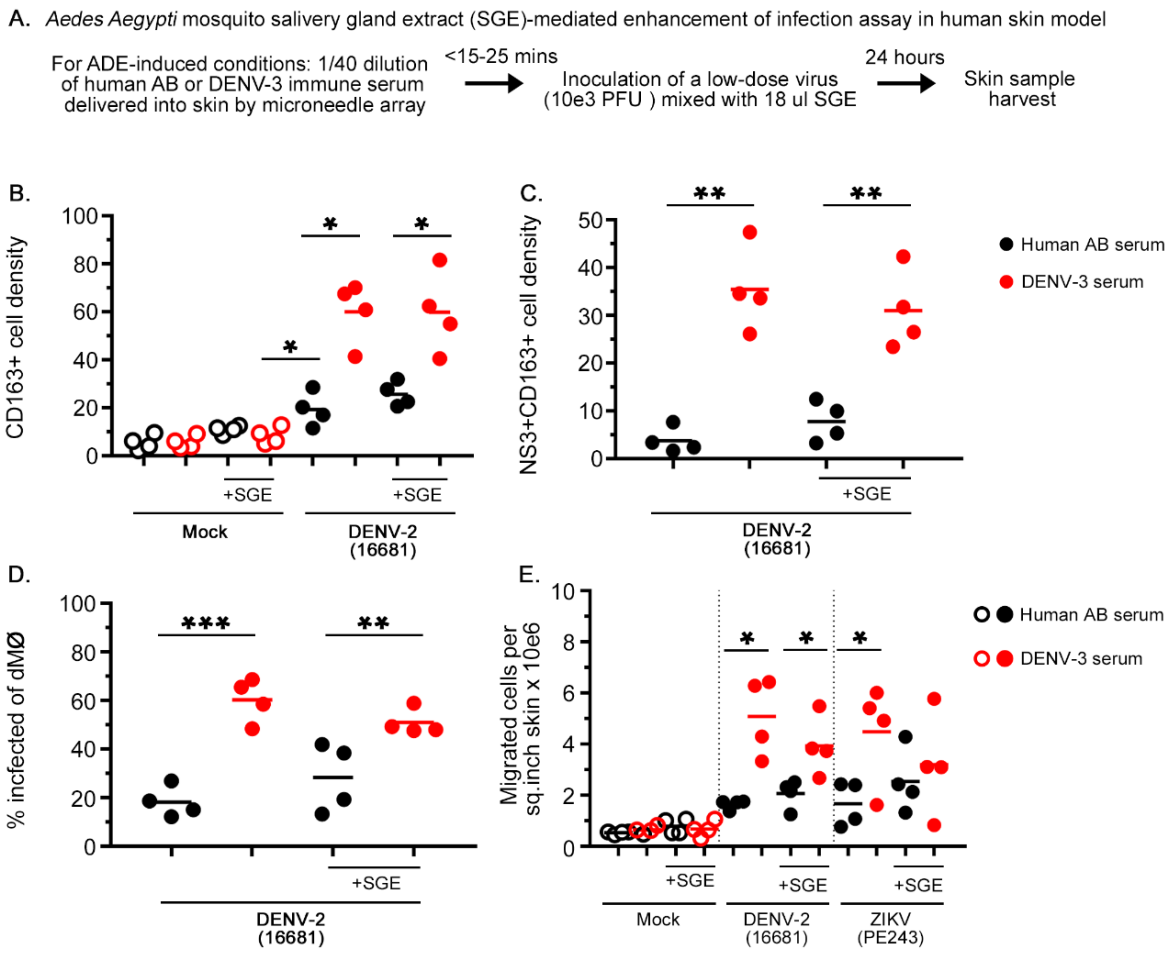

Figure 9. Female *Aedes aegypti* mosquito salivary gland extracts did not exacerbate DENV-2 infection or DENV-ADE infection of macrophages, or enhance dissemination of the virus.

(A.) steps in experiments performed to investigate effects of *Aedes aegypti* mosquito saliva extracts using human skin model of DENV or ZIKV infection. (B.-D.) Quantitative data of macrophage infection in dermis at 24 hours: (B.) Density of macrophages (C.) Density of infected macrophages (D.) Percent of macrophages infected. (E.) Total number of migrated cells collected at 24 hours in skin culture media normalized to skin area. Each symbol represents a different donor and horizontal lines indicate means. *P < 0.05, **P < 0.01, ***P < 0.0001 comparing test conditions with DENV-2 serum with the controls with human AB serum.

4.4.4 Blocking FcγRIa or FcγRIIa substantially inhibits DENV-ADE infection of macrophages, cell spread out of the skin, and substantially reduces overall ADE infection in dermis.

To test the role of Fc receptors in ADE-induced infection of Mϕ in human skin, we delivered neutralizing antibodies to FcγRIa (CD64) and/or FcγRIIa (CD32) to skin loaded with or without 1/40 dilution of DENV-3 immune serum prior to DENV-2 inoculation using dissolvable MNAs (Figure 10A). The recruitment of Mϕ in ADE-induced skin was confirmed in this experiment as a 5-fold increase of the total number of Mϕ as well as the number of Mϕ infected were observed following the inoculation of DENV-2 to skin loaded with DENV-3 immune serum (Figure 10B-C). In skin with the presence of DENV-3 immune serum, MNA containing FcγRIa or FcγRIIa alone had no significant effects on the total number of Mϕ in the dermis relative to the isotype control Ab. However, a combination of neutralizing Ab to FcγRIa and FcγRIIa significantly decreased the recruitment of Mϕ as well as the infection of these cell type (Figure 10B-D). The synergistic blockage from a FcγRIa/FcγRIIa cocktail resulted in a 2-fold decrease of the total number of Mϕ, and a 6-fold decreased of Mϕ infected. These accounted for 10% infected of total Mϕ, comparable to levels observed in skin without DENV-3 serum (Figure 10D). The effect of FcγRIa or FcγRIIa alone also significantly reduce the ADE-infected infection of Mϕ; blocking FcγRIIa alone led to approximately 3-fold decrease of amount of Mϕ infected which accounted for 25% Mϕ infection. Likewise, when using neutralizing Ab to FcγRIa the number of Mϕ infected observed in skin was half the levels observed in the isotype control Ab, which accounted for 20% Mϕ infection. These indicated that either FcγRIa or FcγRIIa interventions had an impact on ADE infection of Mϕ, but were not as effective as when both were used together (Figure 10C-D). A significant reduction of the number of cell emigration out of skin in the presence of

DENV-3 serum was found only when neutralizing Ab to FcγRIa and FcγRIIa were delivered (Figure 10E). A combination of neutralizing antibodies to FcγRIa and FcγRIIa had an impact only on the ADE in dermis, but not in epidermis, resulting in a reduction in the total number of DENV-infected cells in the dermis by 70%. The overall infection in the dermis was comparable to observations in skin delivered with the control Ab (Figure 10F).

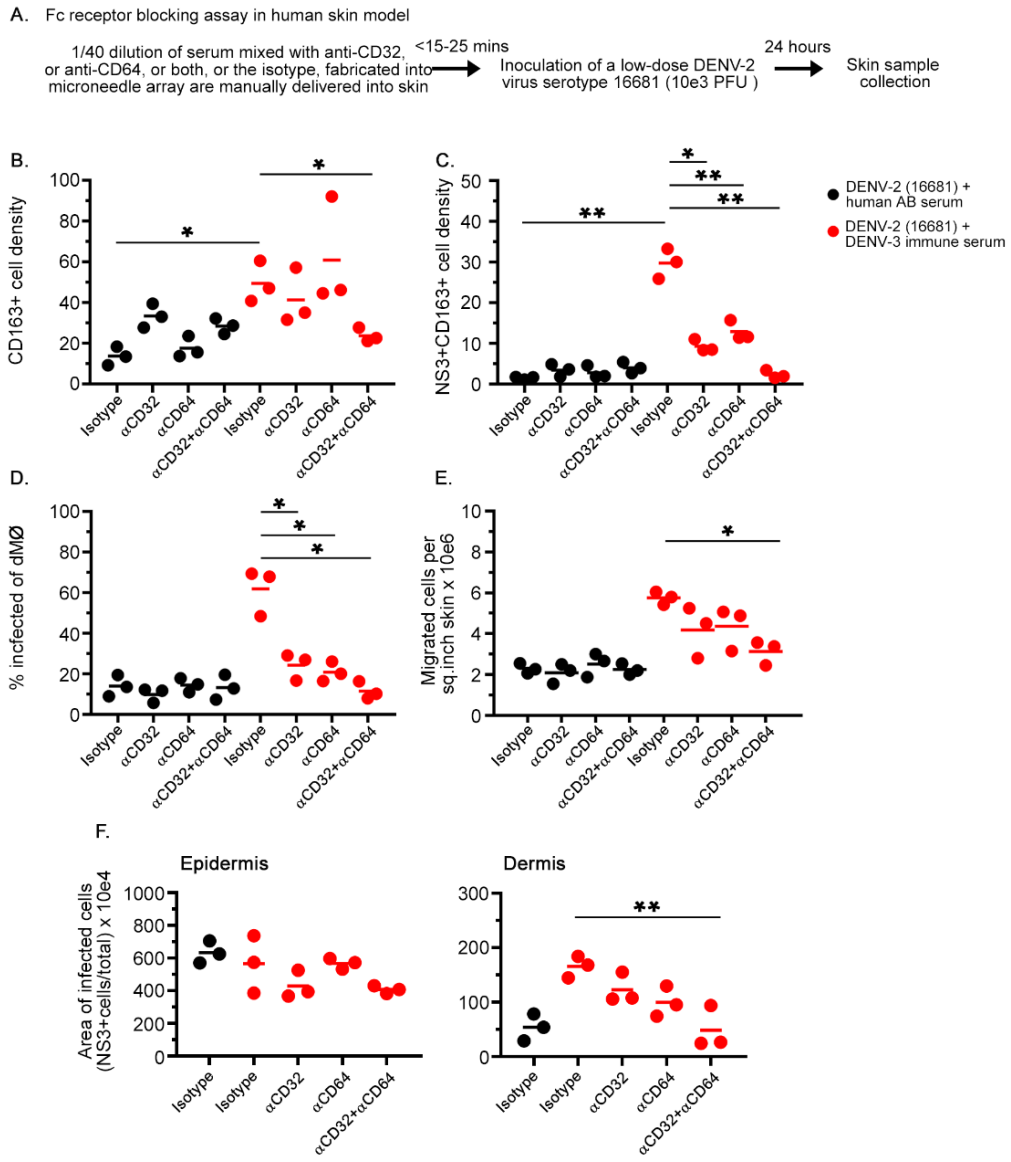

Figure 10. Blocking FcγRIa and FcγRIIa averts ADE infection of macrophage in skin and cell spread out of the skin.

(A.) steps in experiments performed to investigate the role of CD32 and CD64 in ADE infection using human skin model of DENV infection. (B.-D.) Quantitative data of macrophage infection in dermis at 24 hours: (B.) Density of macrophages (C.) Density of infected macrophages (D.) Percent of macrophages infected. (E.) Total number of migrated cells collected at 24 hours in skin culture media normalized to skin area. (F) Total number of DENV-infected cells in epidermis and dermis 24 hours after exposure to isotype control antibodies or antibodies to CD32 or CD64 or both in skin with the presence of DENV-3 immune serum. Each symbol represents a different donor and horizontal lines indicate means. *P < 0.05, **P < 0.01.

4.5 DISCUSSION

Antibody-dependent enhancement is known to occur in experimental conditions using cell lines or animal models of DENV infection, but little is known about this phenomenon in tissues. This is particularly true in human skin which serves as a site of transmission and viral replication. Our data show that pre-existing immunity to DENV-3 in an ex vivo human skin model induces enhancement of skin-resident Mϕ infection with a low-titer DENV-2. This is consistent with findings showing monocytes and Mϕ support ADE in vitro (265). Severe dengue occurs when neutralizing Ab titers against the original infecting strain are more than 1:100, which was consistent with the Ab range between 1:21-1:80 that has been reported to increase the likelihood of ADE in children in a long-term cohort study (266, 267). In our study, DENV-3 immune serum used was pooled from 7 sera confirmed to be homotypic DENV-3 immune, with Ab titer range of 1:340-1:1320. The mean Ab titer of the pooled serum was 1:786. All 3 dilutions (1/4000, 1/400, and 1/40) were found to significantly enhance the infection of DENV-2 in a dose-dependent manner. The highest enhancement of infection was observed when the 1/40 dilution of immune serum was used.

In the presence of DENV-3 serum, the percent of Mϕ infected increases to 50-70%, which is similar to the infection level yielded in our skin model infected with a 3-log higher DENV titer (10^6 PFU). This can be speculated that the peak enhancement may yield up to a 1000-fold increase of progeny virions produced. Similar observations have been made in FcγR-bearing cell lines and the blood of severe dengue patients that maximum peak viremia titers are found to increase up to 1000-fold (103, 104, 268). We found that DENV infection in the presence of Ab promotes a 2- to 3-fold increase in the number of cells emigrating out of the skin relative to virus

alone. The link between the increase in viremia titer or cell migration to skin-draining lymph nodes and disease severity has been described before (104, 168). Our results further strengthen the notion that under ADE conditions, skin cell emigration is a key process in the spread of infection and the determination of disease outcomes.

Two distinct pathways, extrinsic and intrinsic, have been proposed to mediate ADE (178). An extrinsic model for ADE is mediated by an increase in internalization of immune complexes, while an intrinsic ADE of infection pathway involve various intracellular mechanisms including the suppression of type I IFN system or an increase in viral fusion activity. Although the results shown in our study cannot determine which pathway the antibody promotes ADE, our data indicate that the significant increase in viral output in Mϕ, characterized by NS3 expression, occurs in conjunction with a substantial recruitment of Mϕ in the presence of immune serum. In line with previous ADE hypothesis (269), the in situ immunofluorescence reveals that ADE of infection in skin leads to a large infected cell mass that contains several cell types including Mϕ observed in clusters, and increased virus production (burst size). Close proximity of cells within the cluster may promote concentration and targeting of immune complexes to FcR-bearing cell's surface. A study revealed that Ab-DENV complexes activate extensive actin ruffle formation of the Mϕ membrane which facilitates the uptake of opsonized DENV into the cells during ADE (270). It can be speculated that ADE of DENV infection of Mϕ may be more driven by an extrinsic pathway. This finding indicates that trafficking of antibody-opsonized DENV into cellular compartments likely differs from DENV infection without effects of ADE. Furthermore, we have previously shown that type I IFN responses were suppresses in the absence of immune serum within 12 hours post DENV inoculation (264). This suggests that the intrinsic ADE of

DENV infection in Mϕ is also possible. Nevertheless, molecular mechanisms of ADE pathways in which FcγRs aid in DENV entry remain to be revealed. Further investigations are needed to characterize cell entry or closely investigate type I IFN expression within the early course of ADE infection.

It is well-known that flaviviruses are closely related; DENV and ZIKV share a high degree of amino acid sequence overlap with one another, between 55.1-56.3% (271). Ab to these viruses are thus highly cross-reactive; for example Ab to the fusion loop epitope on both virus particles induce poorly neutralizing but strongly enhance infection (236, 239, 268). Our data show that pre-existing DENV immune serum enhances ZIKV infection in Mϕ in skin, which is in agreement with several in vitro studies using FcγRIIa-expressing K562 cell lines or immune-deficient mouse models (240, 272, 273). Using a similar ZIKV stock and enhancing Ab, the ADE observed in human skin for ZIKV was 1.5 fold lower than the observation in vitro. In contrast, studies using monkeys confirmed that DENV-immune serum induces in vitro ADE of ZIKV infection, but did not enhance the severity of disease in animals (274). Similarly, a recent cohort study revealed that ADE of ZIKV infection did not occur in DENV-immune individuals who were subsequently exposed to ZIKV infections (275, 276). These findings indicate discrepancies observed between experimental studies and real-world incidence of ADE of ZIKV infection. In contrast to DENV infection, only a relatively low quantity of total Mϕ as well as ZIKV-infected Mϕ are observed in skin in the absence or presence of the serum. No differences to the number of Mϕ are observed between mock infection or ZIKV infection with or without the DENV-3 enhancing serum. This suggests low inflammatory responses induced by ZIKV inoculation. In line with our observations, ZIKV infected patients who had or had not

experienced a previous DENV infection were presented with comparable low-level cytokine profiles including IFN-γ, IL-1β, IL-6, and IL-8 (275). Our data show that inoculation with ZIKV into skin in the presence of DENV immune serum facilitates more cell spreading out of the skin by a 2-fold increase, compared to normal ZIKV infection. Currently, the ADE of ZIKV infection induced by DENV or other flaviviruses is not well understood. Our results highlight the possibility of the enhancement of ZIKV infection occurring in humans. However, compared to DENV or DENV-ADE infections, it may be less likely to render a large clinical impact. Further epidemiologic evidence in humans would be important to understand the impact of DENV immunity for ZIKV induced disease and sequelae.

Previous studies that examined whether mosquito-derived factors directly modulate DENV infection are controversial. Some have demonstrated that certain identified components from *Aedes aegypti* show blocking activities against DENV infection or target cell binding in vitro and in vivo (277), while others suggest that mosquito saliva augments infection of DENV as well as other arboviruses (261, 263). Currently, it remains unknown which of these mosquito factor contributes most to enhancing infection. Since mosquito saliva is a complex mixture of diversely functioned elements, it is likely that reactions to saliva vary between studies or individuals. In our ex vivo skin model, SGE has no significant effects in enhancing the primary infection of Mϕ, consistent with recent findings in mice, that exacerbated dengue outcomes are observed only in the presence of enhancing antibodies, but not in the first virus exposure (221). In contrast, our data suggest that SGE does not further boost the enhancement of Mϕ infection with DENV-2 even though serotype cross-reactive Ab to DENV-3 is delivered into the skin. We previously have shown that, following DENV-2 inoculation, activated keratinocyte-derived IL-1β drives

recruitment of myeloid cells and spread of virus out of human skin (264). In murine models of infection with other arboviruses including Semliki Forest virus, mosquito bites have been shown to induce multi-steps of cell recruitment beginning with mast cell degranulation, which recruits neutrophils that express high levels of IL-1β. Neutrophil-derived IL-1β subsequently plays a role in coordinating host responses to mosquito bites by promoting an influx and infection of additional myeloid cells such as monocytes and skin-resident Mϕ, leading to enhanced disease severity (218). Although the function of neutrophils during DENV infection in skin is poorly defined, it has been shown in studies with a related West Nile virus (WNV) that early neutrophil influx contributes to WNV replication and spread and worsen the outcome of infection (278). Mosquito feeding induces the release of histamine from mast cells, and the infiltration of neutrophils into the dermis. Both events are known to mediate vascular leakage, suggesting the contribution of mast cells and neutrophils in mosquito-mediated enhancement (279, 280). It has been speculated that vascular leak would allow more access to cross-reactive Ab into tissues. However, there was no need for more accessibility of Ab in our system as a sufficient concentration is controllably delivered into skin by MNA. Furthermore, lack of neutrophils and monocytes infiltrating from the blood into skin explants may also explain the undetectable effect of SGE to further promote the outcome of DENV infection despite of the presence of enhancing DENV immune serum.

Opsonized DENV gains entry into cells by exploiting Ab-dependent cellular phagocytosis, which are triggered by the localized clustering of cell membrane Fc receptors (FcR) through binding to the Fc portion of Ab-coated (opsonized) DENV (281). However, ADE of DENV infection of Mϕ can occur independent of FcγR-mediated internalization, but through enhanced fusion activity

per cell, resulting in augmented infectious viral particle production (282). Our data show that in human skin, ADE of DENV infection in Mϕ is mediated through the FcγR pathway, notably by FcγRIa (CD64) and FcγRIIa (CD32). FcγRIa is a high affinity receptor capable of binding human IgG in monomeric form, whereas FcγRIIa are low-affinity receptors which bind IgG in complexed or aggregated form. Both FcγRIa and FcγRIIa can enable ADE in vitro, but ADE is more efficiently triggered through Ab engagement of FcγRIIa (246, 283). The higher permissiveness to DENV ADE of FcγRIIa is believed to depend on its relatively higher affinity to opsonized viruses (101). In contrast to in vitro studies, the presence of high levels of neutralizing Ab to either FcγRIa or FcγRIIa alone significantly reduces the infection of Mϕ, with a comparable impact, but not the cell migration. However, a simultaneous blockage of FcγRIa and FcγRIIa efficiently diminishes infection and migration of Mϕ in the skin, as well as cells spreading out of the skin. They were observed to be reduced to the level observed in non-ADE induced infection. These findings indicate that ADE of DENV infection of Mϕ in skin is dominantly mediated by FcγRIa and FcγRIIa. Among these activating FcγRs, FcγRIa is present only on mononuclear phagocytes, but FcγRIIa is expressed on a much broader range of cell populations including monocytes/ Mϕ, granulocytes, platelets and B cells (284). Human Mϕ constitutively express high levels of FcγRIIa, whereas FcγRIa is highly upregulated during cell activation (245). Importantly, the activation of Mϕ by immune complexes induces FcγR-dependent caspase-1 and inflammasome activation, resulting in the release of functional IL-1β (285). IL-1β is a chemotactic factor for myeloid cells and is known to be abundantly produced, mainly by infected keratinocytes in response to primary DENV infections (264). In the presence of DENV-3 immune serum, DENV infection of keratinocytes also occurs but without enhancement. This occurs despite an ability to express functional FcγRs (286). The enhancement

of Mϕ infection would promote further IL-1β production at the inoculation site, which in turn brings in additional target cells and facilitates dissemination of DENV-infected cells out of the skin. Our data show the implication of the FcγRIa/IIa-dependent ADE functions in impacting the initial events in DENV transmission and dissemination in skin. These findings lay the groundwork for further mechanistic studies in human skin.

It would be interesting to know whether other FcγR -bearing cell types in skin such as LC and DC actively support ADE. It is well-defined that DENV infects LC and DC, and that these cells can rapidly spread the virus to other tissues and circulation due to their highly migratory ability (160, 166, 287). An ADE study by Xu et al. demonstrated that a specific antibody to the DENV fusion loop successfully enhanced DENV-2 infection of human skin isolated DC and Mϕ, but no enhancement was observed in LC, which is similar to our preliminary data on LC in skin explants (288). DC-SIGN, a dengue receptor that is predominantly expressed by DC, mediates internalization of the virus in an absence of immune sera (164, 289). However, high expression of DC-SIGN overrides FcγIIa-mediated enhancement of infection in the presence of enhancing immune sera (169). This explains why only mature DC which have downed-regulated surface molecules including DC-SIGN in response to maturation stimuli can undergo ADE infection, whereas immature DC do not exhibit ADE in vitro (290, 291). DENV exploits mannose receptors on Mϕ to facilitate its entry (176). In contrast to DC, Mϕ's expressing DC-SIGN are resistant to DENV replication (175). Furthermore, expression of mannose receptors does not only interfere with antibody-mediated entry of DENV but also amplifies the ADE of infection in Mϕ, resulting in impaired type I IFN and increased viral load (176, 178, 282, 292). Because the ADE phenomenon in DC is limited by several determinants such as activation and expression of

DENV entry receptors, these findings suggest that monocyte/ Mϕ are likely main contributors to ADE of DENV infection.

In conclusion, our study introduces an ex vivo human skin model to study in situ ADE infection in tissues. Focusing on Mϕ, we show that this cell type plays a crucial role in increasing viral production in the dermis when pre-existing DENV immunity is locally present. Our results provide valuable clues that the pre-existence of immunity to different serotypes of DENV does not only enhance the DENV infection in cells, but also induces the recruitment and spread of cells from human skin to culture media. The enhancement occurs through the combination of FcγRI and FcγRIIa receptors. The impact of mosquito saliva does not worsen the outcome of ADE infection in Mϕ. In addition, DENV-immune serum boosts the infectivity of ZIKV infection in Mϕ, suggesting the likelihood of ADE incidence following ZIKV infection in DENV-immune populations. Results from our work demonstrated that the ADE phenomenon in humans can locally occur in skin where a primary route of viral replication and dengue vaccine administration is, and may have an impact beyond the site of inoculation as reflected by the cell emigration out of the skin. Our data highlight the importance of skin in initiating and supporting ADE of DENV infection, a major risk factor for severe dengue form, and highlights the necessity of further studies on the role of skin cells in augmenting dengue disease severity.

4.6 MATERIALS AND METHODS

4.6.1 Viruses

The prototype DENV-2 strain 16681 was provided by Dr. Jared Evans at the University of Pittsburgh. The low-passage DENV-2 strain K0049 was isolated in 1995 from an individual in Thailand diagnosed with dengue hemorrhagic fever (83), and was obtained from BEI Resources (Manassas, VA). The low-passage Zika virus strain PE243 was isolated in 2015 from a Zika-infected patient in Recife, Brazil and was obtained from FIOCRUZ. DENV was cultured and propagated in C6/36 insect cells (ATCC, Manassas, VA). C6/36 cells were routinely grown in Dulbecco's modified eagle medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% of the following: penicillin/streptomycin, L-glutamine and sodium pyruvate solution. Tissue culture supernatant was pooled at day 5, 10, and 15 of culture (when cytopathic effect was evident), clarified of cell debris by centrifugation, and concentrated using standard methods. Virus titers were determined by a modified focus forming unit immunoperoxidase assays using Vero cells as described previously (225).

4.6.2 Skin processing and virus inoculation

Large blocks of anonymized full-thickness skin that were being donated following elective aesthetic abdominoplasty or mammoplasty at the University of Pittsburgh were used. Identifiable private information regarding skin donors was not provided and no interaction or intervention with donors was possible. The research did not constitute human subject research as per the University of Pittsburgh Institutional Review Board. Any residual adipose tissue was trimmed

from the underside of the skin explant before virus inoculation. A volume of 50 µl of RPMI 1640 media containing 5 x 10^3 plaque-forming units purified virus was placed in a 4 square-inch that was demarcated in the center of the surface of the skin explant. Bifurcated allergy skin testing needles (Precision Medical Product Inc., Denver, PA) were used to repeatedly puncture the skin surface through the 50-µl virus inoculum and deliver virus into the epidermis and dermis. Our preliminary experiments indicated that this inoculation method delivered approximately 10 µl of virus suspension (or 10^3 plaque-forming units virus) into the skin. Inoculated explants were incubated at 37˚C for 2 hours, then the skin surface was washed with phosphate buffer saline and wiped with sterile gauze pads to remove any remaining virus. Tissue was placed dermis-side down onto mesh grids in 60 x 15 mm culture dishes, and continued incubating at the liquid-air interface in RPMI 1640 complete media. Explants were collected at 24 hours after virus inoculation and the virus-delivered central region was isolated. The samples were immediately submerged in 30% sucrose overnight at 4˚C, and then frozen for subsequent immunohistochemistry. At the time skin was harvested, media was also collected to harvest and count the number of migratory cells.

4.6.3 ADE assay

Seven sera from a study by Castanha et al. (255) were aliquoted, pooled and used in our study as DENV-3 immune serum. These samples were collected from DENV immune pregnant women and identified by a PRNT50 assay to have DENV-3 monotypic immunity. Ab titers to DENV-3 from all sera range between 380-1320 (the mean Ab titer 1:786), with low serotype-specific neutralizing Ab response to DENV-1, -2, and -4 (Ab titer < 20). Pooled DENV-3 immune serum was diluted to 1/40, 1/400 and 1/4000 with phosphate buffer saline, and then formulated into tip-

loaded dissolvable 3:2 carboxymethyl cellulose/trehalose MNA as described previously (206). Human serum off clot sterile type AB (MP Biomedicals, USA) was diluted to 3 similar dilutions as tested serum, and was fabricated into MNAs as the control serum, which was designated in our study as human AB serum. For ADE assay, MNAs were manually applied to skin explants for at least 15 minutes until needle tips dissolved, leaving loading reagents in the skin. Following the removal of MNA patches, virus inoculation was performed immediately at the region of MNA application on the skin surface. A low-dose inoculum of virus, DENV-2 strain 16681 or DENV-2 strain K0049, or Zika virus, was used at 1 x103 plaque-forming units per 10 µl delivered virus. Tissue culturing was performed as described above. Skin was collected at 24 hours after virus inoculation and the MNA-applied region was isolated, submerged in 30% sucrose overnight at 4˚C, and then frozen for immunohistochemistry.

4.6.4 Female *Aedes aegypti* mosquito SGE-mediated enhancement of virus infection assay

SGE from naïve female *Aedes aegypti* mosquitoes was provided Dr. Nikos Vasilakis at the University of Texas, Galveston. Briefly, SGE was prepared by dissecting the heads and thoraces from mosquitoes for the appearance of salivary glands. A pair of salivary glands were removed from a mosquito and were immediately transferred to PBS on ice. 50 salivary glands were collected, pooled in 450 µl sterile PBS and sonicated. The supernatant was then collected and used as SGE. *A. aegypti* mosquitoes inject a small volume of saliva (< 5 µl) during blood probing and feeding (293). In our study, 18 µl SGE which is equivalent to a pair of salivary gland per a mosquito, was used. Given the efficiency of a volume delivered into the skin by bifurcated needle method is 20-27%, using 18 µl SGE as a starting volume would lead to a delivery of an estimated volume an infected mosquito inoculates during the natural transmission to humans. We

mixed SGE with the infecting virus in a micro-centrifuge tube and placed a mixed inoculum on the skin surface. Bifurcated needles were used to puncture through a bubble of inoculum, delivering the virus and SGE into the skin. Tissue culture was performed as described above. Skin was collected at 24 hours after virus inoculation and the MNA-applied region was isolated, submerged in 30% sucrose overnight at 4˚C, and then frozen for immunohistochemistry. Media was also collected at 24 hours post infection to harvest and count the number of migratory cells.

4.6.5 FcR blocking assay

Neutralizing Ab to human CD32 (IV.3, Stemcell Technologies, Seattle, WA), or CD64 (10.1; Biolegend), either alone or together, or isotype control Ab were formulated into tip-loaded dissolvable 3:2 carboxymethyl cellulose/trehalose MNA as described previously (206). MNA contained 10 μg of Ab to CD32 or CD64 or the combination of these 2 Ab. The concentration of these Fc receptors blocking antibodies was 2- to 3- times more than the concentration shown in in vitro studies to neutralize surface expression of CD32 and to saturate all surface CD64 molecules (247, 294). In MNA preparation, the Ab concentration was multiplied by a factor of 2.3 to compensate for dilution following dispersal within skin. Control MNAs contained 10 μg each of mouse isotype control IgG1 (MAB002; R&D systems, Minneapolis, MN) and IgG2b Ab (MPC11; Stemcell Technologies, Seattle, WA). MNAs were manually applied to skin explants for 15 minutes prior to inoculation of virus.

4.6.6 Immunohistochemistry

Six-µm frozen skin sections on microscope slides were rehydrated with phosphate buffer saline and fixed in cold acetone for 5 minutes at 4˚C. Slides were stained with polyclonal rabbit anti-pan DENV NS3 Ab (provided by Sujan Shresta, La Jolla Institute for Allergy and Immunology) and monoclonal mouse anti-human Ab directed against specific cell surface anti-CD163 (5C6FAT; Acris Antibodies GmbH) overnight at 4˚C, then incubated with relevant secondary Ab for 30-45 minutes. Slides were stained with Hoechst dye (prepared by the CBI, University of Pittsburgh) for nuclear visualization. Antibody specificity was determined by replacing the primary antibody with an isotype-matched control. Cover slips were attached with Gelvatol mounting medium. Images were on an Olympus Fluoview 1000 confocal microscope (Olympus, Tokyo, Japan).

4.6.7 Quantitative image analysis

Nikon NIS elements AR 4.40 software was used to convert imaging observations in skin into measurable data, which was performed in a similar manner to that previously described (295). A region of epidermis or dermis or both was circumscribed. Thresholds for red, green, and blue fluorescence (representing staining of specific cell surface marker or cytokine, viral NS3 and nucleus, respectively) were set up corresponding to fluorescent detection in confocal microscopy. Data for each individual skin specimen were calculated from a minimum of 12 confocal images taken from 3 skin sections collected from different sites of virus-inoculated region. Means from each individual were presented as an individual data point. Data are presented from 3-4 individuals per experiment.

4.6.8 Statistical analyses

Statistical analyses were performed using GraphPad Prism, version 7 (GraphPad Software, La Jolla, CA). A paired t test was used for two-group comparisons. P values < 0.05 were considered significant.

5.0 CHAPTER FIVE: FIRST DEMONSTRATION OF ETHNIC DIFFERENCES IN SKIN IMMUNITY TO DENGUE VIRUS

5.1 PREFACE

This chapter aims to use the human skin model and microneedle array technology described previously to expand the knowledge of differences in innate skin immunity to dengue virus between Caucasian and African American populations. Data herein will be incorporated into a manuscript for future publication

5.2 ABSTRACT

Every year an estimated 100 million individuals worldwide become infected with DENV but only a small proportion develop severe disease. Ethnic background is one of the host factors believed to determine a patient's risk in developing severe dengue. African ancestry has been well reported to exert a strong protective effect on DENV infection when observed in admixed populations. However, current evidence of ethnic differences was gathered solely from empirical and genetic variation research; thereby the mechanisms of protection against DENV infection remain poorly understood. Here we studied the dynamics of DENV infection in the skin of healthy, DENV-naïve Caucasians (CA) and African American (AA) donors using quantitative in situ imaging techniques. DENV established the infection in AA skin at a significantly lower degree than that in CA skin. Low-level infection in AA led to less recruitment and infection of DENV-permissive target cells including LC and Mϕ, which subsequently promoted decreased amounts of emigrated cells out of skin. Viral infection in AA skin induced a delayed but sustained production of IFN-α between 24-48 hours, whereas the IFN-α induction in CA skin was prompt by 2 hours but then rapidly inhibited within 8 hours. Infection of epidermis stimulated the expression of IL-1β in both skin types but the level observed in AA skin was significant lower than in CA skin. Macrophages from AA skin show resistance to the effect of enhancing serum, indicating a less likelihood of antibody-dependent enhancement of DENV infection in AA skin. These data demonstrated for the first time that innate immune response restricts DENV infection in AA skin and may point to the skin as an important determinant of African-ancestry protection against severe dengue.

5.3 INTRODUCTION

Dengue is the most important mosquito-borne viral disease affecting humans worldwide; 50-100 million dengue infections are estimated to occur each year. Currently, half of the world's population is at risk of contracting DENV because the disease is endemic in at least 125 countries, mainly in the tropics (296). WHO reported that the Americas, South-East Asia and Western Pacific regions, but not Africa, are the most seriously affected (WHO). Although estimates from a recent model by Bhatt et al. have suggested that a likelihood of dengue burden in Africa (16%) equals that of the Americas (14%) (21), in fact, little has been known or reported about dengue situations in Africa, where almost the entire continent is located geographically within the tropical region. Of 54 African countries, dengue is endemic in 34 countries with multiple virus serotypes circulating simultaneously and the presence of a year-round survival of *Aedes aegypti* mosquitoes; locally acquired dengue cases have only been documented in 22 of these countries, whereas confirmed cases of dengue in the remaining 12 countries were all travel-related (8, 296). In addition to low-level local dengue transmission, dramatic outbreaks of DHF/DSS to date have not been reported in Africa. It has been suggested that the limited recognition of dengue in Africa was due to a lack of an on-site laboratory-based surveillance system, which could result in a misdiagnosis with malaria. Also, there were other hypotheses suggested to explain the low occurrence of dengue in Africa including host genetic factors, dengue transmission inefficiency, and cross protection from other flaviviruses.

The earliest observation of ethnic differences in dengue infection outcomes was a study during DENV-2 epidemics in Cuba; black Cubans were hospitalized with severe dengue states of DHF/DSS at a significantly lower frequency (a risk ratio at 1 to 5.5) than whites (19). A similar

finding was confirmed at later times by several epidemiological studies of dengue in countries of highly admixed populations such as Brazil, Colombia, Cuba, Haiti and Trinidad (89, 90, 297-299); all show that African descendant populations have a strong protective effect against severe dengue disease. A study has shown that the presence of human serum with Ab to DENV-1 did not enhance the DENV-2 infection of blood monocytes isolated from black Cubans, whereas an increased viral multiplication was exhibited in cells from white Cubans (300). This indicates that antibody-dependent enhancement of dengue infection, a phenomenon suspected to be a key driver of a person's risk of developing severe dengue, may be less likely to occur in blacks when compared to whites. These findings support the notion that African ancestry may influence disease severity. Recently, evidence from a genome-wide association (GWA) study has indicated that the parameter of innate immune cells, but not adaptive immune cells, is preferentially driven by genetic factors (301). Using the same technology, a study has identified retinoid X receptors (RXR) as an African dengue resistance gene that confers a protection against severe dengue (92). However, mechanisms of the resistance against dengue have not yet been understood. RXR function as transcription factors binding to specific sequences in the promoter of target genes and regulating signaling pathways including NF-kappa B (302). Thus, RXR are likely to regulate innate immune genes such as the human leukocyte antigen (HLA), FcγRIIa (CD32), IL-1RA and CD209. Regardless of race, the polymorphism (alleles or SNPs) of such genes have been suggested by genetic variation studies for their potential association with severe dengue (303). An in vitro study of dengue infection showed that the expression of RXR suppresses the induction of type I IFN response (304), suggesting the regulatory role RXR play in modulating innate immunity. Their regulation occurs through a LXR/RXR pathway in macrophages (305), one of major DENV-infected cell populations which long-lasting reside in large quantities in

almost all tissues and organs including skin. These findings support that the mechanisms of dengue resistance in Africans may be the result of altered early innate antiviral response.

Because the natural mode of dengue transmission to humans requires mosquitoes, skin is a primary site that plays an important role in protection from dengue-mediated pathology. In addition to offering the first-line defense to hosts, skin is also a site for disease manifestation as dengue patients are often presented with skin rash (2). Skin employs various defense mechanisms including physiology, chemicals and host immunity; all are important in determining the fate of DENV as well as disease outcomes in affected individuals. Biological differences in ethnic skin types are evident and not only limited to visible skin colors. Black skin appears to have a stronger barrier function than white skin due to its greater thickness, lipid levels and water retaining contents (306, 307). Stratum corneum from pigmented skin contains more cell layers and require a greater number of tape stripping to remove than the layer of white skin (308), suggesting epidermis of black skin has an increased intercellular cohesivity and resistance to stripping. In comparison to whites, a decreased rate of irritation or allergic contact dermatitis as well as a significantly lower percutaneous penetration of testing cosmetics were reported in black volunteers (307). These robust characteristics apparently provide black skin a great potential to withstand various physical and chemical stimuli, but are likely to be insufficient for establishing a full protection against dengue. DENV is delivered into the skin during mosquito's blood meal, a process involving piercing the skin and probing for blood. It is plausible that the virus surpasses the stratum corneum, the core protective layer of epidermis. Therefore, effective skin defense mechanisms against dengue require host immunity, particularly from cells conferring skin immune surveillance for rapid responses. Innate immunity constitutes

a large part of skin immune surveillance system and primarily contributes to early defenses against DENV (57, 134). Previously, we showed that interplay between skin-resident cells and innate immunity promotes DENV spread in Caucasian skin and presumably renders hosts susceptible to DENV infection (264). The study highlights the significant role innate immune responses play in dictating dynamics of DENV infection. Even though many clinical case observations have strongly suggested overall dengue protective outcomes in African ancestry (19, 89, 90, 298, 299), together with the relevance of identified African innate immune genes in dengue resistance (92), there have not been experimental studies that aim to understand the mechanism host immune responses use to control the DENV infection in black individuals.

In this study, we used an ex vivo model of DENV infection in human skin explants to study infection and innate immune responses of Caucasian (CA) and African American (AA) skin to dengue. We demonstrated that inoculation of DENV in AA skin results in a low degree of DENV replication, sustained type I antiviral responses, and reduced production of inflammation mediators, leading to less infection and recruitment of cell targets, which then emigrate out of the skin in low quantities. We show that AA skin is resistant to the effect of ADE of DENV infection. Our findings give an insight into the role innate immune responses plays in controlling the dynamic and spread of DENV infection in AA skin. This is the first report that shows differences of dengue infection between ethnic groups in skin.

5.4 RESULTS

5.4.1 DENV replicates at relatively lower levels in African American skin

To investigate DENV replication in African American (AA) skin, we obtained abdominal skin from healthy African American individuals undergoing plastic surgery. Using a procedure where we have established for Caucasian (CA) skin, a high-titer DENV serotype 2 strain 16681 (6.4 x106 plaque-forming units/10 µl delivered virus) was introduced into the skin by a bifurcated needle. Skin was harvested at 24 and 48 hours after inoculation and stained with antibody to DENV NS3. Inoculation of DENV into skin of African American donors resulted in productive infection in cells in epidermis and dermis (Figure 11A). NS3 expression was detected in a large number of cells in epidermis, suggesting that keratinocytes from AA skin were largely susceptible to DENV infection. Quantitative image analysis showed that replicating virus was detected in a significantly increased number of epidermal cells in CA skin at a 24-h interval, but no difference in virus-infected cell number was observed at 24 and 48 hours in AA skin (Figure 11B, left). The increase in viral replication in CA skin resulted in a significant difference between CA and AA epidermis at 48 hours; the number of infected cells in CA epidermis was shown to be approximately 3-4 times greater than that in AA epidermis. In dermis, AA skin contained a significantly lower number of NS3-expressing cells within 24-48 hours post infection than CA skin inoculated with a similar dose of DENV (Figure 11B, right). These findings suggested that DENV was able to invade and replicate in skin cells from both ethnicities, but the skin from AA appeared to support virus replication to a much smaller degree.

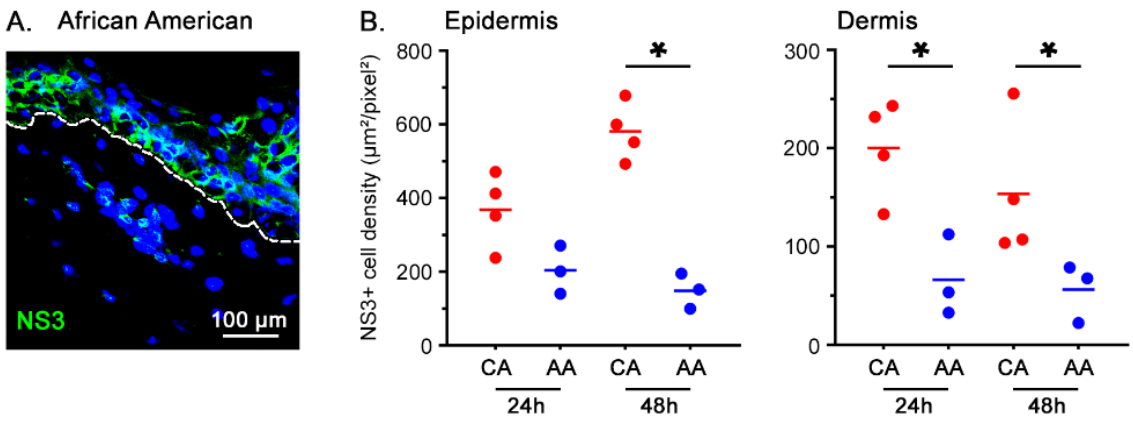

Figure 11. DENV replicates at relatively lower levels in African American skin.

(A.) NS3 (green) expression in African American skin at 48 hours after DENV-2 16681 infection. Blue staining in images represents nuclei and dotted lines indicate epidermal-dermal junction. (B.) Quantification of DENV infection in Caucasian (CA) skin (n=4) or African American (AA) skin (n=3) at 24 and 48 hours in epidermis (left) and dermis (right). Each symbol represents one individual and horizontal lines indicate means. *P < 0.05 comparing CA with AA skin, or comparing a degree of infections at different time points.

5.4.2 DENV fails to induce recruitment of myeloid target cells in African American skin

It has been shown in previous experiments using CA skin that DENV infects myeloid cells and promotes influx of these cells to be cellular targets (see chapter 2). To investigate this process in AA skin, we stained sections with antibody to NS3 and to certain myeloid cell subsets in skin, CD207/Langerin for Langerhans cells (LC) in the epidermis and CD163 for dermal macrophages (Mϕ) in the dermis. While a substantial increase in numbers of LC or dermal Mϕ was observed in the presence of virus infection, we found no significant differences in these cell populations between the skins infected with DENV as compared to mock infection, suggesting that infection with DENV in AA skin did not drive the recruitment of LC and dermal Mϕ close to the site of inoculation (Figure 12A-D, left). In addition to less recruitment, DENV infection of LC and dermal Mϕ in AA skin was significantly lower than the infection in CA skin within 48 hours; about a half of LC or dermal Mϕ from CA skin was succumb to viral replication, whereas only 30% of LC and 20% of dermal Mϕ became DENV-infected (Figure 12B&12D, right). A strong correlation between the number of LC and dermal Mϕ and their infection with DENV was observed in skin from both racial groups (data not shown), suggesting that DENV spread is dependent on an amount of target cells available in skin microenvironment reachable for the virus regardless of skin types.

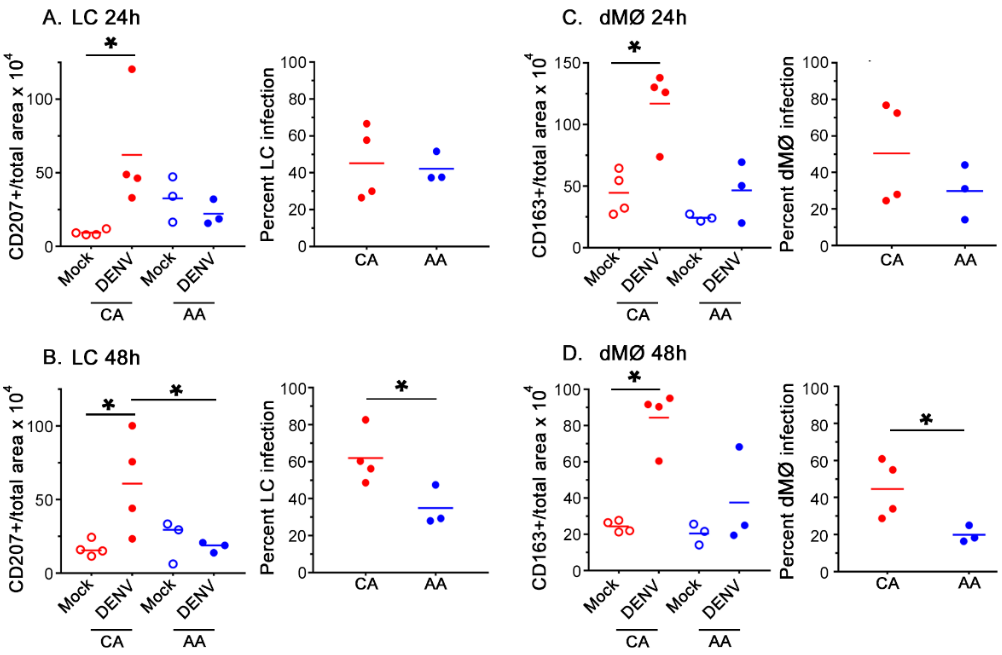

Figure 12. DENV infection promotes myeloid cell recruitment in Caucasian skin, but not in African American skin.

(A, B) Density and infection of Langerhans cells (LC) in epidermis at 24 (A.) and 48 hours (B.) in CA and AA skin. (C, D) Density and infection of dermal macrophages (Mϕ) in dermis at 24 (C.) and 48 hours (D.) in CA and AA skin. Each symbol represents a different donor and horizontal lines indicate means. *P < 0.05 comparing CA with AA skin, or comparing mock and DENV-infected skin from the same race.

5.4.3 DENV infection in AA skin triggers relatively less IL-1β production in epidermis and inefficiently facilitates emigration of cells out of AA skin.

We next sought to investigate the mechanistic basis of this low-level response to DENV infection in AA skin relative to CA skin. Our previous findings in CA skin suggest that DENV spread is mediated mainly by keratinocyte-derived IL-1β within 48 hours. To elucidate ethnic differences on DENV infection with respect to this proposed mechanism, we quantified the induction of IL-1β in epidermis between 0-48 hours after DENV inoculation in CA and AA skin. Our results showed that DENV induced the production of IL-1β in skin when compared to baseline levels in uninfected or mock infected skin; no differences in IL-1β levels were observed in different ethnic skin types from 2 to 12 hours post infection. There was a 6-fold increase in IL-1β expression of epidermal cells in infected CA skin at 24 hours post infection, and these increased levels continued for at least 48 hours. In contrast, IL-1β levels in the presence of DENV infection did not vary over time in AA skin, indicating that the virus appeared to have no impact on IL-1β-mediated inflammation in AA skin. Importantly, IL-1β expression of cells in epidermis was significantly higher (a 2-3-fold) in CA skin with DENV infection during 24 to 48 hours (Figure 13A). This was accompanied by the emigration of cells out of the skin. DENV infection caused a large number of cells, identified to be myeloid cells such as LC, dermal dendritic cells (DC), and dermal Mϕ (264), to migrate out of CA skin at 24 and 48 hours, whereas the virus as well as mock infection promoted only a low quantity of cells out of AA skin (Figure 13B). These findings clearly indicated the importance of DENV-induced IL-1β regulation in the spreading of DENV cell targets, which resulted in distinctively poor responses against DENV infection, reflecting on the low-grade inflammation and reduced cell emigration, observed in AA, but not CA skin.

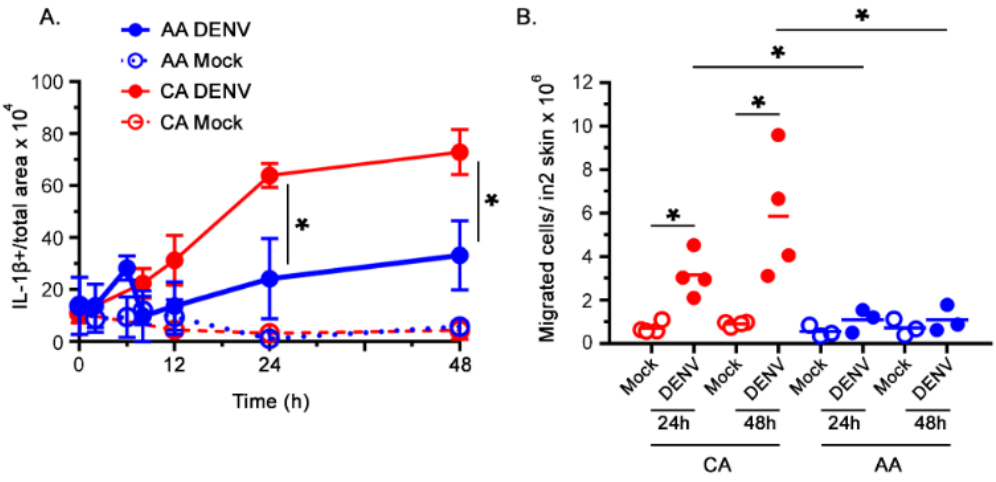

Figure 13. DENV stimulates less production of IL-1β in cells in epidermis of AA skin, and promotes inefficient levels of cell migrated out of the skin.

(A.) Expression of IL-1β in epidermis of CA and AA skin in an absence and presence of DENV infection. Data expressed as mean ± standard error of mean from four CA individuals and three AA individuals. *P < 0.05 comparing CA with AA skin with DENV infection. (B.) Total number of migrated cells collected within 24 and 48 hours in skin culture media normalized to skin area in an absence and presence of DENV infection. Each symbol represents a different donor and horizontal lines indicate means. *P < 0.05 comparing CA with AA skin with DENV infection, or comparing mock with DENV infection in the race.

5.4.4 Infection with DENV induces a striking difference of the IFN-α response between CA and AA skin

Our findings apparently show the distinct dynamics of skin response to DENV in skin from different ethnic groups in terms of degrees of productive infection, cytokine production and virus dissemination. These provide strong evidence for the involvement of a preferential immune mechanism in AA skin, which may consequently confer resistance to the effect of DENV infection among African descents. To determine the protective immune response in skin for differential observations of DENV infection, we did in situ immunofluorescence staining for IFN-α. Represent images illustrated that cells from epidermis and dermis were both responsible for the source of IFN-α in CA and AA skin (Figure 14A). There was a distinct temporal difference in the DENV-mediated induction of the IFN-α response observed in CA and AA skin. In CA skin, IFN-α expression peaked at 2-8 hours and then rapidly returned to baseline levels within 12 hours. Conversely, DENV induced a delayed response of IFN-α in AA skin, which was evident after 12 hours. An induction of IFN-α was steadily increased and sustained to 48 hours. As DENV infection had no effect on IFN-α in CA skin at 24-48 hours when the IFN-α response persisted at relatively high levels in AA skin. This resulted in a significance difference of IFN-α expression during 24 and 48 hours in CA and AA skin (Figure 14B). It has been demonstrated in previous reports that certain MHC class II alleles served as a protective allele for preventing progress to severe dengue. Using our skin model, we observed a significant increase of HLA-DR expression in cells in the dermis of AA skin infected with DENV. However, no difference in HLA-DR induction was found in cells from epidermis (Figure 14C).

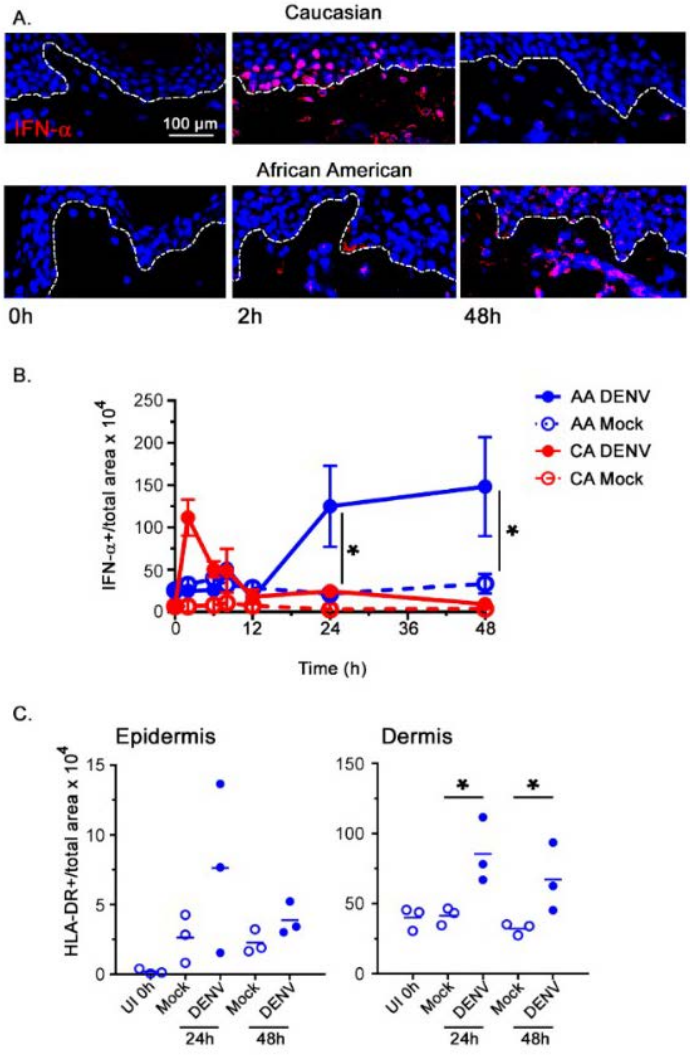

Figure 14. Differential expression of IFN-α in CA and AA skin following DENV infection and MHC class II expression in AA skin.

(A.) Expression of IFN-α at 0 hour, 2 hours and 48 hours post infection in CA (top) and AA skin (bottom). Blue staining in images represents nuclei and dotted lines indicate epidermal-dermal junction. (B.) Quantification of IFN-α-expressing cells in CA and AA skin in an absence and presence of DENV infection. Data expressed as mean ± standard error of mean from four CA individuals and three AA individuals. *P < 0.05 comparing CA and AA skin in the presence of DENV infection. (C.) Quantification of HLA-DR-expressing cells in AA skin in an absence and presence of DENV infection at 24 and 48 hours. Each symbol represents a different skin donor and horizontal lines indicate mean. *P < 0.05 comparing an area of marker expression in mock and DENV infection.

5.4.5 Evidence that AA skin may resist ADE of DENV infection

It has long been a concern that the ADE of DENV infection is a key factor in driving severe dengue outcome. The phenomenon has been well reported in many in vitro and animal studies. However, clinical observations and experimental studies in humans remain limited. To modify our human skin model for the ADE of DENV infection study, we delivered 1/40 dilution of DENV-3 immune serum to skin using dissolvable microneedle arrays (MNAs). This concentration of the immune sera was demonstrated to promote the highest enhancement levels of dermal Mϕ infection in previous experiments (data shown in chapter 3). Following 15-25 minutes after MNA application, skin was inoculated with a low-dose inoculum of DENV serotype 2 (strain 16681) at a titer of 10^3 plaque-forming units for 10 µl delivered virus. Skin was harvested at 24 hours after virus inoculation (Figure 15A). Skin sections were stained with an antibody (Ab) to DENV NS3 together with Ab to CD163 to identify and assess DENV infection of macrophages in dermis. The presence of DENV-3 immune sera in CA skin induced a significant higher number of dermal Mϕ in relative to those detected in DENV infection with human AB serum added (Figure 15B, left). Similarly, a potential of dermal Mϕ recruitment was likely to occur at a much lesser level in AA skin. Importantly, we found that the pre-existing DENV-3 immune sera substantially enhanced the infection of dermal Mϕ. The enhancement activity of DENV-3 immune sera in CA skin was exhibited by a significantly larger number of NS3-expressing dermal Mϕ and a higher percentage of infected dermal Mϕ, compared to CA skin infected with DENV in the presence of control serum. We showed that DENV infection of dermal Mϕ was increased from approximately 25% to 50%, which was a similar proportion of dermal Mϕ infection observed in skin infected with a 3-log higher titer of the same virus (264). In contrast to CA skin, no enhancement effect was observed with the infection with immune sera

delivered in AA skin. Although more dermal Mϕ were recruited, there were no apparent changes in numbers or percentage of infected dermal Mϕ when the DENV-3 immune serum was added. Only 15% of dermal Mϕ proportion were found to be infected in DENV infection with DENV-3 immune or control serum (Figure 15B, middle-right). We next investigate the influence of the enhancing Ab on emigration of cells out of skin. While an increased number of cells were driven out of CA skin with an effect of immune sera, cells from AA skin appeared to be unaffected as numbers of migrated cells were not different across skin with or without DENV infection or DENV-3 immune serum (Figure 15C). Due to a short supply of AA skin, we were able to perform ADE experiments on skin from an AA individual. Nevertheless, observations between CA and AA skin were strikingly different. Further investigations on ADE effect in AA skin are certainly needed for valid comparisons.

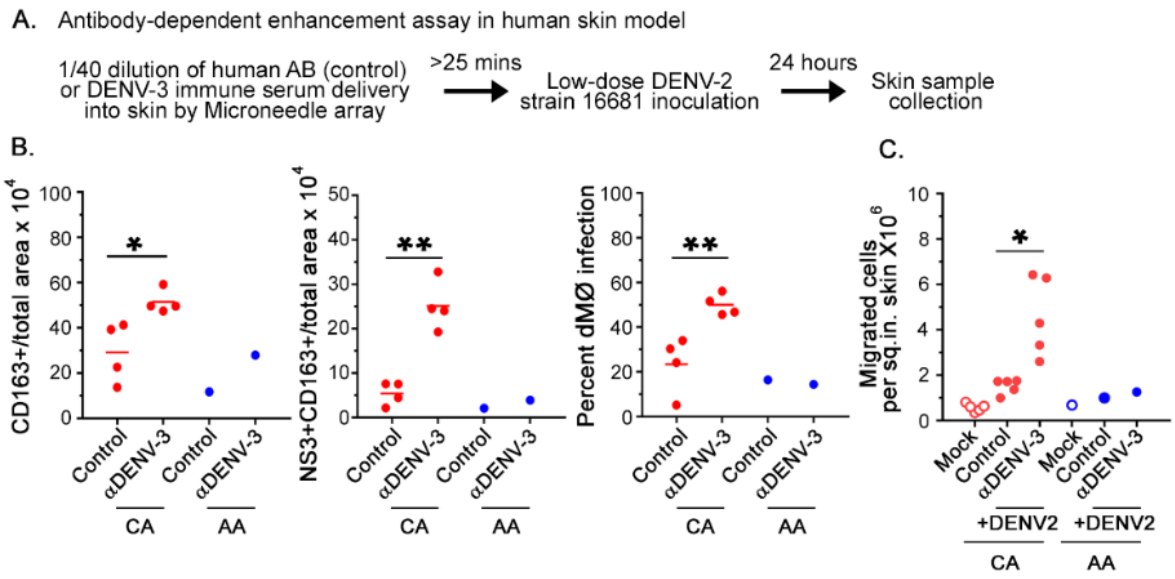

Figure 15. Primed AA skin did not exhibit characteristics of antibody-dependent enhancement of DENV infection.

(A.) A simple schematic showing fundamental steps in an antibody-dependent enhancement assay in human skin model. (B.) Density of dermal Mϕ (left), density of DENV-infected dermal Mϕ (middle), and infection of dermal Mϕ (right) in dermis at 24 hours in DENV- infected CA or AA skin in the presence of either control or DENV-3 immune serum. *P < 0.05, **P < 0.01 comparing the effect of control serum with the effect of DENV-3 immune serum added to CA skin in the presence of DENV infection. (C.) Total number of migrated cells collected within 24 hours in skin culture media normalized to skin area in an absence and presence of DENV infection, which contained either control or DENV-3 immune serum. Each symbol represents a different donor and horizontal lines indicate means. *P < 0.05 comparing the effect of control serum with the effect of DENV-3 immune serum added to CA skin in the presence of DENV infection.

5.5 DISCUSSION

Our study demonstrates for the first time that a difference in ancestry substantially influences different characteristics of DENV infection and dissemination in human skin. In AA skin, the degree of viral replication in epidermis and dermis is significantly less than that in CA skin. A low-level infection in AA skin is associated with reduced recruitment and infection of Langerhans cells (LC) in epidermis and macrophages in dermis. This is consistent to findings in murine models that show the recruitment of monocytes, closely related myeloid cells to dermal Mϕ, is of great importance to supply new cellular targets for DENV which results in an increase viral replication (168). Unlike CA skin, no increase in cell emigration out of AA skin, indicating that less dissemination of the virus and infected cells from skin to other tissues. Due to a natural mode of dengue transmission, skin is a major site for initial viral replication. An *Aedes* mosquito delivers a majority of virus extravascular, leaving more than 99% of mosquito-delivered virus in the skin (149). High viral loads in multiple visceral organs such as spleen, liver and gastrointestinal tracts have been shown to strongly associate with dengue disease severity in murine studies; immune deficient mice receiving higher dose of virus rapidly developed vascular leakage and thrombocytopenia, which is a clinical hallmark of severe dengue, and die (309). Therefore, viral dissemination of virus is a critical process in dengue pathogenesis for achieving high virus titer throughout the body. Taken together, the lower viral burden and less active responses to DENV infection in AA skin in relative to white skin suggest the benefit in dengue clinical outcomes for African ancestry. Our data strongly support the findings from epidemiological studies in countries with highly mixed ancestry populations such as Brazil, Haiti, Colombia, Trinidad and Cuba (19, 90, 297-299).

Our data show that overall DENV infection in AA skin is significant lower than CA skin. This in part can be correlated to differences in skin physical properties. Evidence show that the epidermis of black skin is notably much stronger than that of white skin due to its increased thickness, reduced resistance and percutaneous penetration (306, 307). These features possibly compromise an efficiency to deliver the virus into AA skin. However, it is not always the case that having a strong composition of dark pigmented skin would directly provide a protection against DENV infection as increasing prevalence and high incidence of dengue have been evident in Sri Lanka (310-312), where most of its population share similar ranges of skin pigment with African Americans (313). Instead, a protective effect on DENV infection is profoundly linked to the influence of African heritage. The Colombian cohort study showed that the chance of severe dengue escalated 44-fold when African ancestry was reduced from 100% to 0% (298). These findings give an explanation to a high number of infections in Sri Lanka. The people of Sri Lankans are likely to have a close genetic connection with South Indians (314, 315), whereas African Americans are predominantly the people of African descents with the 73.2% of African ancestry in the genome (316). A resistance to dengue virus in African ancestry has been suggested to be an attribute to ancestral origins of endemic yellow fever or other closely related flaviviruses throughout Africa. Yellow fever is a disease with high mortality rate (50-89%), however, several historical field surveillances observed a relatively low death rate in African descendants (317, 318); of all deaths during YF endemic in Memphis, USA prior to a YF mass vaccination era, only 7% of blacks died in contrast to 75% of whites (319). From an evolutionary standpoint, native African ancestors plausibly evolved to better respond to the special needs imposed by their YF-endemic environments. Those who able to survive are

believed to passed on racial genotypes or immunity of a kind that confer an innate protection from other flaviviruses such as DENV in subsequent generations.

Our data show a significantly lower number of IL-1β-expressing cells in epidermis of AA skin when compared to observations in CA skin. In the skin from CA donors, infected keratinocytes are the main source of many pro-inflammatory cytokines including IL-1β which largely mediates recruitment and infection of LC, dermal Mϕ and DC (264). High levels of DENV infection in Mϕ promote the release TNFα (320), which subsequently induces a cascade production of inflammatory cytokines such as IL-6, or acts in conjunction with IL-1β to drive maturation and migration of dendritic cell subsets to skin draining lymph nodes (212). These findings suggest that a decrease in recruitment and infection of LC and dermal Mϕ as well as cell emigration in AA skin were the result from the low production of IL-1β in keratinocytes. The additive effect of TNF-α and IL-1β actions has been reported to play a role in driving inflammation in several skin-related diseases such as psoriasis (321). In the context of DENV infection, these inflammation-driven mediators can be speculated to induce a cycle of increasing infection rates, which results in abundant viral loads. The critical role of TNF-α on causing endothelium damage has been demonstrated in a mouse model and its relationship with DHF and DSS has been well recognized (322-324). IL-1β recently stands out as an underrated but extremely potent mediator found at elevated levels in serum cytokine or gene expression profiles of severe dengue patients (325). IL-1β increases vascular permeability, especially when acts in concert with TNFα and IFN-γ (326). A recent mouse study of severe dengue suggested the use of antagonists for IL-1ß and caspase-1, or genetic knockout of caspase-1 and NLRP3 protect animals from developing disease (327). High serum levels of IL-1β is clinically correlated with plasma leakage in severe

dengue patients compared to dengue fever patients (328-331). These findings support that the increased level of IL-1β-derived keratinocytes generated in response to the infection in CA skin is related to the severity of dengue. The low-level production of IL-1β in AA skin is thus a host immunological process for DENV control through limiting infection rates and dissemination of target cells, which contribute to a better clinical outcome among African ancestry.

It is well reported that DENV infection effectively stimulates a production of type I IFN in infected skin cells such as keratinocytes, dendritic cells, and fibroblasts (150, 185, 186, 332). Our data reveal the similar findings that IFN-α expression is induced in cells in epidermis and dermis following DENV infection in skin from ethnic groups. However, ethnic differences display in a pattern of IFN-α expression. In CA skin, IFNα expression is remarkably induced within 2 hours but rapidly suppressed within 12 hours. As suggested by the findings of type I IFN blockade in many in vitro studies, DENV is able to expand its productive infection between 24-48 hours because of the ability to antagonize IFN-α (333). Previously, we showed that keratinocytes are accounted for approximately 60% of total infection in CA skin. In in vitro studies, DENV infection in primary human keratinocytes and fibroblasts leads to type I IFN production which lasts for 48 hours (150, 185). This is consistent with our data in AA skin; the IFN-α induction is slowed but sustained out to 48 hours. Previous studies showed that IFN treatment after the infection does not block viral replication, indicating that DENV infection bypasses the IFN actions (208). In contrast, our data suggest that a delayed but strong induction of IFN-α is associated with much lower levels of viral replication, compared to the levels without highly induced IFN-α in CA skin, suggesting an essential role IFN-α plays in keeping DENV infection under control in AA skin. The importance of type I IFN system for recapitulating dengue

outcomes of humans is well described in murine models. Immunocompetent mice do not develop dengue disease, whereas characteristics of dengue disease can display only in mice lacking several components of type I IFN system or humanized mice that are reconstituted with human hematopoietic cells (334). Mice lacking the IFN receptor only on CD11c-expressing dendritic cells and LysM-expressing macrophages succumbed completely to DENV infection (332), indicating that IFN responses in skin are crucial for the resolution of dengue. Genome wide studies suggest that suppression of type I interferon stimulating genes is associated with dengue hemorrhagic fever (331, 335). Thus, a striking difference in IFNα expression kinetics between CA and AA skin following DENV infection would affect viral replication and subsequent cellular processes which contribute to differences in clinical outcomes.

It is well described that DENV undergoes ADE in response to cross-reactive, sub neutralizing concentrations of heterologous anti-DENV Ab. A short assay window to observe the enhancement of infection was suggested in previous studies using cell lines. In our human skin model of infection, we use a low-dose DENV-2 at 103 PFU and a 1/40 dilution of anti-DENV3 serum, and can observe the enhancing activity of anti-DENV-3 serum in CA skin at 24 hours post infection, which is a same detection time in other studies using ADE sensitive cell lines such as murine macrophages P388D1 and K562 (273, 336). In CA skin, the presence of anti-DENV3 serum doubles the number of recruiting macrophages and enhances the DENV-2 infectivity in macrophages, resulting in an 8-fold increase in the quantity of infected macrophages and a 2- to 4- fold enhancement of % infected of macrophages. This enhancement results in a proportion of macrophage infected observed when a 3-log higher virus titer is used in the same model. Similar observations have been shown in primary human monocytes or

macrophages under ADE condition showed a 2- to 7-fold increase in cell infection rate associated with a 2-log increase in production of viral RNA and virions (337). It is strongly suggested that the presence of enhancing antibodies correlates with increased DENV viremia and disease severity (104, 231, 233). The activated cellular process, characterized by cell recruitment and spread out of skin, and the increased quantity of cells infected in CA skin would consequently drive the patient to develop deteriorating clinical outcomes. On the contrary, no differences in the quantity of the number of macrophages infected and the percentage of macrophages infected are detected between skin under ADE or non-ADE (direct infection) conditions. This indicates that cells in AA skin are resistant to the effect of serum enhancing activity and would contribute to a diminished severity of dengue disease in the people of African ancestry, as evidenced by epidemiologic studies (19, 90, 297-300, 316).

Though the cause of severe form of dengue has been primarily identified to be ADE, the development of severe dengue in individuals is a complex multifactorial process in which the presence of pre-existing heterologous antibodies poses an initial risk. A study showed that ADE of DENV-2 16681 infection leads to an increased IL-1β secretion in primary human monocytes (338-340). Together with an abundance of IL-1β produced in epidermis following the infection without serum effect (264), these findings support a significant higher number of macrophages or cells emigrated out of skin in response to the ADE-induced infection in CA skin. Furthermore, DENV ADE has been shown to manifest in conjunction with suppression of antiviral states in infected cells induced by type I IFN system (282). Studies show that DENV-ADE infection inhibits activation of STAT-1/2 and expression of IRF-3, which results in a decrease in production of Nitric Oxide species, potent inhibitors of DENV replication, in THP-1 cells (341).

Taken together, these results suggest the complexity of ADE infection and the involvement of host innate immune responses including IL-1β and IFNα in directing the progression and the severity of dengue. Correspondingly, differences in skin innate immunity against DENV infection shown in our data, in which IL-1β-mediated inflammation favoring DENV replication and spread in CA skin, and antiviral responses of IFN-α favoring DENV control and clearance in AA skin, contribute to ancestry-related differential clinical outcomes. However, whether ethnic differences in type I IFNs response and IL-1β production in skin play a role under influence of ADE remains to be confirmed.

Further investigations are needed to understand mechanisms underlying these differences across ethnicities that influence DENV infection and the disease outcome. To date, RXRα and OSBPL10 are the only two genes that have been recently identified as African protective genes conferring African ancestry protection against DHF (92). Although these genes share the LXR/RXR activation pathway, OSBPL10 mainly functions in a cholesterol/lipid metabolism, whereas RXRα acing as a transcriptional regulator exerts multiple effects in skin biology, particularly keratinocytes in epidermis, and the control of cytokine production in myeloid cells, mainly macrophages (302, 305). A faster control of RXRα expression has been speculated to provide protection against severe dengue in Africans, indicating a relationship of RXRα and fast-acting innate immune responses (92). Following DENV infection, murine models control optimal levels of IFN expression by down-regulation of RXRα expression in an IRF-3-dependent manner, whereas ligand activation of RXRα inhibits IFN and increases susceptibility to infection when over-expression (342). RXRα directly regulates the transcription of chemokines in macrophages such as CCL6 and CCL9 that facilitate the recruitment of leukocytes into inflamed

tissues (343). Exposure to IL-1β remarkably suppresses nuclear levels of RXRα within 30 minutes, which leads to a reduced RXRα heterodimer binding to DNA in nucleus and inhibition of RXRα functions (344). It is important to investigate the interplay between IL-1β and RXRα expression following DENV infection in skin as it has potential to be one of the key mechanisms for African ancestry protection against severe dengue. Regardless of races, RXRα heterodimers are involved with the control of factors governed by genetic polymorphism effects such as vitamin D receptors or MHC molecules (345). Certain variations in these genes may protect or predispose individuals to severe dengue (346).

5.6 MATERIALS AND METHODS

5.6.1 Dengue virus

A prototypical strain of dengue virus serotype 2 (16681) was provided by Jared Evans at the University of Pittsburgh. DENV was cultured and propagated in C6/36 insect cells (ATCC, Manassas, VA). C6/36 cells were routinely grown in Dulbecco's modified eagle medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% of the following: penicillin/streptomycin, L-glutamine and sodium pyruvate solution. Tissue culture supernatant was pooled at day 5, 10, and 15 of culture (when cytopathic effect was evident), clarified of cell debris by centrifugation, and concentrated using standard methods. Virus titers were determined by a modified focus forming unit immunoperoxidase assays using Vero cells as described previously (225).

5.6.2 Skin processing and virus inoculation

Large blocks of anonymized full-thickness skin that were being discarded following elective aesthetic abdominoplasty or mammoplasty at the University of Pittsburgh were used. Ethnicity of donors was evaluated by skin colors and confirmed by a coordinator. Identifiable private information regarding skin donors was not provided and no interaction or intervention with donors was possible. As such, the research did not constitute human subject research as per the University of Pittsburgh Institutional Review Board. Any residual adipose tissue was trimmed from the underside of the skin explant before virus inoculation. A volume of 50 µl of media containing 3.2×10^7 or 5×10^3 plaque-forming units purified DENV 16681 was placed in a 4 square-inch that was demarcated in the center of the surface of the skin explant. Bifurcated allergy skin testing needles (Precision Medical Product Inc., Denver, PA) were used to repeated puncture the skin surface through the 50-µl virus inoculum and deliver virus into the epidermis and dermis; our preliminary experiments indicated that this inoculation method delivered approximately 10 µl of virus suspension into the skin. Inoculated explants were incubated at 37˚C for 2 hours, then the skin surface was washed with phosphate buffer saline and wiped with sterile gauze pads to remove a remaining virus. Tissue was placed dermis-side down onto mesh grids in 60 x 15 mm culture dishes and were continued incubating at the liquid-air interface in RPMI 1640 complete media. Explants were collected at 24 and 48 hours after virus inoculation and the virus-delivered central region was isolated, immediately submerged in 30% sucrose overnight at 4˚C, and then frozen for subsequent immunohistochemistry. At same intervals, media were collected to harvest migratory cells, which were treated with DNase, counted and frozen for further flow cytometric analysis.

5.6.3 Antibody-dependent enhancement assay

Seven sera from a study by Castanha et al. (255) were aliquoted, pooled and used in our study as DENV-3 immune serum. These samples were collected from DENV immune pregnant women and identified by a PRNT50 assay to have DENV-3 monotypic immunity, Ab titer ranging between 1:380-1320 (the mean Ab titer is 1:786), with low serotype-specific neutralizing Ab response to DENV-1, -2, and -4 (Ab titer < 20). Pooled DENV-3 immune serum was diluted by 1:40 with phosphate buffer saline and then formulated into tip-loaded dissolvable 3:2 carboxymethyl cellulose/trehalose MNA as described previously (206). Control MNAs contain 1/40 dilution of human AB serum (Fisher Scientific, USA). For antibody-dependent enhancement, MNAs were manually applied to skin explants for at least 15 minutes until needle tips dissolved in the skin, and then were removed. Virus inoculation was performed immediately at the region of MNA application on the skin surface. A low-dose inoculum of DENV was used at 1 x106 plaque-forming units per 10 µl delivered virus. Tissue culture was performed as described above. Skin was collected at 24 hours after virus inoculation and the MNA-applied region was isolated, submerged in 30% sucrose overnight at 4˚C, and then frozen for subsequent immunohistochemistry. Media were collected to harvest migratory cells, which were treated with DNase, counted and frozen for further flow cytometric analysis.

5.6.4 Immunohistochemistry

Six micron frozen skin sections on microscope slides were rehydrated with phosphate buffer saline and fixed in cold acetone for 5 minutes at 4˚C. Slides were stained with polyclonal rabbit anti-pan DENV NS3 Ab (provided by Sujan Shresta, La Jolla Institute for Allergy and

Immunology) and monoclonal mouse anti-human Ab directed against specific cell surface or cytokine markers: anti-Langerin/CD207 (DCGM4; Beckman Coulter), anti-CD163 (5C6FAT; Acris Antibodies GmbH), IL-1ß (6E10; Novus Biologics) and IFN-α (MMHA-2; PBL Assay Science, Piscataway Township, NJ), overnight at 4˚C, then incubated with relevant secondary Ab for 30-45 minutes. Slides were stained with Hoechst dye (prepared by CBI, the University of Pittsburgh) for nuclear visualization. Antibody specificity was determined by replacing the primary antibody with an isotype-matched control. Cover slips were attached with Gelvatol mounting medium. Images were on an Olympus Fluoview 1000 confocal microscope (Olympus, Tokyo, Japan).

5.6.5 Quantitative image analysis

Nikon NIS elements AR 4.40 software was used to convert imaging observations in skin into measurable data, which was performed in a similar manner to that previously described (295). A region of epidermis or dermis or both was circumscribed. Thresholds for red, green, and blue fluorescence (representing staining of specific cell surface marker or cytokine, viral NS3 and nucleus, respectively) were set up corresponding to fluorescent detection in confocal microscopy. Data for each individual skin specimen were calculated from a minimum of 12 confocal images taken from 3 skin sections collected from different sites of virus-inoculated region. Means from each individual were presented as an individual data point. Data are presented from 5-7 Caucasian and African American individuals per experiment.

5.6.6 Statistical analyses

Statistical analyses were performed using GraphPad Prism, version 7 (GraphPad Software, La Jolla, CA). A paired t test or Mann-Whitney U test was used for two-group comparisons. P values < 0.05 were considered significant.

6.0 CHAPTER SIX: OVERALL DISCUSSION

6.1 AN EX VIVO HUMAN SKIN MODEL OF DENV INFECTION

An infected *Aedes aegypti* mosquito transmits DENV to humans as it probes and feeds on blood under the skin. Although skin blood vessels are target sites for the mosquito, only a small amount of virus is directly delivered into the blood and more than 99% of the virus is deposited in the skin, based on studies with WNV (149). The skin is a specialized microenvironment that contains different cell types working together dynamically to protect the host. Because many skin cells are targets of DENV infection, it is hypothesized that the initial replication of the virus in skin leads to subsequent migration of infected cells out to draining lymph nodes. This results in bloodstream dissemination. Evidence in support of this hypothesis includes the presence of DENV in skin and lymph nodes of infected monkeys, in human cadaveric skin explants, and in skin biopsies from skin rashes in individuals receiving a live attenuated experimental dengue vaccine (160, 205, 347). Autopsy reports of children who have died after DHF have described a high expression of non-structural DENV antigens in dendritic-appearing macrophages, a prominent morphology of dermal Mϕ in skin, within lymph nodes and spleen (348). Skin is not only a primary site for DENV transmission but also a site for dengue pathology. Skin manifestations include a maculopapular rash that occurs in a large population of dengue patients (50-82%), and a hemorrhagic petechiae which is commonly seen in patients with severe dengue

but more rarely in patients with dengue fever (73). The complete comprehension of DENV pathogenesis requires a better understanding of the dynamics of an early event of DENV infection in human skin.

To date, much understanding of DENV biology regarding infection of skin cells is shaped through the use of permissive cell lines in vitro or genetically modified animal models. Studies using primary cells isolated from human skin explants require disassembling the tissue to obtain comprehensive analyses, with methods consisting of subjecting isolated skin cells to enzymatic digestion. Thus, this raises questions about the biological relevance of these models to the in vivo dynamics of early infection. We developed an ex vivo human skin model of DENV infection using microneedle arrays (MNA)-based delivery to control and manipulate infection processes in skin, coupled with digital imaging analyses for in situ quantification. The goal was to obtain high-resolution observations on viral infection, while maintaining the skin microenvironment to preserve skin biological complexity, connectivity of cells and crucial contextual responses. The resulting technology enables visualization and robust measurement of the degree and dynamics of DENV infection. It also allows for insight into DENV-induced immune responses as well as a characteristic ADE phenomenon in human skin.

The natural mammalian hosts for DENV are humans and nonhuman primates. Exposing intact human skin to the virus provides the closest laboratory model attainable to the in vivo environment where the host's natural defense takes place. Typically, the host immune response towards infectious agents is a key element of infection biology. In the case of DENV, the innate immunity, particularly antiviral type I IFN, impedes the development of immune competent

murine models of DENV infection (349). DENV counteracts STAT2, a requisite factor involving in type I IFN signaling, by degradation that consequently allows the virus to establish infection efficiently. However, the ability of DENV to mediate STAT2 degradation is species specific due to distinct N-terminal STAT2 sequences. DENV NS5 binds and degrades STAT2 in humans, but not in mice, resulting in the higher sensitivity of DENV to antiviral activity of IFN in murine cells than in human cells (350). Mice are not permissive to DENV infection, in particular with human clinical isolates, unless they have deficiencies in the IFN system. Use of mouse strains lacking IFN receptors such as the AG129 mouse strain renders a complete susceptibility to DENV infection (351). Without modifications of the model, our study shows that IFNα is a critical determinant of the infection outcome. As a result, a degree of productive DENV infection is observed with a reverse correlation with expression of IFNα. Furthermore, expression of IFNα in Caucasian skin is detected as early as 2 hours, prior to the initial viral replication at 6 hours, indicating that counteracting mechanisms rapidly occur in the skin. Differentially regulated type I IFN responses can influence contextual differences that the virus primarily encounters and thus determine outcome of infections. Lack of an intact IFN system in mice may profoundly alter host responses to infection with DENV, resulting in a poor predictive power of murine models. In addition to the IFN system, physiological differences in FcγR expression between human and mouse complicate interpretation of murine studies. Our data indicate that both FcγRI and FcγRII facilitate antibody-dependent enhancement of DENV infection. While human macrophages express all types of FcγR, mice do not express FcγRII (245, 352). Although it has been suggested that mouse FcγRIII is an orthologous receptor for human FcγRIIa, they may not retain identical functions, as the mouse FcγRIII does not contain its own ITAM motif (353). These are critical

differences between mouse and humans that suggest limitations of translating ADE research from mice to humans.

Not only would the use of intact human skin tissues lead to a better translation into DENV behaviors in natural host settings than that of in vitro or animal models, but it also allows us to look at differences in susceptibility to DENV infection across ethnic groups. These differences have never been experimentally investigated beyond the genetic level before (354). All skin specimens were speculated to be dengue naïve because the sample procurement was exclusively from heathy individuals undergoing surgeries in Pittsburgh, Pennsylvania, a region with no reports of locally-acquired dengue cases nor active distributions of the vector (64, 355). Therefore, no serological tests were performed to screen the immune status of samples obtained. A small sample size dictated by the limited access to patient specimens, particularly from African Americans, presents one of the limitations in our study.

One of the challenges in the investigation of dengue tropism is the identification of DENV replication. Entry of DENV into host cells occurs through receptor mediated-endocytosis or FcγR-mediated internalization in the presence of heterotypic Ab (356). However, following an introduction of the virus, non-infectious forms of DENV such as degraded virions, dispersed viral proteins, or non-engulfed immune complexes, or the remnant virus may also present with infectious virions on skin structures or cell surfaces despite no involvement of an active infection. Previously, the main method of DENV detection has been focused on the use of antibodies to DENV structural proteins such as envelope or pre-membrane proteins (160, 164, 357), which may confound findings on host cells or tissues that actively support the viral

replication. For this study, our technique to identify DENV infected cells includes immunohistochemistry by using a panel of specific Ab against cell-identity markers together with an Ab to pan DENV-NS3 antigen. The detection of NS3 expression defines the actively synthesized DENV inside the target cells which is truly an indicative of a site of DENV replication. This system overcomes challenges in addressing a definite site of DENV replication in cells as well as tissues in previous dengue works. Furthermore, the similarities of amino acid sequences between DENV and ZIKV provides advantages in using to developed system to detect ZIKV replication in skin cells (271). Our findings confirm the previous hypothesis that skin is the primary site of DENV and ZIKV replication.

During mosquito probing, local tissues in the epidermis and the dermis are being physically damaged while mandibles and maxillae, parts of the proboscis, are sawing through the skin. It may be possible that the piercing primarily damages the dermis because of blood vessel locations, whereas needle stabs are likely to cause more damage to the epidermis. Bifurcated needles can pick up a 1-2 μl drop of the virus suspension between their two prongs. This ensures a consistent administration of the virus to skin when multiple punctures are applied on an area of 1x1 square inches, and thus reduces variations in imaging observations on a frozen tissue sample. It is unclear whether a mosquito deposits the virus into the epidermis, the dermis or both. Although we did not inoculate a dye or fluorescent-conjugated beads to monitor the penetration depth at the inoculation site, a bifurcated needle is expected to deliver most of the virus into the papillary (upper) dermis due to the length of two prongs (1-3 mm). The amount of the virus inoculum being delivered into the full-thickness skin is approximately 12.5 μl out of the total 50 μl, which has been determined by subtracting it from the gross skin weight before and after the

inoculation. Though the delivery efficiency is only 25%, the virus delivery dose is controlled to support the purpose of experiments. For example, a low-dose virus (10^3 PFU /10 μl) was used to investigate the effect of mosquito-related factors or patients' immune serum, according to the fact that mosquitoes inoculate 1 X 10^3- 1x 10^5 infectious particles of WNV per bite (149, 263).

It is important to note that data collection and image analysis are confined to a specific area covering the epidermis, the epidermis-dermis junction, and the 500-micron depth of the dermis (the upper third of the reticular dermis). While this region does not include the whole depth of dermis, it is justifiable considering a number of reasons. First, the virus on the needle is expected to reach the upper dermis, indicating the likelihood of an establishment of infection in such areas or above. Second, our research deliberately focuses on early events following virus inoculation such as the first target of DENV infection and corresponding host responses. Lastly, skin explant samples, solely consisting of epidermis and dermis, lack additional cells infiltrating from blood supply, resulting in a constant or decreased number of cells observed in total. Any cellular dynamics driven by the virus and/or other procedures involving changes in cell density must be measured within a specific skin region. Our results suggest that dynamics in the tissue samples occur near the delivery site where the driving force (virus, SGE, Ab, or serum) are most concentrated, in relation to the whole system.

It is critical to recognize that the deposition of the virus by a bifurcated needle to skin might bypass significant interactions between the host and the mosquito, or the impact of mosquito-related factors on skin environment. Studies have shown that mosquito bites worsened disease outcomes in mice infected with the pathogens such as malaria, WNV, Chikungunya virus and

DENV, when compared to needle infections (263, 358, 359). The exact mechanisms of mosquito bite-driven increases in disease severity remain unknown, but current findings suggest the involvement of polarization toward Th2 responses and subversion of IFN-γ stimulated antiviral mechanisms (360). This suggests that DENV under the influence of the mosquito would encounter a permissive skin setting for viral infection, rather than the antiviral (Th1 response) environment induced by a needle inoculation alone. To replicate these mosquito-related effects for the investigation, it is clear that the bite of an intact, infectious mosquito provides the closest natural transmission in laboratory settings as it incorporates physical damage (due to bites, blood probing, or other unknown blood-feeding biomechanics), the release of saliva and its components, and the virus. However, this approach leads to difficulties in calculating the absolute quantity of the viral inoculum delivered by the vector. Also, the technique requires a local mosquito research facility to maintain and supply infectious mosquitoes in their foraging period. Alternatively, studies using a related WNV have shown that SGE or mosquito saliva enhanced viremia of a mouse model in a dose-dependent manner, and their effects were localized and potent as a single mosquito bite (equivalent to 0.01 μg of SGE) was sufficient for enhancing activity (361). These factors make attractive alternatives as they offer a more convenient tool for implementing in our skin model, more quantitative control over the viral inoculum as well as the amount and concentration of whole saliva or SGE, and an ability to analyze and compare data with previous findings as the method of inoculation remains the same . In our study, we were unable to demonstrate an effect of SGE when co-inoculated with DENV. However, the finding remains preliminary due to several potential limitations. We did not incorporate collected mosquito saliva or salivary proteins of known functions to the system. Among factors released from an intact mosquito, saliva represents the closest natural route of exposure as it is subjected

to less process of collection as well as less possibility of bioactive molecules to degrade or modify when compared to SGE or proteins. Co-incubation of DENV with SGE and saliva would thus lead to more specificity of immune response modulation (362). Furthermore, purified salivary protein, or a mixture of proteins, shown to enhance dengue disease severity should be added as a positive control along with saliva/SGE to validate whether the effects of mosquito-related factors are detectable in our model.

6.2 KERATINOCYTES: THE PARADIGM SHIFT OF EARLY DENV INFECTION IN SKIN

Although it is well known that DENV enters the body through the skin, the study of the earliest stage of DENV infection in this organ has been largely unexplored in humans. This is mainly because the onset of dengue symptoms starts with fever within 4-7 days after a bite of an infected mosquito. At this phase, the virus is no longer detectable or is in a decline in the blood and presumably in the skin (23). Accordingly, the study on samples collected from hospitalized patients will likely preclude initial interactions between the virus and host cells in situ. Based on limited data from human skin tissue staining, DENV infection initiates at the site of inoculation in skin LC, Mϕ and DC (160, 205, 363). Among these myeloid cells, epidermal LC have been indicated as the first target cells of DENV infection based on their infection in a skin biopsy obtained from the recipient of a live attenuated tetravalent DENV vaccine (160). Thus, it remains unclear whether LC infection occurs during natural DENV infection. The selectivity of DENV in these myeloid cells has been supported by loss of virus spread in immune compromised mice when infection in hematopoietic cell populations was restricted (253). Furthermore, intracellular

DENV RNA copies detected in patients' PBMCs during the acute phase of infection have suggested the spread of actively replicating virus to the blood by immune cells following infection in the skin (364). Because T and B cells are less permissive to DENV infection than monocytes when examined together, mononuclear phagocytes are thought to be the principal cell types supporting DENV replication in vivo (252, 365). While it is unknown precisely how the infection in skin causes systemic infection, it is largely believed that the migratory myeloid cells spread virus to regional lymph nodes as well as other organs via the lymphatic and circulatory systems.

DENV has been shown to utilize multiple attachment factors and receptors to enter host cells, including heparan sulfate (356). Heparan sulfate, which are highly sulfated glycosaminoglycan molecules (GAGs), are ubiquitously expressed on the surface of many cell types. These molecules render a diverse range of cells permissive to DENV entry by mediating an electrostatic attraction between a dengue E glycoprotein and the negatively charged carbohydrate moieties present in GAGs (53, 54). Factors mediating more specific cell entry for DENV has been identified in DC and Mϕ. These cells bear putative DENV receptors including DC-SIGN, mannose receptor and CLEC5A (164, 175, 289, 366). DC-SIGN is the calcium-dependent lectin that are expressed on DC and Mϕ. While the receptor facilitates DENV infection in DC, Mϕ expressing DC-SIGN have been shown to be resistant to the infection (367). Whether DC-SIGN is the only receptor responsible for DENV entry or replication in DC is largely unknown. Infection of human Mϕ occurs through other lectins, including mannose receptor and CLEC5A. Besides DENV entry, CLEC5A participates in the release of IL-1β (179, 368), one of pro-

inflammatory mediators important in the pathogenesis of severe forms of dengue, indicating the importance of Mϕ in dengue disease progression.

Although cells of hematopoietic origins are shown to be a requirement for DENV replication (253), non-hematopoietic cell types including endothelial cells, fibroblasts, and epithelial cells have been shown during the natural infection (in an absence of enhancing antibodies) (150, 185, 369, 370). However, infection of endothelial cells detected in mice and humans remain questionable because autopsy reports have described undetectable levels of viral RNA in endothelial cells in response to natural infection of DENV (363). In vitro DENV is able to infect a number of epithelial cell lines and epithelial cells from tissues including human lung epithelium or primary human keratinocytes (150, 371). While keratinocytes have been identified in animal and human skin models as cell targets for other mosquito-borne viruses such as ZIKV and WNV, DENV infection of keratinocytes has only been described in vitro studies (150, 372, 373). The detection of DENV-infected cells in the basal layer of epidermis of human skin explants cultured for 5 days have been described as rare events (205). Based on the location and morphology of infected cells, the authors speculated that keratinocytes were infected and underwent apoptosis following DENV inoculation. Overall, DENV infection of keratinocytes has been perceived in the past as making a relatively small contribution to dengue pathogenesis.

While the current hypothesis has suggested that myeloid cells such as monocytes/Mϕ and DC are implicated as predominant reservoirs for DENV replication (165, 374), it remains unclear which cell populations primarily permit DENV replication in vivo and are responsible for virus spread. This is largely because previous research has focused on investigating each cell target of DENV

separately. Though these prior findings are very useful in establishing the basis of DENV biology, significant knowledge gaps remain. Because skin contains the majority of cell populations known to support DENV replication (177), we have used a quantitative in situ imaging approach to examine the relative importance of cell targets in DENV infection in intact human skin. We found that DENV infection in seven distinct skin cell populations, including keratinocytes, LC, dermal DC, Mϕ, fibroblasts, mast cells and lymphatic endothelial cells, which account for the majority of infected cells in human skin (75-85% of total infection). Nevertheless, infection of keratinocytes alone made up 60% of all infected cells. Keratinocytes not only principally support DENV infection in the skin, they are also the first cell type infected by the virus. Our findings highlight the dual role of keratinocytes as the portal of entry and the main supporter for DENV infection in humans. Furthermore, the infection of keratinocytes has been notably observed in all conditions tested in the skin (across the ethnicity, the absence or presence of enhancing serum, and the use of a low or high titer of the virus), indicating the central role these cells play in DENV infection.

The delivery of virus by the mosquito probing for blood vessels or by the bifurcated needle clearly reaches to the upper dermis, which may account for why infection of keratinocytes occurs primarily in the suprabasal and basal layer of epidermis. These basal cells move down and form ridges along with the dermal papillae valleys containing capillaries, end arterioles and veins that nourish the epidermis via diffusion. The homeostasis of continuously renewing basal cells of epidermis is likely to assist the virus replication (375). Keratinocytes have not been reported to be infected with DENV in murine models, although keratinocytes are primary targets of infection with the related WNV in mice (207). It is possible that differences in skin properties between

human and mice may impact the extent of keratinocyte infection in intact skin between the two species. Despite the similar structural organization, the epidermis of rodent skin consists of only a total of two to three layers with turnover rate at every 8-10 days, indicating a much thinner skin with a 4-times faster differentiation process than that of the human epidermis (376-378). The human dermis is highly vascularized with both a rich papillary network and a deep dermal network, whereas rodent dermis is poorly vascularized and ridge formation at epidermis-dermis junctions is present only in footpads. Although the impact of skin properties on DENV infection has never been explored, these morphological differences between mouse and human skin are worthwhile noting when interpreting murine studies of DENV infection.

Keratinocytes are equipped with a diverse array of innate pathogen recognition receptors and defense mechanisms including induction of inflammatory responses (128). In our study, DENV infection significantly modulated the expression of keratinocyte-produced cytokines including IL-1, CCL20 and CXCL8, known to be involved in the pathogenesis of inflammatory skin-related diseases, including psoriasis. Some of these keratinocyte-derived proteins have been suggested to be predictors for severe dengue due to their increased presence in blood of patients (328). However, because of limited pathological data on tissues, these pro-inflammatory cytokines and chemokines have never been linked to the initiator role of keratinocytes in the skin. Our study for the first time highlights the importance of keratinocytes both in establishing DENV infection and in recruiting virus-permissive myeloid cells into inflamed skin, as well as driving them out of the skin. Altogether, our in-situ findings have shifted the paradigm of the earliest events in DENV infection to include a fundamental role of the keratinocytes.

6.3 IL-1B AS A KEY MEDIATOR IN DENV INFECTION AND SPREAD

Among keratinocyte-induced cytokines during DENV infection, IL-1β alone exerts an effect on the recruitment and infection of all three myeloid cell subsets studies, LC, DC, and Mϕ. IL-1β was as strong as all potential mediators combined (IL-1α, IL-1β, CCL20 and CXCL8). This suggests that IL-1β is a key mediator in DENV infection. The recruitment of myeloid cells to the superficial dermis is an important characteristic of DENV infection as it brings in additional target cells to the site of virus inoculation. Studies in mice lacking the IFN-α/β receptor have suggested that DENV infection in dermis occurs in two phases: initial infection of resident DC and Mϕ, followed by infection of CCR2-expressing monocytes recruited from the blood within 48 hours of viral inoculation. Some of these monocytes differentiate into monocyte-derived DC, providing additional cellular targets for the virus (168). Our data show that the infection of IL-1β-recruited LC, DC and Mϕ occurs within 12-24 hours of infection, consistent with the interval of the first infection phase reported in mice. Although an absence of the influx of blood cells in human skin explants make it impractical to investigate whether DENV-induced IL-1β activity recruits monocytes, previous studies have shown that IL-1α/β expression promotes the accumulation of monocytes at the inflamed site. Furthermore, it has been shown that mosquito saliva-induced IL-1β expression resulted in the early recruitment of neutrophils, which can promote monocyte recruitment (218, 358). Biopsies obtained from skin rashes of patients with severe dengue showed that infected Mϕ were more frequent around the blood vessel wall in dermal papillae (379), indicating the recruitment skin-resident Mϕ and the infiltration of monocytes, which later can differentiate into dermal Mϕ, into the site of virus inoculation. Disrupting IL-1β significantly reduced the infection in epidermis and dermis. These findings together highlight the previously unidentified role of IL-1β in mediating the spread of DENV in

skin through the recruitment of myeloid cells from deeper layers of the dermis, and presumably from the blood. The substantial increase in number of newly virus-permissive myeloid cells at the site of virus introduction further supports and amplifies DENV replication, resulting in higher viral loads in the skin.

Because many cell types that support DENV replication including LC and Mϕ can produce IL-1β (380, 381), it is possible that keratinocytes are not the exclusive source of IL-1β throughout the course of infection. Following DENV infection in our explant model, the expression of IL-1β was mainly in the epidermis. While the triple labeling of IL-1β with AE-1 and NS3 staining strongly indicated that infected keratinocytes were the major IL-1β producer, it is unclear whether LC were also involved in the IL-1β production. Nevertheless, the release of IL-1β at the earliest stage of DENV infection in the skin is mainly from infected keratinocytes, given their large quantity in the epidermis and their role as the first responder to the virus. In dermis, the production of IL-1β has been well described in monocytes and Mϕ (382), which are also known to respond to IL-1β, creating a positive feedforward loop. IL-1β induces monocytes to differentiate into DC-SIGN-expressing Mϕ (383), and these cells are known to resist to DENV infection, at least in vitro (367), suggesting that monocytes recruited to the skin are less likely to become infected and release IL-1β. Elevated levels of IL-1β in primary monocytes have been demonstrated during ADE of DENV infection (339). In our model, Mϕ-expression of IL-1β may occur in the presence of immune serum. Compared to DENV infection without immune serum, the increased infection of Mϕ in the presence of enhancing serum suggests that these infected Mϕ may upregulate IL-1β as well as other unidentified mediators in response to ADE-induced infection. Taken together, our results suggest that IL-1β expression following primary DENV

infection is mainly from keratinocytes, whereas IL-1β or other factors released from highly activated Mϕ in ADE of infection is likely to magnify the actions of DENV-infected keratinocytes-derived IL-1β.

The abundant release of biologically active IL-1β from DENV-infected keratinocytes indicates that these cells may undergo programmed cell death processes such as apoptosis and pyroptosis (384). Both types of cell death have been observed after DENV infection in previous studies (95, 385). Unlike IL-1α, the cytokine IL-1β is an inducible protein that requires proteolytic cleavage to become active (382). Although it is not clear how IL-1β is secreted from cells , it is well described that the activation and secretion of IL-1β by UVB (ultraviolet B)-irradiated human keratinocytes require caspase-1 activity (386), which is activated by incorporation into the NLRP3 inflammasome complex (387). Caspase 1 is a key driver of pyroptosis, and it is therefore likely that caspase-1-driven IL-1β secretion by keratinocytes following DENV infection leads to pyroptosis. This notion is supported by a recent study of ZIKV using patient sera and mice, which has shown that the virus infection promoted the assembly of NLRP3 inflammasome and the secretion of IL-1β (388). Correspondingly, the presence of increased IL-1β levels has been reported in cytokine profiles of patients infected with ZIKV during acute and recovery phases compared to levels observed in healthy individuals (389). This is consistent with significantly elevated serum levels of IL-1β observed in dengue patients during acute to defervescence phases or patients present with severe dengue (328, 390).

The mechanism of pyroptosis involving pore formation at the plasma membrane causes rapid loss of plasma membrane integrity and may lead to cell lysis (391). These pyroptosis pores allow

the release of active IL-1β from the cytosol (392). Following DENV infection, the IL-1β–mediated recruitment of myeloid cells would occur through IL-1β acting on IL-1 receptors type 1 (IL-1R1) present on the cell to initiate cell movement toward cytokine gradients to the upper dermis. However, it is possible that the virus-mediated pyroptosis facilitates the release of other intracellular contents such as pro-inflammatory mediators or senescent cells and debris. This would attract more myeloid cells with phagocytic activity to the site of infection, and further promote efficient DENV replication and sustain abundant viral loads in the skin prior to the systemic spread. High levels of apoptosis have been reported in DC and hepatocytes that were infected in vitro with DENV (393, 394). However, apoptosis has generally been defined by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay (205), but this assay does not accurately discriminate between apoptosis and pyroptosis. The importance of NLRP3 inflammasome and IL-1β in DENV infection encourages further studies on cell death in response to the infection.

The counter-regulation of IL-1 and type I IFN-mediates inflammatory responses have been described in autoimmune diseases and infections with various types of pathogen including viruses (395, 396). Although our study did not demonstrate the direct cross talk between two system responses, the IL-1β production has been found to inversely relate to the IFN-α induction in the epidermis in the course of DENV infection. The potential counteracting mechanism was further confirmed by significant differences of DENV replication in two ethnic groups; the relatively low DENV replication observed in African American (AA) skin has been observed in conjunction with the lessened IL-1β production and the sustained expression of IFN-α. Interestingly, not only AA skin was maintained, but were also increased to the higher levels than

the maximal IFNα induced in Caucasian American (CA) skin. This indicates the protective effect of African ancestry on DENV infection, as consistent to many previous studies that have established a beneficial effect for type I IFN signaling in response to DENV infection including the inhibition of IL-1 production. In addition to the role of recruiting cells to inflamed tissues, IL-1β is a highly potent pro-inflammatory mediator that induces vasodilation (397). Failure of type I IFN induction in controlling the magnitude and function of IL-1β during DENV infection will likely increase the severity of dengue through vascular leakage. Although DENV has developed many strategies to evade type I IFN signaling (136), such mechanisms with the involvement of IL-1β have yet to be explored. Altogether, our study suggests that the IL-1β plays a key role in driving dynamics of virus infection in human skin through the recruitment of myeloid cell targets, which sustains and spreads the virus. The existence of a complex interplay between IL-1β and type I IFN responses determine the regulation of the response to DENV infection, whether a skin environment that DENV will encounter favors early viral replication (IL-1β influence) or contributes to anti-viral responses (IFN-α influence).

6.4 PROPOSED MECHANISMS OF DENV SPREAD IN HUMAN SKIN

Our findings in an ex vivo human skin model of DENV infection reveal for the first time, that DENV-infected keratinocytes drive virus spread by orchestrating the release of pro-inflammatory chemokines and cytokines, most critically IL-1β, that attract virus-permissive myeloid cells which subsequently disseminate virus. The protective effect on DENV infection in African ancestry has supported counteracting interactions between IL-1β and type I IFN responses,

represented by IFN-α in our study. Overall, our findings in intact human skin has led to the new mechanism of early DENV infection illustrated in figure 16.

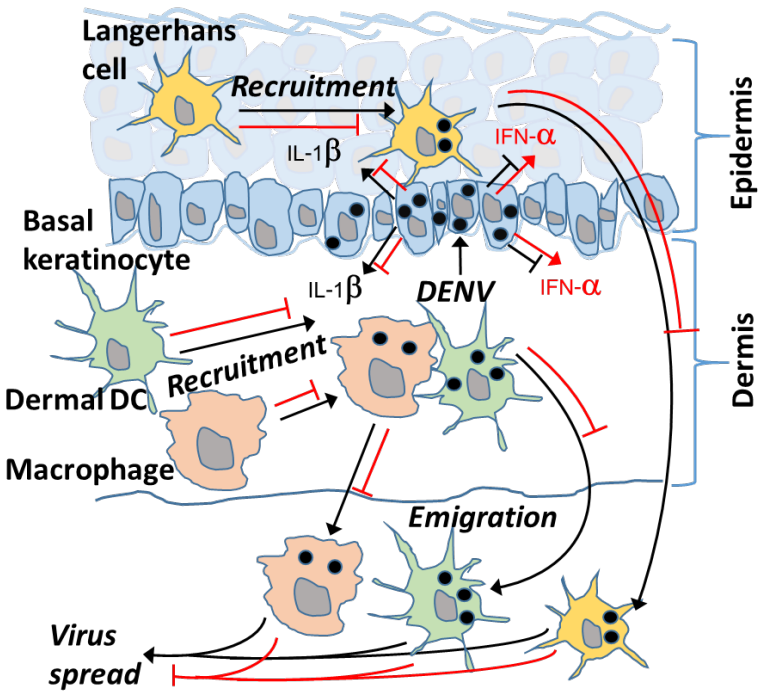

Figure 16. The proposed mechanism of DENV infection in human skin from two ethnic groups.

DENV infection in Caucasian skin induces inflammatory responses by infected keratinocytes and IFN blockade to promote virus replication and spread via infected myeloid cells (black arrows). In contrast, DENV infection in African American skin appears to favor antiviral responses that limit infection and spread (red arrows).

Together with previously related findings in murine models (168, 218, 358), it is possible that the success invasion of DENV human skin occurs in multiple stages (illustrated in figure 17): (1) in epidermis, DENV initially establishes its infection in keratinocytes, which leads to the production and release of pro-inflammatory chemokines and cytokines, including IL-1β. Mosquito bites and saliva components induce the infiltration of neutrophils into the dermis. (2)

DENV infects resident myeloid cells such as LC, DC, and Mϕ, resulting in LC migration from epidermis into dermis, and subsequent emigration of myeloid target cells out of the skin to lymph nodes. These infected cells are likely to play a role in inducing pro-inflammatory mediators, which would act synergistically with IL-1β-derived keratinocytes and neutrophils to attract addition resident myeloid cells from a deeper dermis and circulating monocytes from the blood into the upper dermis. DENV infection also occurs in other skin cell populations including mast cells and fibroblasts. (3) Proportion of infiltrating monocytes will differentiate into monocytes-derived DC or Mϕ. Together with newly recruited resident myeloid cells, these cells become new cellular targets for DENV, leading to enhanced viral loads in the skin and more cell emigration to skin-draining lymph nodes. Migrating cells carrying replicating virus would have potentially effects on spreading the virus to the circulation and other organs such as brain, liver, lungs, and spleens.

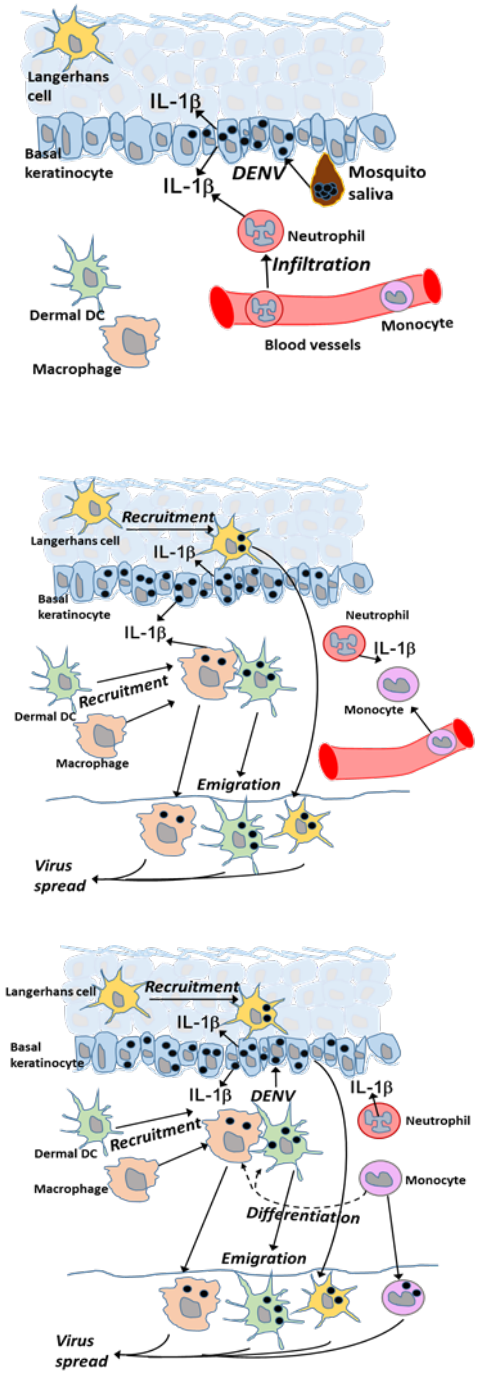

Figure 17. The 3-stage process of DENV infection in human skin.

(Top) First stage is the infection and release of IL-1β by keratinocytes and the recruitment of neutrophils by effects of mosquito bites and saliva. (Middle) Second stage involves subsequent DENV infection in skin-resident myeloid cells and multiple steps of cell recruitments that become infected. DENV infection drives viral spread through cell emigration out of the skin. (Bottom) The final stage includes monocyte differentiation to DC and Mϕ that can become targets for virus replication.

In addition to the primary DENV infection, our study has shown that the presence of immune serum in the skin enhanced DENV infection in Mϕ, indicating the occurrence of ADE phenomenon. The blockade of FcγRI and FcγRII significantly reduced the enhancement of infection in the dermis as well as the number of emigrant cells out of the skin. Here we proposed the mechanism of ADE of DENV infection in the skin (Figure 18).

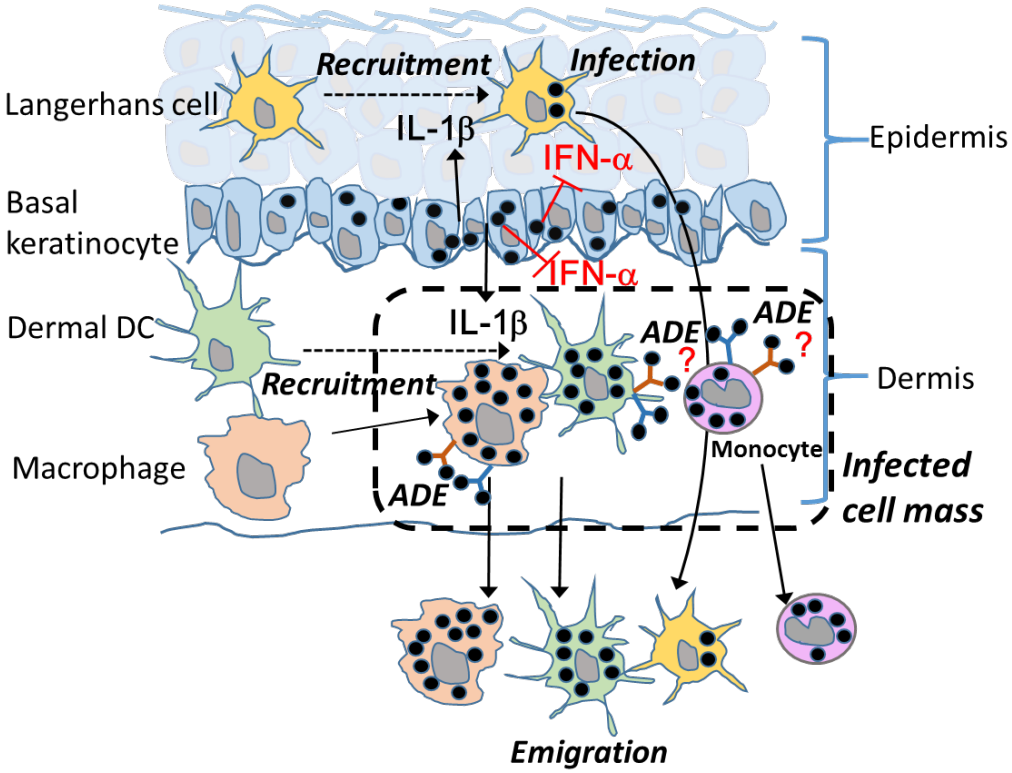

Figure 18. The proposed mechanism for ADE of DENV infection in human skin.

Immune serum enhances the recruitment and infection of cells expressing FcγRI and FcγRII including Mϕ, DC, and monocytes. In situ ADE of DENV infection displays clustering of the immune cell in the upper dermis. The spread of a large amount of the infected cells out of the skin facilitates high viral loads present in the blood and organs of dengue patients.

In vitro ADE infection has shown to reduce type I IFN responses. Consistent with the suppression of IFN-α which corresponds with the progressive replication of DENV in our previous results, it is likely that ADE of DENV infection in skin also inhibits type I IFN systems. Although no enhancement of infection occurs in keratinocytes and LC, DENV infection in keratinocytes and resulting pro-inflammatory cytokines and chemokines would promote the infection and recruitment of myeloid cells including LC, and the spread of the cell into the media. The immune serum has the significant impact on cells in dermis, particularly Mϕ. FcγRI and FcγRII facilitate the ADE of Mϕ infection, resulting in the increase in the number of infected Mϕ, and the substantial elevation of the percentage of Mϕ infected from 20-30% to 50-70%. Mϕ, infected and uninfected cells, are accumulating in large numbers in response to the enhancing activity, together with other cell populations that are also infected. As a result, ADE infection displays clustering of infected cells located in the upper dermis, which has confirmed the increase of infected cell mass suggested in the current ADE hypothesis. Together with the drastic reduction of ADE infection in dermis through the simultaneous inhibition of FcγRI and FcγRII, our findings suggest that other cell populations bearing these FcRs (including DC and infiltrating monocytes) are likely to participate in the ADE phenomenon in skin. Altogether, the ADE of DENV infection in skin would drive the virus replication in the cell in dermis, particularly Mϕ, to a much higher levels. Consequently, a large amount of these highly infected cells migrates to lymph nodes and distributes the virus to other organs. High viral loads present in the blood and organs of dengue patients are one of the major risk for developing severe dengue.

6.5 THE INFLUENCE OF SKIN ON DENGUE DISEASE PROGRESSION

Since DHF and DSS were first identified in the 1950s, the pathogenic mechanism to explain severe and fatal dengue remains elusive. The presence of increased plasma leakage, the hallmark of DHF, occurs late during the transition from acute infection to defervescence, which is coincident with the clearance of virus (23, 398). Together with the strong association of severe dengue with heterologous secondary infections and high cytokine levels, evidence have led to a compelling view that severe dengue is primarily mediated by immunological responses (94, 399). On the other hand, the virulence hypothesis suggests that the innate properties of different genotypes or strain of DENV profoundly control the outcome of disease. It is clearly that this hypothesis cannot fully explain how severe dengue develops when considering that dengue pathogenesis is complex and can be affected by many host factors (400). Although the involvement of virus in dengue pathogenesis has not been widely appreciated in the field, many studies have reported that dengue patients with more severe symptoms had higher viremia levels and slower viral clearance in the first days of illness (103, 104). Furthermore, viral loads during defervescence can predict progression to DHF (401). These findings suggest the importance of early DENV infection as well as the initial interplay between the virus and the host response in determining dengue disease severity.

In addition to immune risk factors, race is also a determinant for disease severity (89). Consistent with the epidemiological observation in mixed-ancestry populations (19, 90, 298, 299, 316), our findings clearly demonstrate that ethnic differences result in the differential host response and outcome of infection in the skin; protective effects of African ancestry lead to a lesser extent of viral replication, lower levels of IL-1β, and higher and sustained antiviral type I IFN responses.

These strongly suggest that the establishment of DENV infection in skin, the first anatomical site of DENV transmission, plays a critical role in dictating the disease progression.

DENV infection in skin leads to the abundant production of pro-inflammatory cytokines and chemokines including IL-1α, IL-1β, CCL20, and CXCL8 (328, 402, 403). Among these factors, IL-1β provides the strongest effect on increasing the recruitment and infection of myeloid cells in the skin site of infection. Importantly, the elevation of IL-1β plasma levels has been reported in DHF patients and can be used to predict the progression to severe disease. Additionally, an increased level of IL-1β is well known in Chikungunya virus infection and rheumatoid arthritis as well as other diseases caused by systemic inflammation (404-406). Considering the vasoactive function of IL-1β (407), this suggests that the production and release of IL-1β from the skin to the bloodstream may potentially lead to physiologic abnormalities of the endothelial lining of blood vessels in multiple organs, resulting in vascular leak.

It is well known that in vitro DENV-immune complex infection of monocytes/Mϕ boosts DENV replication approximately 100-fold in association with the suppression of type I IFN (178, 282, 290, 408). In support of the ADE hypothesis, our findings have demonstrated that the immune serum significantly enhanced DENV replication in skin-resident Mϕ and possibly DC. This results in much higher viral loads in the dermis and the larger number of target cells disseminating out of the skin, compared to the infection without the enhancement. In support of our proposed mechanism of ADE infection in skin, a previous study found that caspase-1 mediated IL-1β secretion by DENV-infected primary monocytes was elevated during ADE (339, 340), suggesting its association with the severity of dengue. Taken together, the ADE

phenomenon originating in the dermis of the skin and high virus loads and IL-1β produced by keratinocytes have potential to contribute to an early magnitude of viremia, reported to drive the serious dengue complications.

Consistent with our cell emigration findings, the skin biopsy report of patients during the first 24 hours of DSS has reported the significant decrease of migratory skin-resident CD1a+ dermal DC compared to healthy skin controls (409), indicating the association of skin cell dissemination and dengue severity. The emigration of myeloid cells out of skin could play a key role in promoting severe dengue in several ways. First, these myeloid cells, particularly monocytes/Mϕ, become activated following DENV infection and release large quantities of cytokines and chemokines (such as IL-1β, TNF-α, IL-8 and IL-6), histamines, and reactive oxygen species (180, 340). These secreted proteins can induce alterations of the vascular endothelium. Second, one of the main function of these migratory immune cells is to activate T cells, either locally in the skin or after migrating to the draining lymph nodes. In secondary infection cases, activation of T cells is may occur rapidly in skin because of the pre-existing of cross-reactive, DENV-specific skin-resident T cells (T_{RM}) from the primary infection. T_{RM} have reduced cytotoxic activities without diminishing cytokine production and expansion predominantly during DENV infection (410-412). The activation of skin CD8+ T cells in DSS suggests that aberrant interactions of T_{RM} cells and innate cells in the skin can promote pathogenic responses of cross-reacting, low-affinity T cells, which is known as original antigenic sin (409). Along with the potent inflammatory effect of IL-1β from keratinocytes and activated myeloid cells, these selectively defective T cell responses can lead to a plethora of cytokines and chemokines (cytokine storm) that together cause damage to endothelial cells and lead to vascular leak in DHF/DSS (113, 399). Lastly, the

emigrant cells carry actively replicating virus out of the skin supposedly spread the virus in the circulation and tissues. Although the critical phase of DENV infection (plasma leak) typically occurs at the time when the viremia titer is declining or undetectable, some clinical observations reported that plasma DENV loads in DHF, but not DF patients, remained at high levels during fever and defervescence (413). Furthermore, a study using autopsy tissues from dengue fatal cases (DHF/DSS) has reported DENV replication, characterized by anti-NS3 staining, in Kupffer cells and hepatocytes in liver, mononuclear phagocytes in spleens and lymph nodes (348). This provides a strong evidence for the viral spread from the skin and the viral replication continuing in dengue affected tissues despite of the viral clearance in the blood. Due to high serum levels of IL-1β, it is possible that DENV constitutes the local infection in various tissues through exploiting existing cellular processes such as cell death mechanisms, which involve the release of IL-1β (285, 328). As DENV was able to infect lymphatic endothelial cells in the skin model, once the virus enters specific tissues, the infection of endothelial cells may occur readily and cause vascular leakage.

In summary, several features of DENV infection in skin can influence dengue disease severity including the high viral load induced by ADE infection, the production of potent cytokines and chemokines, the aberrant activation of T cell responses, and the spread of myeloid target cells to other tissues.

7.0 CHAPTER SEVEN: PUBLIC HEALTH SIGNIFICANCE

Dengue has emerged as an increasingly worrisome mosquito-borne viral disease, with 2.5 billion people currently living in areas at risk of disease, and countless others exposed through travel. There is currently no effective vector control or vaccines. Thus, there is thus an urgent need to understand the biology of DENV, the mechanisms underlying the interaction between DENV and immune responses to explain factors mediating fatal complications and support the development of efficient therapeutic and preventive approaches.

7.1 TISSUE-ASSOCIATED EVIDENCE OF AN EARLY DENV INFECTION

The findings of this study will redound to the benefit of dengue research field considering that skin is the primary site of DENV transmission and replication. Previously, there has been an incomplete understanding of cellular mechanisms that DENV exploits to invade skin. Much of the knowledge of cellular tropism of DENV has shaped through the use of primary human cell lines and immune-deficient mouse models. Together with access issues to human specimens, the understanding of tissue-associate pathophysiology of dengue fever and severe dengue is limited. While certain immune cell types such as LC and DC have been implicated as the main targets of DENV infection (160, 166), the relative contribution of skin target cells to DENV infection is one of the most intriguing questions in the field, as studies remain in conflict. Our study

deciphers an early infection following DENV transmission into skin, and demonstrates that the contribution of keratinocytes to DENV infection accounts for 60% of overall viral burden in human skin. We show that DENV exploits the production of pro-inflammatory cytokines and chemokines by activated keratinocytes to recruit additional myeloid cells for replication, which leads to higher viral loads in skin. Our findings highlight the previously unidentified role of keratinocytes during DENV infection, and greatly advance the understanding of early dynamics of DENV infection in human skin. Also, the study lays the groundwork for the use of an established ex vivo human skin model as a system for studying DENV infection of cells as well as host innate immune responses, with an advantage of manipulating and controlling biological processes through MNA delivery system.

7.2 IL-1B AS A PROMISING THERAPEUTIC TARGET OF DENGUE

We demonstrated that DENV infects at least 7 different cell populations in the epidermis and the dermis shown in our skin model. The ability of DENV to infect a wide range of cell types suggests difficulties in using an appropriate dose of direct-acting antiviral agents alone to effectively combat with high viral burden. However, this could be one of the reasons to explain the preclinical failure of multiple anti-DENV agents under development (414). Alternatively, the compact 11 kb genome of DENV forces DENV to primarily rely on existing cellular factors and interactions for its replication. This viral characteristic can be exploited in attempts to arrest viral replication at some point in the viral life cycle through deprivation of these required host factors. Our findings have identified that the release of IL-1β from the DENV-infected cell is a dependent host factor for DENV spread in human skin. Blocking keratinocyte-derived IL-1β

reduced the overall infection of major skin-resident myeloid cells by 75-90%, and reduced the total number of infected cells in epidermis and dermis by 33% and 65%, respectively.

The use of IL-1β inhibitors to interfere with DENV infection will likely reduce DENV transmission locally in skin and limit the spread of DENV beyond the skin, resulting in low viremia levels and reduced risks of severe dengue. In fact, targeting IL-1 or IL-1β therapeutics have long been used in other chronic inflammatory diseases such as rheumatoid arthritis and type 2 diabetes mellitus (415, 416). This ensures safety and efficacy in individuals. The characteristics of IL-1β antagonists available in the market, including a short half-life (ranging from days to <1 month) and a subcutaneous route of administration, are suitable for acute care beyond the earliest hours of DENV infection (417, 418). Therefore, IL-1β is not only a conceptually promising host target for anti-DENV activity, but it also comes with opportunities for rapid investigations and fast-track approvals in the search for safe and effective therapeutics against dengue. Clearly, effects of IL-1β inhibitors would only intervene with mechanisms of DENV transmission, but not with the virus. In practical terms, the agent may be used in association with other antivirals and/or by early treatment to strengthen the overall treatment.

7.3 IMPLICATIONS FOR DENGUE VACCINES

While the DENV-ADE hypothesis has been proposed for decades and the phenomenon can be readily demonstrated in vitro (100, 231). Whether sub-neutralizing levels of the pre-existing Ab from previous infections truly intensify the subsequent DENV infection in severe dengue cases remains questionable. This is because not all individuals develop severe dengue in the secondary

or subsequent infections. Although there is still no definitive proof of ADE of DENV infection in humans, dengue experts have raised concerns that the only licensed dengue vaccine produced by Sanofi Pasteur may be triggering ADE and increase the risk of DHF/DSS in dengue naive recipients (419). Currently, the vaccine has been suspended in some countries following the company's official announcement that its vaccine could worsen the disease in dengue naïve recipients. Given the current scenario, with no specific treatments available for dengue and the low-efficacy dengue vaccine, the field of dengue is in need of rigorous ADE investigations in humans. ADE, while it is still a theory, could lead to life-threatening complications. Our study in human skin explants has demonstrated that the presence of immune serum strongly enhanced the DENV infection and recruitment of Mϕ, one of the cell types known to play a key role in ADE, and induced more cells spreading out of the skin. Although the skin model of infection does not fully represent the in vivo system of humans, the new finding supports that Ab can enhance DENV infection at least in human skin and drive more target cells out of the skin to contribute to systemic dengue pathogenesis. Our study necessitates evaluations of enhancing Ab or other possible ADE-driven potentials when developing and implementing vaccines or therapeutics against dengue.

7.4 IMPLICATIONS FOR DENGUE PREVENTION AND CONTROL IN ETHNIC GROUPS

Our study is the first demonstration for ethnic differences in DENV infection and spread in human skin. Consistent with epidemiological evidence in various regions with highly admixed populations (19, 90, 298, 316), our findings have experimentally provided a proof of concept in

human skin and should serve as a basis for future investigations in skin. This could be further implemented in other organs to reveal the mechanisms behind the protection against severe dengue in African ancestry. Whether these important differences are driven by biological or genetic causes or both, they will open new avenues for the development of novel strategies in the prevention and treatment of dengue diseases. This could be a major turning point in the fight against severe dengue infections. This will also allow for creation or support of effective evidence-based decisions to maximize dengue interventions and immunization programs for people of different ethnic backgrounds.

APPENDIX: PUBLICATION LIST

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