CD8+ T Cell and NK Responses to a Novel Dengue Epitope: A Possible Role for KIR3DL1 in Dengue Pathogenesis: A Dissertation

Elizabeth Townsley
University of Massachusetts Medical School

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CD8^+ T CELL AND NK RESPONSES TO A
NOVEL DENGUE EPITOPE: A POSSIBLE ROLE FOR KIR3DL1 IN
DENGUE PATHOGENESIS

A Dissertation Presented

By

ELIZABETH TOWNSLEY

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
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April 3, 2014

M.D./Ph.D. Program
CD8$^+$ T CELL AND NK RESPONSES TO A NOVEL DENGUE EPITOPE: 
A POSSIBLE ROLE FOR KIR3DL1 IN DENGUE PATHOGENESIS

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By
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April 3, 2014
I feel very lucky to have spent the last four years working with a caring, fun, supportive group of fellow researchers. I have grown as a scientist thanks to their constant willingness to provide feedback and advice. I leave the lab excited for what lies ahead, but also sad to leave behind an exciting project and a wonderful group of friends and colleagues.

I would like to especially thank my PI, Anuja, for encouraging me to pursue interesting findings, her help in forming crucial collaborations, and for always reminding me to maintain a healthy balance between research and life. I appreciate her constant support, guidance, and encouragement throughout the last four years. I would also like to thank Alan for introducing me to the field of dengue research and sharing his insights and ideas.

A number of vibrant collaborations have made the last four years very rewarding. I would like to thank the group of students and post-docs at the Ragon Institute who invited me to join in on discussion of ongoing research and have helped me technically as I expanded outside the scope of my work.

I would also like to thank lab members both current and past who helped me in innumerable ways. I would like to thank the former students especially Heather, Derek, Rachel, and Jenny who took the time to help me learn lab techniques and improve my presentation of the data. My project never would have gotten off the ground if Kim had not taught me how to baby cryopreserved cells frozen over 20 years ago and the ever
elusive art of primary cell culture. I will be ever thankful for the many hours I have been lucky to spend learning the ins and outs of flow cytometry from Marcia. She always encouraged me to take the time to generate the best data possible, and was always willing to spend as much time as necessary working with me to realize this goal. I especially appreciate Pam’s help these last few months repeating experiments as I prepared this Thesis.

Lastly, I would like to thank my family for their constant encouragement and support. I will forever be grateful to them for nurturing my love of learning and encouraging me to find a career I would always find interesting and challenging.
ABSTRACT

Variation in the sequence of T cell epitopes between dengue virus (DENV) serotypes is believed to alter memory T cell responses during second heterologous infections contributing to pathology following DENV infection. We identified a highly conserved, novel, HLA-B57-restricted epitope on the DENV NS1 protein, NS1_{26-34}. We predicted higher frequencies of NS1_{26-34}-specific CD8^+ T cells in PBMC from individuals undergoing secondary, rather than primary, DENV infection due to the expansion of memory CD8^+ T cells. We generated a tetramer against this epitope (B57-NS1_{26-34} TET) and used it to assess the frequencies and phenotype of antigen-specific T cells in samples from a clinical cohort of children with acute DENV infection established in Bangkok, Thailand. High tetramer-positive T cell frequencies during acute infection were seen in only 1 of 9 subjects with secondary infection. B57-NS1_{26-34}-specific, other DENV epitope-specific CD8^+ T cells, as well as total CD8^+ T cells, expressed an activated phenotype (CD69^+ and/or CD38^+) during acute infection. In contrast, expression of CD71 was largely limited to DENV-specific CD8^+ T cells. In vitro stimulation of CD8^+ T cell lines, generated against three different DENV epitopes, indicated that CD71 expression was differentially sensitive to stimulation by homologous and heterologous variant peptides with substantial upregulation of CD71 detected to peptides which also elicited strong functional responses. CD71 may therefore represent a useful marker of antigen-specific T cell activation.
During the course of our analysis we found substantial binding of B57-NS1_{26-34} TET to CD8⁻ cells. We demonstrated that the B57-NS1_{26-34} TET bound KIR3DL1, an inhibitory receptor on natural killer (NK) cells. NK sensitive target cells presenting the NS1_{26-34} peptide in the context of HLA-B57 were able to dampen functional responses of only KIR3DL1⁺ NK cells. Analysis of the activation of an NK enriched population in our Thai cohort revealed peak activation during the critical time phase in patients with severe dengue illness, dengue hemorrhagic fever, compared to people with mild illness.

Our data identified CD71 as biologically useful marker to study DENV-specific CD8⁺ T cell responses and highlighted the role of viral peptides in modulating NK cell activation through KIR-MHC class I interactions during DENV infection.
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ABBREVIATIONS

$^{51}$Cr radio-labeled chromium

ADE antibody dependent enhancement

APC allophycocyanin

APCs antigen presenting cells

A2-E$_{213-221}$ TET A2 tetramer loaded with the E peptide position 213-221

(FLDLPLPW, FLDLPLPW, FFDLPLPW, FFDLPLPW)

A11-NS$_{133-142}$ TET A11 tetramer loaded with the NS3 peptide 133-142

(GTSGPIVNRE, GTSGSPIVDR, GTSGSPIIN)

B57-LF9 TET B57 tetramer loaded with the self peptide LF9 (LSSPVTKSF)

B57-NS$_{26-34}$ TET B57 tetramer loaded with the NS1 peptide position 26-34

(HTWTEQYKF)

B57-TW$_{10}$ TET B57 tetramer loaded with the HIV-Gag peptide TW10

(TSTLQEQIGW)

B57-TW$_{10n}$ TET B57 tetramer loaded with the HIV-Gag peptide TW10 mutated at position 2 (TNTLQEQIGW)

B-LCL B-lymphoblastoid cell line

BV brilliant violet

C capsid protein

CMV cytomegalovirus

CTL cytotoxic T lymphocyte
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>DC-specific intercellular adhesion molecule-3-grabbing non-integrin</td>
</tr>
<tr>
<td>DENV</td>
<td>dengue virus</td>
</tr>
<tr>
<td>DF</td>
<td>dengue fever</td>
</tr>
<tr>
<td>DHF</td>
<td>dengue hemorrhagic fever</td>
</tr>
<tr>
<td>DSS</td>
<td>dengue shock syndrome</td>
</tr>
<tr>
<td>E</td>
<td>envelope protein</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein</td>
</tr>
<tr>
<td>gMFI</td>
<td>geometric mean fluorescence intensity</td>
</tr>
<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP</td>
<td>inducible protein</td>
</tr>
<tr>
<td>ITIM</td>
<td>immunoreceptor tyrosine-based inhibition motif</td>
</tr>
<tr>
<td>JEV</td>
<td>Japanese encephalitis virus</td>
</tr>
<tr>
<td>KIR</td>
<td>killer cell immunoglobulin-like receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>L-SIGN</td>
<td>liver/lymph node specific ICAM-3 grabbing non-integrin</td>
</tr>
<tr>
<td>LTA</td>
<td>lymphotoxin alpha</td>
</tr>
<tr>
<td>LTB</td>
<td>lymphotoxin beta</td>
</tr>
<tr>
<td>MDA5</td>
<td>melanoma differentiation-associated protein 5</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MICA/B</td>
<td>major histocompatibility complex class I-related chains</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>macrophage inflammatory protein 1β</td>
</tr>
<tr>
<td>NC</td>
<td>nano crystal</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor-κB</td>
</tr>
<tr>
<td>NIAID</td>
<td>National Institute of Allergy and Infectious Disease</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NS</td>
<td>no stimulation</td>
</tr>
<tr>
<td>NS[#]</td>
<td>non-structural protein [#]</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>pMHC</td>
<td>peptide-MHC complex</td>
</tr>
<tr>
<td>prM</td>
<td>precursor form of M protein</td>
</tr>
<tr>
<td>Qdot</td>
<td>quantum dot</td>
</tr>
<tr>
<td>RCS</td>
<td>reduced chi square</td>
</tr>
<tr>
<td>rh</td>
<td>recombinant human</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Rosewell Park Memorial Institute cell culture medium</td>
</tr>
<tr>
<td>SS</td>
<td>single stranded</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter associated with antigen processing</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>UMMS</td>
<td>University of Massachusetts Medical School</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WNV</td>
<td>West Nile virus</td>
</tr>
<tr>
<td>WRAIR</td>
<td>Walter Reed Army Institute of Research</td>
</tr>
<tr>
<td>YFV</td>
<td>yellow fever virus</td>
</tr>
</tbody>
</table>
Parts of this thesis have appeared in separate publications:

**Chapter III: CD8$^+$ T cell Responses to a Novel DENV Epitope During Acute Primary and Secondary DENV Infection**

**Chapter IV: The B57-NS1$_{26-34}$ TET Interacts with the Inhibitory Receptor KIR3DL1 on NK cells**

Other work performed during thesis studies that is not discussed in this thesis has appeared in separate publications:

Mathew A, Townsley E, Ennis FA. Elucidating the role of T cells in protection against pathogenesis of dengue virus infections. *Future Microbiology* 2014;9 (3).
CHAPTER I
INTRODUCTION

A. Dengue Virus

Dengue virus (DENV), a member of the family *Flaviviridae*, genus *Flavivirus,* consists of four distinct serotypes numbered 1-4\(^1,2\) with ~70% identity in both the nucleotide and amino acid sequences\(^3\). DENV is an enveloped, positive-sense ribonucleic acid (RNA) virus with three structural proteins (capsid [C], membrane [M], envelope [E]) and seven nonstructural (NS) proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5). The RNA genome is translated as a single polyprotein, which is co- and post-translationally modified by NS2B-NS3\(^4,5\).

The DENV virion is comprised of a nucleocapsid formed by the structural protein C, containing the viral genome, surrounded by a viral envelope which contains the structural proteins precursor form of (pr) M and E. The immature prM is later cleaved in the Golgi apparatus by the convertase furin into pr and M proteins\(^6\). This cleavage results in a conformational change of the E protein, and the generation of mature infectious virions\(^7,8\). Even after cleavage pr binds to E at acidic pH, preventing membrane fusion\(^7,9\).

Cellular entry of DENV is thought to occur in multiple ways, depending on the cell type and available receptors. Direct fusion of virus to the cell membrane, clathrin-mediated endocytosis\(^10-16\), E-mediated binding of virus to both lectin-type receptors (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin [DC-SIGN] and liver/lymph node specific ICAM-3 grabbing non-integrin [L-SIGN])\(^17,18\) and
aminoglycan-type adhesion molecules are possible mechanisms of viral entry. Virus tropism for DC-SIGN or L-SIGN can be modified through variation in N-linked glycosylation of E. Dejnirattisai et al. demonstrated that differences in glycosylation patterns between mosquito and human cells switch the tropism of DENV from DC-SIGN expressing cells, such as dendritic cells (DCs) and macrophages, to L-SIGN expressing cells, such as liver sinusoidal endothelial cells.

In vitro studies have demonstrated that many cell types can be infected by DENV, although confirming in vivo targets of DENV infection is difficult due to limitations in the types of tissue samples available from humans with dengue disease. Previous reports suggest B cells, monocytes, endothelial cells, and DCs as sites of viral replication in vivo, with monocyte lineage cells as the primary targets of infection. DENV has also been detected in hepatocytes, perivascular cells in the brain, and endothelial cells of the spleen when such tissues have been collected from fatal cases. However, DENV fatalities generally occur after the initial febrile illness, when viremia has been resolved, and therefore may not be reflective of viral replication during the early stages of infection.

Once a cell is infected, the NS proteins are responsible for viral replication, viral translation, and suppression of innate antiviral responses. While our understanding of the exact functions of these seven NS proteins has advanced greatly over the last decade, their roles have not been fully elucidated. NS2A, NS3, NS4A, and the NS5 proteins are known to be involved in replication and translation. NS3, in addition to participating as a serine proteinase with NS2B, functions as an RNA helicase and RNA triphosphatase. NS5 stimulates and modulates the enzymatic activities of NS3. NS2A, NS4A, NS4B,
and NS5 are involved in suppression of the innate immune response by suppressing type I interferon (IFN) production or signaling\textsuperscript{31-34}. This suppression of the type I IFNs, IFN-α/β, maintains high levels of viral replication.

While the function of NS1 is not fully elucidated, it may be involved in viral replication\textsuperscript{35}. NS1 is secreted from infected cells, based on its glycosylation status\textsuperscript{36}, and its presence in serum has been used to diagnose DENV infection\textsuperscript{37-39}. During DENV infection NS1 can exist in monomeric, dimeric or hexameric forms\textsuperscript{40}. Levels of NS1 in plasma correlate with disease severity\textsuperscript{41-43}, and soluble and cell-associated NS1 have been reported to activate the complement cascade\textsuperscript{44}. Avirutnan et al. found high levels of NS1 and the complement protein C5a in pleural fluids of patients with dengue shock syndrome (DSS)\textsuperscript{44}. Additionally, antibodies formed against NS1 may be cross-reactive with important human hemostatic proteins leading to hemostatic disruption when cross-reactive antibodies bind fibrinogen, thrombocytes, endothelial cells, and human clotting factors\textsuperscript{45-48}.

**B. Dengue Virus Infections: The Global Burden, Clinical Picture, and Vaccine Strategies**

_\textit{i. Global Burden}_

It is believed that DENV originally circulated in monkeys via a sylvatic transmission cycle and jumped to humans over 200 years ago\textsuperscript{49}. While monkeys can be infected, they no longer play a role in the transmission cycle of most DENV strains detected in the human population\textsuperscript{49}. A fifth serotype was recently identified which does
not yet appear to have sustained transmission in humans. The first reports of dengue may have been as early as 1635 when a dengue-like epidemic was recorded in Martinique, and in 1780 there was a well described outbreak of “bilious fever” in Philadelphia which was likely caused by DENV.

DENV is transmitted to humans through mosquitoes, primarily via the vector Aedes aegypti but it can also be transmitted by Aedes albopictus. Aedes aegypti are found primarily in residential areas, breed commonly in water that has accumulated in man-made containers, and primarily feed on humans. Eradication programs began in 1947 to eliminate Aedes aegypti in the Western hemisphere and have changed the global picture of DENV and yellow fever virus (YFV). While the initial eradication efficiency varied between countries, many areas have subsequently been re-infested by Aedes aegypti. In the last decade, the territory of Aedes aegypti has continued to spread with the appearance of DENV in many South American countries.

Efforts have continued to limit the breeding of Aedes mosquitoes primarily through education aimed at reducing standing water in urban areas. Despite these efforts, however, the continued increase in the number of DENV cases suggests increased transmission of the virus. DENV is now endemic in over 100 tropical and subtropical countries, and the number of reported infections in these countries has increased over the last few years. In 2010, 2.3 million cases of DENV infection were reported to the World Health Organization (WHO). In 2013, Bhatt et al. estimated that 390 million people become infected each year with DENV of which 96 million are clinically apparent.
Increased urbanization is a major factor proposed to explain the increase in dengue cases.

**ii. Clinical Picture**

Dengue is an acute infection with no reports of chronic cases. While serotype and strain differences in virulence appear to exist, all serotypes have the same transmission cycle and cause similar clinical symptoms during acute infection\(^5\). DENV infection causes a broad spectrum of clinical symptoms ranging from inapparent to acute febrile illness, to a more severe clinically significant change in hemodynamics. While DENV infection can be determined serologically, the classification of dengue disease severity relies on criteria established by the WHO, which has recently undergone extensive changes. The goal of the new classification system was to improve identification of patients with severe disease in an attempt to limit DENV mortality. This new classification refers to clinically apparent cases of dengue as either probable or laboratory-confirmed dengue, dengue with warning signs, or severe dengue\(^4\). The benefits of this new system have yet to be demonstrated and many research groups still use the old system for classifying patients\(^5\). For the purpose of the work presented here, the 1997 WHO classification system, which categorizes dengue patients as having inapparent illness, uncomplicated dengue fever (DF), dengue hemorrhagic fever (DHF), or DSS, will be used\(^5\).

The majority of DENV infections are subclinical. Of clinically significant infections, most present as an acute febrile illness, DF, while only approximately 3% of DENV-infected patients develop DHF\(^5\). DHF is characterized by high fever, plasma
leakage, thrombocytopenia, and bleeding tendency, which are coincident with the resolution of fever and viral clearance\textsuperscript{58-60}. Hepatomegaly is also present in over 90% of Thai children with DHF\textsuperscript{57}. The presence and extent of pleural effusion, an indication of plasma leakage, can be measured using chest X-ray\textsuperscript{57}. Shock is a rare but serious complication of plasma leakage and is known as DSS. Currently, medical therapy for dengue disease is purely supportive.

\textit{iii. Vaccine Strategies}

The earliest vaccines against DENV infection were developed in the 1940s, but concerns about vaccine purity stopped further development even though these vaccines produced neutralizing antibodies and appeared to provide protection against subsequent infection\textsuperscript{61}. In the 1980s, the Walter Reed Army Institute of Research (WRAIR) developed an attenuated live DENV vaccine that was based on viruses isolated from patients\textsuperscript{61}. Several of these viruses were discontinued during clinical trials due to unacceptable reactogenicity in humans. The attenuated DENV strains which WRAIR proceeded with for phase I testing were sold to GlaxoSmithKline\textsuperscript{61} who completed Phase II trials.

Sanofi Pasteur is now testing a YFV-DENV chimeric tetravalent vaccine that is in phase III clinical trials. These chimeras replace some of the YFV structural proteins with those of DENV. YFV live-attenuated vaccine was chosen as the backbone for the chimeras due to its immunogenicity and ability to induce long term immunity\textsuperscript{62}. One chimeric flavivirus vaccine, for Japanese encephalitis virus (JEV), has been licensed and is currently in use\textsuperscript{63}. The phase II trial of Sanofi Pasteur’s dengue vaccine showed two to
three fold increases in anti-DENV antibodies against DENV-1, -2, -3, and -4. While protection was observed to DENV-1, -3, -4 the efficacy of the vaccine against DENV-2 was poor. A number of other candidate dengue vaccines built off various platforms are also in clinical trials. Live-attenuated vaccines have also been developed by the National Institute of Allergy and Infectious Diseases (NIAID) via deletions in the 3′ untranslated region of the DENV genome.

All of the DENV vaccines currently in clinical trials aim to induce protective immunity to all four DENV serotypes in the hope of eliminating the possibility of immunopathology following DENV infection in a partially-immune host. DENV vaccine trials are complicated by pre-existing immunity both to DENV and to other circulating flaviviruses or previous flavivirus vaccines such as the YFV vaccine and the JE vaccine, which are routinely given in many DENV-endemic areas.

C. Risk Factors for Developing Severe Disease in Humans

A number of risk factors for developing severe disease have been identified epidemiologically, including weight, age, nutritional status, viral strain, and immunologic genotypes, as well as, most strikingly, a subsequent DENV infection with a second serotype. Early studies of DENV infection linked secondary infection with a heterologous serotype to a final outcome of DHF and this pattern has continued to hold true. Additionally, it has been suggested that the sequence of infecting serotypes modulates the risk of developing DHF.
The potential for strain variants to affect virulence was highlighted in 1981 when epidemic DHF appeared suddenly in the Americas following a change in the predominant circulating strain of DENV-2. The possibility of a new serotype being introduced raises concerns about the possibility of epidemics in already endemic areas with an increased burden of DHF cases.

The elevation of soluble factors in the serum of DENV-infected patients is thought to play a role in DENV pathogenesis. The few tissue samples obtained from patients who succumbed to DHF show no endothelial damage. The rapid onset and recovery from plasma leakage in most individuals with severe dengue, support the model that soluble factors, rather than direct damage by immune cells, alter the ability of endothelial cells to form an effective barrier. This hypothesis has been further supported by work that demonstrates that cytokines, such as tumor necrosis factor-alpha (TNF-α), which are produced in response to DENV infection, can affect the barrier integrity of cultured endothelial cell monolayers. Coagulation and endothelial markers, including von Willebrand factor, plasminogen activator inhibitor, and tissue factor are other soluble factors that have been associated with more severe disease.

Human leukocyte antigen (HLA) class I genotype has been associated with dengue disease severity in a number of studies. However, the specific associations vary between ethnic populations. A number of other immune-related genes including TNF, lymphotoxin alpha (LTA), lymphotoxin beta (LTB), have also been linked to dengue disease severity. These larger groupings are referred to as extended haplotypes. The
existence of the extended haplotypes can make it difficult to narrow down which immune gene is crucial for protection or pathogenesis epidemiologically.

The major histocompatibility complex (MHC) class I-related chains A/B (MICA/B) have been associated with particular disease outcomes for DENV\textsuperscript{92-94}. These proteins are upregulated in cells under stress and are ligands for an activating receptor on natural killer (NK) cells\textsuperscript{95}.

The combination of host health and genetic risk factors, combined with the timing of plasma leakage in DHF, suggests a role for the adaptive immune response in the pathogenesis of DENV infection. The very pronounced risk of DHF in patients undergoing a secondary infection has focused most effort on understanding how the adaptive immune response could contribute to disease severity. The variety of risk factors and the variation between populations suggests that many factors contribute to the outcome for DENV patients and that the same clinical endpoint can be reached in many ways.

D. Adaptive Immune Responses to Dengue Virus

While only a percentage of DENV infections are clinically apparent, the global burden of DENV is high. With the incidence of DHF on the rise, the underlying determinants of DHF remain a central question of DENV research. Infection with one DENV serotype provides lifelong immunity to that serotype but not to the other three serotypes of DENV\textsuperscript{96}. Moreover, patients undergoing a second infection with a different serotype are at increased risk for developing DHF\textsuperscript{69,70,97}. Protection against homologous
infection, in combination with some newer data generated from mouse models discussed below, suggest that the adaptive immune response likely plays an important role in viral clearance and the subsequent protection against re-infection. However, the association of DHF with secondary infection and the timing of DHF symptoms after the peak of viremia also implicate the adaptive immune response in DHF pathogenesis. Both antibodies and T cells have been proposed to contribute to the development of severe dengue disease\textsuperscript{98}.

\textit{i. Role of Antibodies in Dengue Pathogenesis}

Antibodies to DENV can mediate a number of activities \textit{in vitro}\textsuperscript{67}. Some antibodies are able to neutralize the virus but enhance virus uptake at higher dilutions, while other antibodies do not neutralize the virus but are also able to bind to the virus and Fcy I and II receptors, and mediate more efficient entry into the host cell\textsuperscript{99, 100}. This more efficient viral entry mediated by viral antibodies is referred to as antibody dependent enhancement (ADE)\textsuperscript{101}. DENV-specific antibodies of the appropriate subclasses bound to dengue antigens on the infected cell membrane can bind to complement proteins and promote complement-dependent lysis (CDL) of infected cells and contribute to antibody-dependent cellular cytotoxicity (ADCC) of infected cells\textsuperscript{102, 103}.

After a large outbreak of dengue in Thailand in 1980, a study of antibody responses in children hospitalized with dengue found that all of the patients with DSS had antibody responses consistent with previous DENV infection\textsuperscript{69}. This observation suggests that sub-neutralizing levels of cross-reactive anti-DENV antibodies that were generated during a previous infection increased the risk of developing DHF\textsuperscript{69, 104}. Follow-up studies suggested that non-neutralizing antibodies, via ADE, may enhance viral load and immune
activation during DENV infection\textsuperscript{69,105-107}. However, Libraty et al. did not find any correlation between the ADE activity of maternal antibodies in DENV-infected infants and the development of DHF, though they did find protection from symptomatic DENV in infants with high levels of maternal antibodies\textsuperscript{108}.

ADE may facilitate virus entry and initiate intracellular antiviral responses\textsuperscript{109}. However, ADE-mediated virus entry has also been reported to down-regulate the RIG-I/MDA5 signaling pathway leading to decreased production of type I IFNs\textsuperscript{110,111}. Hence, the effects of ADE on DENV pathogenesis may not be limited to increasing viral burden by increasing the number of infected cells, but may also act by dampening innate and downstream adaptive immune responses, allowing for more robust viral replication\textsuperscript{97}.

While ADE can be observed \textit{in vitro}, demonstrating ADE \textit{in vivo} in humans is more challenging. In mice, passive transfer of low doses of cross-reactive anti-DENV antibodies enhanced DENV infection and features of lethal disease. Mutation of the antibody to prevent Fc\gammaR binding eliminated these effects\textsuperscript{112}. However, in contrast to human disease, lethal features of murine disease, such as vascular leakage, occurred during viremia rather than post-viremia. Huang et al. established the first mouse model with post-viremia disease. They found thrombocytopenia following infection with DENV-2 correlated with the development of anti-platelet antibodies in these mice\textsuperscript{113}. As noted earlier, human antibodies to DENV that are cross-reactive with human endothelial cells and human clotting factors have been reported to play a role in the hemostatic changes observed in patients who develop DHF\textsuperscript{45}. 


**ii. Role of T cells in Dengue Pathogenesis**

Global T cell expansion, expansion of epitope-specific T cells, and markers of T cell activation have been assessed during acute DENV infection in an attempt to elucidate how T cells may contribute to disease severity. The results of these studies have varied based on study design, ethnic group, and the epitope being studied (Table 1.1 and Table 1.2). Other factors, such as virus strain, quality of care, and consistency of diagnosis may have contributed to the variability between these studies.

Studies of total CD8$^+$ T cell responses during DENV infection have shown higher frequencies of CD8$^+$ T cells expressing CD69, and higher levels of immune activation markers, such as in individuals with DHF as compared to those with DF. Not all groups, however, have observed such a pattern of CD8$^+$ T cell activation. Dung et al. reported no evidence of CD8$^+$ T cell activation, as measured by expression of CD38, HLA-DR, and Ki-67, in the peripheral blood of patients until after capillary leakage had begun; they concluded that CD8$^+$ T cells do not play a primary role in DENV pathogenesis, but suggested that T cell activation may amplify DENV pathogenesis. Increased levels of cytokines, which are secreted *in vitro* by DENV-specific CD8$^+$ T cells, have been found in patients with mild disease, and some were increased to higher levels in severe DENV disease, including IFN-γ, interleukin (IL)-6, IL-8, IL-10, IL-18, and TNF-α.
Table 1.1. Dengue CD8\(^+\) T cell association studies

<table>
<thead>
<tr>
<th>Study Location(^a)</th>
<th># of Subjects(^b)</th>
<th>Timing of Enrollment(^c)</th>
<th>DF vs. DHF(^d, e); P vs. S(^e)</th>
<th>Timing of Acute Samples(^f)</th>
<th>Timing of Convalescent Samples(^g)</th>
<th>HLA-Typing Done?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thailand 1999(^22)</td>
<td>51 children</td>
<td>within 72hrs of fever onset</td>
<td>29 DF 22 DHF 22 P 29 S</td>
<td>study day 2 and 1 day after defervescence</td>
<td>NONE</td>
<td>NO</td>
</tr>
<tr>
<td>Thailand 2002(^29)</td>
<td>10 children</td>
<td>within 72hrs of fever onset</td>
<td>5 DF 5 DHF 1 P 9 S</td>
<td>during illness &amp; 8 to 11 days after study entry</td>
<td>6 months 1 year 2 year 3 year</td>
<td>YES</td>
</tr>
<tr>
<td>Thailand 2003(^114)</td>
<td>19 children</td>
<td>5 DF 14 DHF 19 S</td>
<td>4 times during acute illness Fever day -2, -1, 0, +1</td>
<td>14 days after defervescence</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>Vietnam 2005(^330)</td>
<td>48 adults</td>
<td>48 S</td>
<td>day of admission, study day 3, study day 5</td>
<td>2 weeks, 1 month post admission</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thailand 2006(^131)</td>
<td>13 children</td>
<td>13 DF 10 DHF 1 P 12 S</td>
<td>during illness &amp; 8 to 11 days after study entry</td>
<td>6 months</td>
<td>YES</td>
<td></td>
</tr>
</tbody>
</table>

Evaluated total CD4\(^+\) T cell, CD8\(^+\) T cell, NK cell, and γδ T cell responses during acute illness.

FINDINGS: Increased expression of CD69 in DHF compared to DF

Evaluated CD8\(^+\) T cell responses to the HLA-B07 restricted epitope NS3\(_{222-231}\)

FINDINGS: higher freq of B7-NS3\(_{222-231}\) TET\(^+\)CD8\(^+\) T cells in DHF pts

Evaluated CD8\(^+\) T cell responses to the HLA-A11 restricted epitope NS3\(_{133-142}\)

FINDINGS: higher freq of A11-NS3\(_{133-142}\) TET\(^+\) T cells in DHF pts

Evaluated T cell responses to 260 overlapping peptide antigens from DENV-2. Data on hemoconcentration during dengue was available for 24 pts

FINDINGS: IFN-γ ELISPOT responses weakly correlated with hemoconcentration, but not disease severity.

Evaluated responses to the HLA-A24 restricted epitope NS3\(_{556-564}\)

FINDINGS: percentage of TET\(^+\) CD8\(^+\) T cells correlate with DHF and DHF severity.
**Vietnam 2008**  
75 Infants <18 mos. with acute dengue  
192 healthy infants  
Healthy with no DENV IgM at birth. Serum at birth 6, 9, 12 moths or enrolled at one of the above time points.  
2 DF  
67 DHF  
6 DSS  
75 P  
daily for 4 days during hospitalization  
10 to 14 days after hospital discharge  
YES  
Evaluated CD8+ T cell activation in acute dengue using the activation marker CD69. In HLA-A11+ subjects NS3133-142  
FINDINGS: CD69+CD8+ T cells were significantly, but transiently, increased in DHF. Found measurable frequencies of NS3133-142 TET+CD8+ T cells only in convalescence.

**Vietnam 2008**  
103 children  
23 controls  
2 studies:  
1) Pts enrolled w/in 72 hrs of fever onset  
2) Pts enrolled w/in 7 days of illness  
86 DF  
17 DHF  
30 P  
73 S  
daily during acute illness  
2-3 wks after presentation  
Evaluated plasma leakage within 24 hrs of defervescence. Studied CD8+ T cell responses by measuring expression of Ki-67, CD38, HLA-DR and frequencies of TET+ cells (NS3133-142)  
FINDINGS: Peak TET+CD8+ T cell frequencies after plasma leakage commenced.

**Thailand 2010**  
40 children  
18 DF  
22 DHF  
40 S  
2 weeks  
Evaluated the function of CD8+ T cells in response to peptide stimulation.  
FINDINGS: DHF patients had decreased frequencies of CD107a+ CD8+ T cells and increased frequencies of TNF-α and IFN-γ producing CD8+ T cells following stimulation.

**Thailand 2010**  
33 children  
pre-illness (with bleed)  
17 s.c.  
10 Symp  
w/in 7 days of onset of symptoms  
>15 days  
YES  
Evaluated pre-illness responses between subclinical or symptomatic secondary infection.  
FINDINGS: Higher frequencies of DENV-specific TNF-α, IFN-γ producing T cells in children who developed subclinical infection.
<table>
<thead>
<tr>
<th>Study Location</th>
<th>Number of Subjects</th>
<th>Serotypes</th>
<th>Timeline of Sample Collection</th>
<th>Follow-Up</th>
<th>Evaluated Response</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thailand 2011</strong>&lt;sup&gt;134&lt;/sup&gt;</td>
<td>44 children</td>
<td>25 DF, 19 DHF, 17 P, 27 S</td>
<td>daily during acute illness and 1 week after defervescence</td>
<td>6 month, 1 year, 2 years</td>
<td>YES</td>
</tr>
<tr>
<td>Evaluated CD8&lt;sup&gt;+&lt;/sup&gt; T cell responses to the HLA-A11 restricted epitope NS3&lt;sub&gt;133-142&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FINDINGS:</strong> no correlation between frequency of TET&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt; T cells and disease severity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sri Lanka 2012</strong>&lt;sup&gt;135&lt;/sup&gt;</td>
<td>24 adults, 5 controls</td>
<td>24 healthy seropositive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Evaluated ex-vivo responses to peptides from each of the four DENV serotypes to determine serotype specific T cells.</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>FINDINGS:</strong> All immune donors responded to at least two DENV serotypes. Eight individuals responded to DENV-4 peptides even though no DENV-4 had been previously reported in Sri Lanka</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Study location and reference
<sup>b</sup> Number of subjects enrolled in the study, divided into dengue patients and healthy controls when applicable, noted if the study is of children or adult subjects.
<sup>c</sup> When patients were enrolled in the study
<sup>d</sup> Number of subjects with DF versus DHF (or DSS as noted),
<sup>e</sup> Number of subjects with Primary (P) versus secondary (S) DENV infection
<sup>f</sup> Timing of collections of samples taken during acute dengue illness
<sup>g</sup> Timing of collection of samples taken following dengue illness

s.c. = subclinical. Symptomatic = some studies simply classified subjects as symptomatic.
Table 1.2. Dengue genetic association studies

<table>
<thead>
<tr>
<th>Study Location</th>
<th># of Subjects</th>
<th>DF vs. DHF</th>
<th>P vs. S</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thailand 2002</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>263 children</td>
<td>149 DF</td>
<td>54 P</td>
</tr>
<tr>
<td><strong>FINDINGS:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Associated HLA-A<em>203 and HLA-B</em>52 with DF, HLA-A<em>207 and HLA-B</em>51 with DHF, HLA-B<em>44, B</em>62, B<em>76, and B</em>77 with protection from developing clinical disease</td>
<td></td>
</tr>
<tr>
<td><strong>India 2007</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>197 pts, 100 controls</td>
<td>90 DF</td>
<td>109 P</td>
</tr>
<tr>
<td><strong>FINDINGS:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Associated TAP1 333 ILE/VAL and HPA1a/a1 and HPA2a/2b with DHF.</td>
<td></td>
</tr>
<tr>
<td><strong>Thailand 2009</strong>&lt;sup&gt;c&lt;/sup&gt;</td>
<td>435 children</td>
<td>65 s.c</td>
<td>69 P</td>
</tr>
<tr>
<td><strong>FINDINGS:</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Associated TNF-238A together with HLA-B<em>48 or B</em>57 with DHF</td>
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</tr>
<tr>
<td><strong>Vietnam 2011</strong>&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2008 DSS, 2018 controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FINDINGS:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Found MICB and PLCE1 to be susceptibility loci for susceptibility to DSS</td>
<td></td>
</tr>
<tr>
<td><strong>Jamaica 2011</strong>&lt;sup&gt;e&lt;/sup&gt;</td>
<td>50 dengue, 177 healthy</td>
<td>45 DF</td>
<td>5 DHF</td>
</tr>
<tr>
<td><strong>FINDINGS:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Found that HLA-A*24 associated with DF</td>
<td></td>
</tr>
<tr>
<td><strong>Cuba 2011</strong>&lt;sup&gt;f&lt;/sup&gt;</td>
<td>104 adults</td>
<td>68 DF</td>
<td>36 DHF</td>
</tr>
<tr>
<td><strong>FINDINGS:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Found that MICA<em>008 and MICB</em>008 associate with susceptibility to illness but greater likelihood of DF then DHF.</td>
<td></td>
</tr>
<tr>
<td><strong>Brazil 2012</strong>&lt;sup&gt;g&lt;/sup&gt;</td>
<td>109 pts</td>
<td>67 DF</td>
<td>42 DHF</td>
</tr>
<tr>
<td><strong>FINDINGS:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Associated HLA-A*01 with DHF</td>
<td></td>
</tr>
<tr>
<td><strong>Brazil 2013</strong>&lt;sup&gt;h&lt;/sup&gt;</td>
<td>104 pts, 172 controls adults</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FINDINGS:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Associated KIR2DS1, KIR2DS5, KIR2DL5, KIR3DS1-Bw4, KIR3DL1-Bw4, KIR2DL1-C2, KIR2DS1-C2, KIR2DL3-C1/C1 with dengue</td>
<td></td>
</tr>
<tr>
<td><strong>Brazil 2013</strong>&lt;sup&gt;i&lt;/sup&gt;</td>
<td>187 pts with DENV-3</td>
<td>120 DF</td>
<td>66 P</td>
</tr>
<tr>
<td><strong>FINDINGS:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Associated HLA-B*44 with DHF in patients with DENV-3</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Study location and reference

<sup>b</sup> Number of subjects enrolled in the study, divided into dengue patients and healthy controls when applicable, noted if the study is of children or adult subjects.

<sup>c</sup> Number of subjects with DF vs DHF(or DSS as noted),

<sup>d</sup> Number of subjects with Primary (P) versus secondary (S) DENV infection s.c.=subclinical.
HLA class I allele associations with dengue disease severity have been reported by a number of epidemiological studies providing additional support for a role for CD8\(^+\) T cells in contributing to clinical outcome\(^{88-91}\). Stephens et al. found that, in the Thai population, HLA-A*0207, B*51, B*46, and A*11 associate with DHF susceptibility, while HLA-A*0203, B*52, B*44, B*62, B*76, and B*77 associate with DF\(^89\). An extended HLA haplotype that included \(TNF-\alpha\) and \(LTA-3\) alleles together with HLA-B*48 and HLA-B*57 was more prevalent in patients with secondary DHF compared to the general Thai population \(^90\). Recently, Hertz et al. demonstrated that higher HLA class I binding scores for DENV proteomic regions that are conserved among flaviviruses correlates with protection from DHF supporting a role for CD8\(^+\) T cells in protective responses to DENV infection\(^141\).

Recent studies have used peptide-MHC (pMHC) tetramers to investigate the kinetics of expansion and activation of DENV-specific CD8\(^+\) T cells during acute DENV infection and convalescence. However, there are a limited number of CD8\(^+\) T cell epitopes which have been identified (Table 1.3). Friberg et al., looked at frequencies of an HLA-A11 restricted epitope NS3\(_{133-142}\) in subjects with DF versus those with DHF; patients with all four DENV serotypes were represented. They found A11-NS3\(_{133-142}\)-specific T cell expansion did not correlate with disease severity\(^134\). A similar lack of association between the frequency of A11-NS3\(_{133-142}\)-specific T cells and disease severity was reported in two studies in Vietnam\(^{116,117}\). A strength of these studies is the information on clinical profile, viral isolation and HLA typing in individuals with primary and secondary DENV infection\(^{116,117,134}\).
Table 1.3 CD8⁺ T cell epitopes recognized by virus-specific CD8⁺ T cells

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amino acidsᵃ</th>
<th>Sequenceᵇ</th>
<th>MHCᶜ</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>22-31</td>
<td>RVSTVQQLTK</td>
<td>A03/11</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>107-115</td>
<td>CLIPTAMAF</td>
<td>B15</td>
<td>143</td>
</tr>
<tr>
<td></td>
<td>107-115</td>
<td>MLIPTAMAF</td>
<td>B35</td>
<td>143</td>
</tr>
<tr>
<td>prM</td>
<td>133-141</td>
<td>FTLAFLAH</td>
<td>B35</td>
<td>126</td>
</tr>
<tr>
<td>E</td>
<td>211-219</td>
<td>FFDLLPWT</td>
<td>A02</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>297-306</td>
<td>MSYSMCTGKF</td>
<td>B35</td>
<td>143</td>
</tr>
<tr>
<td></td>
<td>414-422</td>
<td>ILGDTA WDF</td>
<td>B07</td>
<td>130</td>
</tr>
<tr>
<td>NS2a</td>
<td>198-206</td>
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<td>KLAEAIFKL</td>
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a Sequence positions vary slightly between strains.

b Sequence as reported by the cited reference. These sequences do not necessarily reflect the minimal epitope. As sequences vary between serotypes and strains these epitopes may not represent the sequence found in prevalent circulating strains.

c HLA restriction was not confirmed in all studies and some were based on peptide binding predictions.

Adapted from Mathew et al. Future Microbiol. 2014
While peak tetramer frequencies during acute infection did not appear to correlate with disease severity based on these studies, other studies have reported higher frequencies of DENV-specific T cells in patients with DHF 2 weeks\textsuperscript{131, 132} and 6 months\textsuperscript{129} post-infection. Differences between these study cohorts such as, timing of sample collection and differences in infection history (e.g., serotype of primary and secondary infection) may explain the differences in results between these studies. These findings are limited by the small number of samples tested during capillary leakage, the inability to look at CD8\textsuperscript{+} T cell responses at sites of infection outside the blood, and the processing and freezing of samples often required which could eliminate cells of interest from the sample. However, the lack of a correlation with disease severity, and the timing of peak TET\textsuperscript{+} T-cell frequencies in early convalescence rather than at the time of plasma leakage, suggest that the frequency of A11-NS3\textsubscript{133-142} TET\textsuperscript{+} T cells may not be the principal determinant of disease. Data from Friberg et al. suggest that responses to other epitopes, such as the B7 restricted epitope NS3\textsubscript{222-230}, may contribute to disease severity to a greater extent than the response to NS3\textsubscript{133-142}.

Other characteristics of the DENV-specific T cell response, such as the quality of the effector response, may be more important than the quantity of epitope-specific cells\textsuperscript{144}. Evaluating the quality of effector responses in peripheral blood mononuclear cell (PBMC) samples obtained from dengue patients can be difficult. Many groups have used the expression of surface markers such as CD69, CD38, HLA-DR, in an attempt to identify qualitative differences in T cell responses. In the study by Friberg et al. no significant correlations were seen between the expression of CD38 (a marker of
activation) or phenotypic markers on A11-NS3\textsubscript{133-142}-specific T cells and disease severity\textsuperscript{134}. It appears that neither the quantity nor the quality of responses to NS3\textsubscript{133-142} associated with disease severity\textsuperscript{117, 134}.

Studies focused on DENV-specific T cells have found varying levels of cytokine production and cytotoxic activity in CD8\textsuperscript{+} T cells. The production of IFN-\(\gamma\), TNF-\(\alpha\), and macrophage inflammatory protein 1\(\beta\) (MIP-1\(\beta\)) by CD8\textsuperscript{+} T cells was dependent on the peptide sequence of the stimulating epitope which typically varies between DENV serotypes\textsuperscript{118, 120, 144}. Stimulation of CD8\textsuperscript{+} T cell lines with peptides from different serotypes of the same epitope has highlighted how strikingly different cytokine and cytolytic responses can be to peptides that vary even by a single amino acid\textsuperscript{120}. Simmons et al. demonstrated significant IFN-\(\gamma\) responses to 47 DENV-2 peptides in PBMC of Vietnamese patients during secondary DENV infection, though they found only a weak correlation with the extent of plasma leakage\textsuperscript{130}. Most recently, Duangchinda et al. showed higher frequencies of TNF-\(\alpha\) and/or IFN-\(\gamma\) -producing CD8\textsuperscript{+} T cells in response to DENV peptides in PBMC collected during acute dengue illness from patients with DHF versus those with DF\textsuperscript{132}. These studies suggest that not only high levels of T cell activation but also the effector response may contribute to DENV pathogenesis.

\textit{iii. Role of T cells in Protection against Dengue Virus Infections}

Very few studies have examined the role of T cells in the protection of DENV infections. Determining the role of CD8\textsuperscript{+} T cells in protecting humans from DENV infection and subsequent dengue disease is complicated by the need for PBMC to be
collected prior to infection and limited by the inability to manipulate CD8$^+$ T cells in human subjects. Therefore, whether CD8$^+$ T cells contribute to protection against DENV infection and dengue disease remains unknown, though several studies have assessed the T-cell frequencies and responses in PBMC collected prior to a secondary DENV infection.

Mangada et al. compared the T-cell responses of the pre-secondary infection PBMC responses of patients who were hospitalized during their subsequent DENV infection to those of patients who were not hospitalized$^{149}$. IFN-$\gamma$ production in response to the infecting serotypes was significantly more common among patients who were not hospitalized. In a study performed by Hatch et al. the level of CD8$^+$ T-cell activity in pre-illness PBMC was compared between subjects who subsequently developed a subclinical secondary DENV infection who had a symptomatic secondary infection$^{133}$. They found higher frequencies of cytokine-producing (TNF-$\alpha$, IFN-$\gamma$, IL-2) CD8$^+$ T cells in patients who did not develop symptomatic infection. Gunther et al. studied cellular immune responses in recipients who received a candidate tetravalent vaccine and were subsequently challenged with infectious DENV. They found that in vitro IFN-$\gamma$ responses mediated by DENV-specific CD8$^+$ T cells in the peripheral blood were associated with protection against fever and/or viremia$^{150}$.

It is not completely clear how these findings of higher cytokine producing potential in patients who go on to develop mild cases of dengue can be reconciled with the studies discussed above which found higher levels of many of the same cytokines, such as IFN-$\gamma$ and TNF-$\alpha$, in subjects with DHF than in subjects with DF. Comparison of
these studies is complicated by differences in the make-up of the cohorts and differences in how cytokines were evaluated, either as measured levels in patients’ serum or as the potential of PBMC to produce these cytokines. There is a need for study cohorts which can address all of the above observations simultaneously.

Though the majority of T cell studies have been performed using samples obtained from human clinical cohorts, mouse models have also been used to study T cell responses to DENV. These models have significant limitations, however, a number of studies in mice have highlighted the importance of T cells in protection from DENV. Immunization of IFN-α/β receptor knockout mice with either CD8⁺ or CD4⁺ T cell epitopes enhanced viral clearance¹⁵¹, ¹⁵². Additionally, depletion of CD8⁺ T cells but not CD4⁺ T cells in mice resulted in higher viral loads¹⁵¹, ¹⁵² and negated protection against a lethal strain provided by prior immunization¹⁵³, ¹⁵⁴.

There is no single metric to identify a protective CD8⁺ T cell response. Nevertheless, it is clear that some CD8⁺ T cell responses will be protective and other CD8⁺ T cell responses likely contribute to dengue pathogenesis. The generation of multifunctional T cells with high-quality responses may be protective, while the generation of T cells with lesser-quality responses is considered suboptimal¹⁵⁵. The presence of cross-reactive DENV-specific CD8⁺ T cells, which have been shown to have quantitative and qualitative differences in degranulation and cytokine responses to variant peptides¹³¹, ¹⁴⁴, suggest the possibility that CD8⁺ T cells with multi-functional responses to the primary infection will mount lesser-quality responses to a secondary infection.
iv. Characterization of $CD^8^+$ T Cells by Flow Cytometry

A number of cell surface markers have been used to characterize the phenotype and function of $CD^8^+$ T cells. Before a T cell encounters antigen and becomes activated, it is considered a “naïve” T cell expressing CD45RA and CCR7, the latter of which allows these cells to traffic into lymph nodes\textsuperscript{156}. After encountering antigen, activated T cells downregulate expression of CCR7 and CD45RA, and new markers become prominent. Some of the markers used to identify activated T cells include Ki-67, HLA-DR, CD69, and CD38. Ki-67 is a marker of cell proliferation present in the nucleus only during cell cycling\textsuperscript{157}. HLA-DR is expressed on cycling cells\textsuperscript{158}. CD69 and CD38 are upregulated on activated $CD^8^+$ T cells\textsuperscript{159-161}. CD69 may function as a costimulatory receptor to enhance proliferation of activated T cells\textsuperscript{162, 163}, though T cells were shown to proliferate normally in CD69\textsuperscript{-/-} mice\textsuperscript{164}. CD38 is thought to have many functional roles for T cells including transmission of activating signals leading to increased intracellular Ca\textsuperscript{2+} and cytokine production\textsuperscript{165-167}.

After resolution of infection some activated T cells will go on to become memory cells. Memory T cells are typically grouped into central or effector memory pools based on their phenotype. CD45RA can reappear on memory T cell populations so it cannot be used alone to identify naïve T cells\textsuperscript{156}. Another marker used to identify memory T cells is CD57. CD57 was thought to be a marker of T cell senescence, but recent work demonstrated that CD57\textsuperscript{+} $CD^8^+$ T cells are capable of expansion\textsuperscript{168}, contain high levels of granzyme and perforin, and have the ability to produce high levels of cytokines\textsuperscript{168}. In neuroinvasive West Nile virus (WNV) infection in humans, an increased percentage of
CD45RA$^+$ CD57$^+$ T cells was observed, compared to those whose WNV infection was not neuroinvasive$^{169}$, suggesting that CD57$^+$ T cells may play a role in flavivirus immunopathology.

While Ki-67 is used to mark proliferating cells, detecting its expression requires permeabilization of the cells, which can affect staining of surface markers$^{170}$. The transferrin receptor, CD71, is also thought to be upregulated on dividing cells since iron is necessary for cell division$^{171}$. For T cells, CD71 has an additional role participating in T cell receptor (TCR) signaling. CD71 is rapidly trafficked to the immune synapse and participates in the phosphorylation of TCR$\zeta$$^{172}$. Hence, CD71 should also mark T cells that have recently encountered antigen. The expression of CD71 on T cells has yet to be studied in acute viral infections. CD71 has, however, been used as a marker of activation when studying CD8$^+$ T cell responses to tumors$^{173,174}$, CD8$^+$ phenotypes in persistent cytomegalovirus (CMV)$^{175}$, and human immunodeficiency virus (HIV)$^{176}$. CD71 has also been used as a marker of CD8$^+$ T cell proliferation in a mixed lymphocyte reaction$^{177}$.

E. Animal Models of Dengue

The lack of a reliable animal model for studying DENV pathogenesis has limited the study of how early immunological responses may affect the outcome of DENV infection$^{178}$. Without an animal model of pathogenesis it has also been difficult to study individual factors that contribute to dengue pathogenesis. Current studies of factors potentially affecting DENV infection outcomes can only be done using ex vivo samples from acutely ill human subjects, or using in vitro cell culture models. While there is not
an animal model of pathogenesis, there are a number of animal models of DENV infection each with significant limitations\textsuperscript{179, 180}.

DENV does not establish viremia in wild-type mice. IFN-α/β receptor-deficient mice can be infected, but only a limited number of adapted DENV strains cause disease in these animals disease occurs during viremia\textsuperscript{181}. More recently, humanized mice have been used to overcome deficiencies in other mouse models. While these mice do become viremic and develop disease that mimics human dengue illness\textsuperscript{182}, human immune responses need to be improved\textsuperscript{183, 184}. Additionally, each type of humanized mouse model has its own limitation(s) with respect to the development of a functional human immune system.

Non-human primates, including several species of monkeys, have also been used to study DENV infection, ADE, and candidate DENV vaccines\textsuperscript{185-190}. Viral replication, neutralizing antibodies, and T cell responses have been routinely observed, but there is only limited evidence of disease or hematologic abnormalities\textsuperscript{191-193}. Non-human primates have been used with some success to study responses to tetravalent vaccines, demonstrating protection against subsequent infections, and allowing the study of vaccines that are too reactogenic to be studied in humans\textsuperscript{194-196}. However, the use of non-human primate models involves significant costs.

**F. Innate Responses to Dengue Virus**

Elucidating the role of the innate immune system in either the resolution of DENV infections or pathogenesis of dengue disease is challenging. DENV, like other
single-stranded (ss)RNA viruses, activates the innate immune system through recognition by toll-like receptors (TLRs) 3, 7, and 8, retinoic acid-inducible gene I (RIG-I), and melanoma differentiation-associated protein 5 (MDA5)\textsuperscript{197}. However, as noted earlier, the DENV proteins NS2A, NS4A, NS4B, and NS5 can prevent the production and/or signaling of type I IFNs\textsuperscript{31-34}. Cytokines associated with T cells, such as IFN-$\gamma$ and TNF-$\alpha$, can also be produced by innate cells, such as monocytes, DCs, and NK cells, during DENV infection\textsuperscript{198, 199}. While innate responses to DENV are probably important and may contribute to disease, these innate responses are not well understood. Innate responses are early responses that are often well underway when patients appear at the clinic. The study of innate immune responses in human subjects would therefore likely require samples taken before they become symptomatic.

Antigen presenting cells (APCs) are a key component of the innate immune system and are targets of DENV infection leading to cytokine production, cell activation, and maturation\textsuperscript{199-201}. The production of interleukin (IL)-6, IL-8, inducible protein (IP)-10, and TNF-$\alpha$ by DENV-infected monocytes has been reported to correlate with maximum virus production\textsuperscript{201}. Interestingly, infection of DCs by DENV can lead to impaired DC maturation and subsequently decreased T cell proliferation, suggesting that innate immune responses are crucial to the development of some adaptive T cell responses\textsuperscript{202}.

\textit{i. NK Responses to Dengue Virus Infection}

NK cells also play a role in the innate response to DENV infection. Kurane et al. identified a sub-population of NK cells which lysed DENV-infected target cells in the
presence of an anti-DENV antibody\textsuperscript{203}. They observed heterogeneity in the NK cell population, but the mechanism by which NK cells recognized DENV-infected cells was not identified\textsuperscript{203}. Subsequent research identified CD16\textsuperscript{+} NK cells as being responsible for antibody-mediated cytotoxicity\textsuperscript{204}. Yoshida et al. transiently depleted tamarins of CD16\textsuperscript{+} NK cells and saw no significant effect on the plasma levels of DENV or the immune response to DENV as assessed by the presence of anti-DENV antibodies\textsuperscript{205}. However, they conducted this experiment in animals undergoing a primary infection, and did not address whether CD16\textsuperscript{-} NK cells may play a role in controlling viremia during acute DENV infection. Other studies have noted the activation of NK cells during acute DENV infection. Azeredo et al. linked increased frequencies of NK cells during acute DENV infection with mild dengue disease\textsuperscript{206}. They reported expression of the activation markers CD69, HLA-DR, and CD38 on NK cells during acute DENV infection. In contrast, Green et al. found higher frequencies of circulating NK cells expressing CD69 among children who developed DHF compared to those with mild disease\textsuperscript{115}. Kuo et al. found elevated serum levels of liver enzymes AST in 93\% and ALT in 82\% of dengue patients in their cohort. Based on these data Sung et al. used a mouse model of DENV infection to determine which cell types might be responsible for this liver damage. They found that NK cell infiltration of the liver peaked one day following infection while CD8 T cell infiltration peaked five days following infection\textsuperscript{207}.

\textit{ii. Upregulation of MHC Class I following Dengue Virus Infection}

One possible mechanism by which DENV-infected cells might escape lysis by NK cells is mediated by inhibitory receptors which bind MHC class I on the surface of
infected cells. Flaviviruses, including DENV, have been reported to upregulate MHC class I expression\textsuperscript{208-210}. Which is in contrast to most other viral infections that downregulate MHC class I\textsuperscript{211}. Libraty et al. reported upregulation of MHC class I on DENV-infected DCs and even more upregulation on uninfected bystander DCs present in the infected culture\textsuperscript{212}. Warke et al. showed that DENV-infected muscle satellite cells decrease MHC class I expression while bystander cells increase MHC class I expression\textsuperscript{213}. The mechanism by which flaviviruses upregulate MHC class I expression is not known. Several possible mechanisms have been proposed, including increased transporter associated with antigen processing (TAP)-mediated\textsuperscript{209} peptide translocation into the endoplasmic reticulum (ER)\textsuperscript{214} and increased HLA promoter activity\textsuperscript{210}. Work with WNV has shown increased nuclear factor-κB (NFκB) activity leading to increased MHC transcription in infected cells\textsuperscript{215}. These studies suggest that MHC class I expression may vary in response to DENV infection through multiple mechanisms and perhaps can vary based on cell type, the HLA genotype of the infected individual, and infectious burden per cell. DENV infection can also trigger activation of NK cells by upregulating the expression of activating ligands in response to the stress of viral infection. Additionally, the flavivirus E protein may activate NK cells directly through binding to the activating receptor NKp44\textsuperscript{216}.

The understanding of NK cell function has expanded dramatically since these studies of NK cell responses to DENV were performed. Of special note are the discovery of NK cell memory\textsuperscript{217-221} and our expanded understanding of how NK cells interact with the adaptive immune response, which are discussed further below.
G. Overview of NK Cell Responses to Acute Viral Infections

NK cells were first identified in the 1970s as ‘non-specific’ lymphocytes capable of killing cancer cells and virally-infected cells\textsuperscript{222-224}. While NK cells are part of the lymphocyte lineage, they are usually considered a member of the innate immune system. As our understanding of NK cells has improved, however, it has become increasingly clear that NK cell responses to viral infections are more complicated than initially recognized and have characteristics consistent with both innate and adaptive immune responses.

NK cells are CD3\textsuperscript{-} lymphocytes identified by CD56 and CD16 expression and can be CD56\textsuperscript{+} and/or CD16\textsuperscript{+}\textsuperscript{225}. Human NK cells are very heterogeneous, with each cell carrying a number of different activating and inhibitory receptors on their surface (Table 1.4), which work in concert to control the response to any given stimulus. A number of surface markers have been used to classify NK cells. CD57 marks fully mature NK cells that are highly cytotoxic but proliferate less robustly\textsuperscript{226}. CD69 and CD38 are markers of activation upregulated on activated NK cells. However, they also function as activating receptors that can further promote proliferation and activation of NK cells\textsuperscript{227-232}. While these receptors mark the ability of NK cells to respond to a given stimulus, they do not necessarily provide information about the current activation status of the cell.

NK cell activation requires signaling through multiple receptors, with the exception of signaling through CD16 (Figure 1.1)\textsuperscript{233,234}. CD16 provides a strong activating signal to NK cells and can independently activate NK cells even in the presence of inhibitory signals\textsuperscript{234}. Inhibitory receptors are thought to be particularly
Figure 1: Activation of NK Cells. NK cells which carry inhibitory receptors are lessened to respond and do so when the activating signals received overwhelm the inhibitory signals received. The viruses infected APCs are believed to activate healthy AcC, while no activation occurs in the absence of activating ligands. Activating ligands include HLA-B57 (self peptide) and activating receptor KIR3DL1.
important in the development of NK cells by “licensing” NK cells to respond to activating signals received later on\textsuperscript{235}. The strength of an inhibitory signal is proportional to the strength of the response to subsequent activating signals\textsuperscript{236}. A major set of inhibitory receptors important for NK cell licensing are the killer cell immunoglobulin-like receptors (KIRs). While only some KIR ligands are known, all are thought to be class I MHCs (Table 1.4).

Epidemiological studies of immunological events, including responses to infectious diseases, autoimmune diseases, multiple miscarriages, and tumor responses, have implicated KIR/MHC interactions in protective or pathological roles\textsuperscript{237-243}. Studies of hepatitis C virus (HCV) highlight this possibility for protective or pathologic relationships during a viral infection. In Brazil, HLA-C and its associated KIRs have been linked epidemiologically to the development of liver damage in HCV patients\textsuperscript{244, 245}. In contrast, in a study in Puerto Rico of intravenous drug users at high risk for contracting HCV, the presence of HLA-C1 and KIR2DS4 and KIR2DL2 and/or KIR2DL3 were found to highly correlate with being HCV negative\textsuperscript{246}. One of the most well studied relationships between a KIR and an HLA molecule is the interaction of KIR3DL1 and HLA-B57 which is associated with long-term non progression in HIV infected individuals\textsuperscript{237, 247-253}. Further details are provided in Chapter IV.

NK cell responses are affected by previous stimulation, either through modification of the receptor repertoire or of the sensitivity of the receptors, and the interplay between signals from different receptors. O’Leary et al. demonstrated that NK
Table 1.4: **Known activating and inhibitory NK cell receptors and their ligands**

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<td>258</td>
</tr>
<tr>
<td>KIR2DS3</td>
<td>HLA-C</td>
<td>Activating</td>
<td>259</td>
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<tr>
<td>KIR2DS4</td>
<td>HLA-Cw4</td>
<td>Activating</td>
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<tr>
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<td>HLA-Bw4</td>
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<tr>
<td>KIR3DL2</td>
<td>HLA-B27, HLA-A3, HLA-A11</td>
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<td>260, 261</td>
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<tr>
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<td>HLA-Cw4</td>
<td>Inhibitory</td>
<td>262</td>
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<tr>
<td>KIR3DS1</td>
<td>HLA-Bw4?</td>
<td>Activating</td>
<td>262</td>
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<tr>
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<td>CD48</td>
<td>Activating or Inhibitory, coreceptor</td>
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<td>LILRB1</td>
<td>HLA class I</td>
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<td>IgG</td>
<td>Activating</td>
<td>266</td>
</tr>
<tr>
<td>CD94-NKG2C</td>
<td>HLA-E</td>
<td>Activating</td>
<td>262, 267</td>
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<tr>
<td>CD94-NKG2E</td>
<td>HLA-E</td>
<td>Activating</td>
<td>267</td>
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<tr>
<td>NKG2D</td>
<td>MICA, MICB, ULBP</td>
<td>Activating</td>
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</tr>
<tr>
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<td>269</td>
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<td>NKp44</td>
<td>DENV protein E, viral hemagglutinins</td>
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<td>216, 270</td>
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<td>271</td>
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<td>CD161</td>
<td>LLT1</td>
<td>Activating and Inhibitory</td>
<td>272, 273</td>
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<tr>
<td>CD38</td>
<td>CD31</td>
<td>Activating</td>
<td>229, 230, 232</td>
</tr>
</tbody>
</table>

<sup>a</sup>Receptor present on the surface of NK cells  
<sup>b</sup>Ligand(s) bound by the NK cell receptor  
<sup>c</sup>Functional effect of receptor signaling in NK cells
cells are responsible for delayed type hypersensitivity responses to haptens in sensitized mice for up to four weeks\textsuperscript{218}. Strikingly, this hypersensitivity was transferred to an unsensitized host through adoptive transfer of NK cells\textsuperscript{220, 221}. Viral antigens can also induce recall responses by NK cells that can protect against subsequent viral challenge\textsuperscript{217-219}. These findings suggest that NK cells are capable of altered responses for a period of time following infection. Notably, this NK cell “memory” is not the classic, recombination-based memory that is the hallmark of adaptive immunity.

NK cells have also been implicated in shaping the adaptive response to viral infections in a number of ways including promoting maturation or elimination of DCs, perforin-dependent elimination of CD$^8^+$ T cells, and cytokine production\textsuperscript{274}. It is unclear whether NK cell lysis of DCs only affects virally-infected cells or is in fact a mechanism of immune modulation. The most extensive work on the potential of NK cells to modulate T cell responses directly or indirectly, apart from lysing virally-infected cells, was done by Waggoner et al.\textsuperscript{275, 276}.

Waggoner et al. used the model of lymphocytic choriomeningitis virus (LCMV), which is not susceptible to direct control by NK cells as evidenced by the lack of change in viral replication in mice devoid of CD$^4^+$ and CD$^8^+$ T cells following NK cell depletion. Normally high dose LCMV clone 13 infection results in minimal pathology and establishment of chronic infection, medium dose challenge results in substantial pathology and 23% mortality, and low dose challenge results in minimal pathology and clearance of LCMV. Waggoner et al. showed that depletion of NK cells prior to high dose infection with LCMV clone 13 led to severe immunopathology and death, rather
than the chronic infection established in the presence of NK cells\textsuperscript{275}. In the presence of NK cells and high levels of antigen CD8\textsuperscript{+} T cells become exhausted minimizing their damage. This CD8\textsuperscript{+} T cell exhaustion was governed indirectly by NK cell-mediated depletion of CD4\textsuperscript{+} T cells. The authors later investigated how depletion of NK cells after the establishment of chronic infection affected CD8\textsuperscript{+} T cell responses, immunopathology, and viral levels. They found that delayed NK cell depletion resulted in increased CD8\textsuperscript{+} T cell activity and resolution of infection with minimal immunopathology. This work suggests that altering NK cell responses in patients with chronic infection may improve the clinical outcome. Waggoner et al. also showed that depletion of NK cells even as late as day 10 or day 13 following high dose infection improved disease outcomes. The data suggest that NK cells continue to participate in immune modulation well after initial infection, when NK cells are traditionally thought to be active. This is important for the treatment of human patients with acute or chronic infections, as they typically present to clinic well after the initial infection, and any therapeutic intervention therefore needs to modulate outcomes at later time points.

Another group demonstrated that rapid innate control of virus can prevent the development of an adaptive response to viral infection\textsuperscript{277}. While this interplay between adaptive and innate immune systems can be very important for certain viral infections that are well controlled by NK cells this is not truly NK cell control or modification of the adaptive response. NK cells can also directly kill activated CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells through a perforin-mediated mechanism\textsuperscript{277}. Lastly, NK cells are important contributors to the overall cytokine profile of the antiviral innate immune response, which subsequently
directs T cell responses\textsuperscript{278}. These studies demonstrate that multiple mechanisms exist by which NK cells can directly shape adaptive immune responses.

NK cells can also alter DC responses to infection and promote DC maturation\textsuperscript{279-281}. Additionally, NK cells can lyse immature DCs, this lysis was decreased if DCs where matured by exposure to a strong stimulant such as LPS prior to incubation with NK cells\textsuperscript{282}. Ferlazzo et al demonstrated that while both immature and mature DCs, as assessed by the expression of CD80, CD86, CD83, and HLA-DR, were able to upregulate the expression of CD69 on NK cells, only NK cells co-cultured with mature DCs gained the ability to autolyse immature DCs\textsuperscript{283}. NK cell contact-mediated lysis of immature DCs is mediated by the upregulation of ligands for NK cell activating receptors such as NKp30 on immature DCs\textsuperscript{284}. This interplay between DCs and NK cells functions to mature DCs and remove DCs which have not fully matured but this relationship may be altered in chronic infections like HIV\textsuperscript{285}. Alter et al. showed that high levels of IL-10 lead to increased susceptibility of mature DCs to NKG2D-dependent elimination by NK cells\textsuperscript{286}. Interestingly, poor NK cell activity leads to poor maturation of DCs and a reduced ability of NK cells to eliminate immature DCs\textsuperscript{287, 288}. This provides another mechanism by which NK cells may affect the adaptive immune response to an acute viral illness such as DENV.
H. Thesis Objectives

An individual’s HLA haplotype has been linked to shifts in the probability of developing DHF during a secondary infection. Extended human HLA haplotypes containing TNF-4 and LTA-3, together with HLA-B*48, HLA-B*57, and HLA-DPB1*0501, were detected only in patients with secondary DHF. This thesis sought to characterize CD8⁺ T cell responses in HLA-B57⁺ individuals to a highly conserved DENV epitope during primary and secondary infection in order to better understand how a conserved epitope affects CD8⁺ T cell responses and dengue disease outcome.

We hypothesized that:

Responses to a highly conserved, HLA-B57 restricted, epitope NS126-34 lead to stronger CD8⁺ T cell responses following secondary heterologous DENV infection since the epitope sequence would be identical to that seen in primary infection.

During the course of the dissertation we found binding of a B57-NS126-34 TET to CD8⁺ cells which lead us to additionally hypothesize that:

HLA-B57 molecules expressing the conserved epitope NS126-34 bind the inhibitory receptor KIR3DL1 on NK cells.
This Thesis assessed CD8\textsuperscript{+} T cell and NK cell responses over the course of DENV infection in donors with mild and severe dengue illness and characterized the binding of an inhibitory receptor KIR3DL1 with HLA-B57 expressing a highly conserved NS1 epitope.

The work is presented in two parts:

Chapter III: CD8\textsuperscript{+} T cell Responses to a Novel DENV Epitope During Acute Primary and Secondary DENV Infection.

Questions:

1. Are tetramer frequencies in PBMC obtained from patients during acute secondary DENV infection higher compared to frequencies in PBMC from patients obtained during acute primary DENV infection?
2. Does activation of tetramer positive cells in secondary DENV infection vary in timing or quality from the activation of tetramer positive cells in primary DENV infection?
3. Are peak frequencies of tetramer positive cells different in patients with DHF compared to DF?
4. Is the total CD8\textsuperscript{+} T cell population more activated in patients with DHF?
Chapter IV: The B57-NS1_{26-34} TET Interacts with the Inhibitory Receptor KIR3DL1 on NK cells.

Questions:

1. Does the B57-NS1_{26-34} TET bind KIR3DL1?

2. Does the binding of the B57-NS1_{26-34} TET to KIR3DL1 result in a physiologically functional interaction and subsequent inhibition of KIR3DL1^+ NK cells?

3. Are B57-NS1_{26-34} TET^+ NK cells activated during DENV infection? How does this compare to the activation of the total NK cell population during DENV infection?
CHAPTER II
MATERIALS AND METHODS

A. Study Subjects and Blood Samples.

The study design for patient recruitment and collection of blood samples has been reported in detail elsewhere\textsuperscript{59, 129, 146, 289}. Briefly, the subjects enrolled were Thai children 6 months to 14 years of age with acute febrile illnesses (<72hrs) diagnosed as DF or DHF according to WHO 1997 guidelines\textsuperscript{57}. Serology and virus isolation were used to confirm acute DENV infections, and primary and secondary infections were distinguished based on serologic responses\textsuperscript{59}. For donors undergoing a secondary infection it is not always possible to accurately determine what the previous serotype(s) were. Blood samples were obtained daily during acute illness, once in early convalescence, and at 6 month to 1 year intervals during late convalescence. Informed assent and/or consent was obtained from each subject and/or his/her parent or guardian and the study was approved by the Institutional Review Boards of the Thai Ministry of Public Health, the Office of the U.S. Army Surgeon General and the University of Massachusetts Medical School (UMMS).

The samples were numbered relative to the day of defervescence (designated Fever Day 0). Days prior to or after defervescence were designated fever days -1, -2, etc. or +1, +2, etc. Serologic HLA class I typing was performed on blood from immune Thai donors or healthy UMMS subjects for use as HLA-B57\textsuperscript{+} dengue-naive controls. HLA typing was performed at UMMS or the Department of Transfusion Medicine, Siriraj
Hospital, as previously described \(^{89,146}\). PBMC were isolated by density gradient centrifugation, cryopreserved, and stored at \(-70^\circ C\).

**B. Healthy Donors and Blood Samples**

Blood samples were obtained from healthy donors at UMMS. Serologic HLA class I typing was performed at UMMS. PBMC were isolated by density gradient centrifugation, cryopreserved, and stored at \(-180^\circ C\).

**C. Generation of Peptides**

Peptides were purchased from 21\(^{st}\) Century Biochemicals (Marlboro, MA) at \(>90\%\) purity or BioSynthesis (Lewisville, TX) at \(>95\%\) purity (Table 2.1).

**D. Peptide-MHC Tetramers.**

Peptide-MHC tetramers (pMHC TETs) were generated at the UMMS and the NIAID Tetramer Cores. The different pMHC multimers (Table 2.3) were conjugated to fluorochromes (APC-A11-NS3\(_{133-142}\) TET or Qdot605-A11-NS3\(_{133-142}\) TET, PE-B57-NS1\(_{26-34}\) TET, APC A2-E\(_{213-221}\) TET, PE-B57-TW10n TET, APC-TW10 TET). pMHC TETs were also generated by Dr. David Price (Cardiff Institute of Infection & Immunity, Cardiff, UK) and Dr. Geraldine O’Connor (National Cancer Institute, Bethesda, MD) (APC and PE B57-NS1\(_{26-34}\) TET)
E. Viruses Used

Vero cells were infected with viruses at an approximate multiplicity of infection of 0.5-1 plaque forming unit (PFU) per cell and cultured in minimal essential medium containing 2% fetal calf serum (FCS). After approximately seven days, supernatants were collected and concentrated using ultracentrifugation. DENV virus strains were titered using a modified plaque assay\textsuperscript{290}.

F. Generation of Monocyte Derived Dendritic Cells

Dendritic cells (DCs) were generated from CD14$^+$ monocytes isolated from PBMC by magnetic bead enrichment for CD14$^+$ cells (MACS, Miltenyi Biotec, Auburn, CA) followed by culture with IL-4 (500U/mL) and GM-CSF (800U/mL) in Rosewell Park Memorial Institute cell culture medium (RPMI-1640) with 10% Fetal Bovine Serum (FBS), hence forth referred to as RPMI-10, at 37°C for 7 days as previously reported\textsuperscript{199}.

G. Dengue Virus Infection Protocol

DCs, were washed with serum-free RPMI-1640, and then DENV (serotype/strain as noted, Table 2.2) was added at a multiplicity of infection (MOI) of 1, 5, or 10, with just enough serum-free media to cover the well. After 1.5 hours incubation at 37°C, RPMI-10 was added to fill the well. When required the percentage of DENV-infected cells was determined by flow cytometry using intracellular staining with an antibody against DENV E or prM protein (Table 2.3).
Table 2.1. **Peptides generated for T and NK cell studies**

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Origin(^a)</th>
<th>Sequence(s)</th>
<th>HLA(^b)</th>
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<tr>
<td>E(_{213-221})</td>
<td>DENV-1, DENV-2, DENV-3, DENV-4</td>
<td>FLDLPLPWT, FLDLPLPWL, FFDLPLPWT, FFDLPLPWL</td>
<td>A2</td>
</tr>
<tr>
<td>NS(_{126-34})</td>
<td>DENV 1-4</td>
<td>HTWTEQYKF</td>
<td>B57</td>
</tr>
<tr>
<td>NS3(_{133-142})</td>
<td>DENV-1, DENV-2, DENV-3,4</td>
<td>GTSGPIVNRE, GTSGSPIVDR, GTSGSPIIN</td>
<td>A11</td>
</tr>
<tr>
<td>LF9</td>
<td>Self</td>
<td>LSSPVTKSF</td>
<td>B57</td>
</tr>
<tr>
<td>TW10</td>
<td>HIV</td>
<td>TSTLQEQIGW, TNTLQEQIGW</td>
<td>B57</td>
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<tr>
<td>TW10n</td>
<td>HIV</td>
<td></td>
<td>B57</td>
</tr>
</tbody>
</table>

\(^a\) Origin of the epitope listed  
\(^b\) HLA restriction of the epitope listed

Table 2.2. **Strains of DENV used for infections**

<table>
<thead>
<tr>
<th>Serotype</th>
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<tbody>
<tr>
<td>DENV-1</td>
<td>BRP 04-00</td>
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<tr>
<td>DENV-2</td>
<td>16681</td>
</tr>
<tr>
<td></td>
<td>S-16803</td>
</tr>
<tr>
<td>DENV-3</td>
<td>CH53489</td>
</tr>
<tr>
<td>DENV-4</td>
<td>341750</td>
</tr>
</tbody>
</table>
H. Generation of B-Lymphoblastoid Cell Lines

B-lymphoblastoid cell lines (B-LCLs) were established by infecting PBMC from the donor with Epstein–Barr virus obtained from an infected marmoset cell line (B95-8) that was purchased from the American Tissue Culture Collection (ATCC). CpG (2.5µg/mL) was added to PBMC and cells were cultured with RPMI-10.

I. Generation and Maintenance of CD8^+ T Cell Lines

T cells were cultured with complete RPMI-10 media or TexMACS media (Miltenyi Biotec, Auburn, CA) supplemented with 50U/mL recombinant human (rh)IL-2 (BD Biosciences). T cell lines were generated by stimulating PBMC with either DENV, at an MOI of 1, or 10µg/ml peptide in the presence of 5ng/mL IL-7 (Peprotech). After one week, T cells were seeded at 10 cells per well in a 96 well plate and re-stimulated. T cells were restimulated every other week with 0.1µg/mL αCD3 (12F6, Dr. Johnson Wong, Harvard University, Cambridge, MA) with 50U/mL IL-2 and PBMC, irradiated with 3500rads, used as allogeneic feeders.

J. Cytotoxicity Assay

Cytotoxicity assays were performed as previously described. Briefly, HLA-B57^+ B-lymphoblastoid cell line (BLCLs) target cells were labeled with radio-labeled chromium (^51Cr) and pulsed with 10µg/mL of the indicated peptides or infected with recombinant vaccinia viruses, expressing DENV proteins, at an MOI of 5. Primary DCs from HLA-B57^+ healthy individuals were generated and infected with DENV-1-4 at a
MOI of 5. Peptide-pulsed or DENV-infected target cells were cultured with T cells at an effector-to-target ratio of 10:1. After 4 hours, supernatants were harvested and the $^{51}$Cr content was measured using a gamma counter (Cobra™ II auto-gamma®, Packard Instrument Company, Downers Grove, IL). Percent specific lysis was calculated as follows: % lysis = (experimental $^{51}$Cr release – minimum $^{51}$Cr release)/(maximum $^{51}$Cr release – minimum $^{51}$Cr release) x 100.

K. Peptide Stimulation of CD8$^+$ T Cell Lines.

At day 16 of culture approximately $2 \times 10^5$ CD8$^+$ T cells were cultured with $2 \times 10^4$ HLA matched B-LCLs at 37°C for 0–24 hours. The B-LCLs were pre-incubated for 30 minutes with peptide at the concentrations indicated (0.01μg/mL to 10μg/mL). Cells were washed in phosphate buffered saline (PBS) and stained with antibodies to CD8, CD19, CD69, CD38, and CD71 for 30 minutes at 4°C (Table 2.3). Finally, cells were washed and placed in BD Stabilizing Fixative™ (BD Biosciences) diluted 1:3 and kept at 4°C for analysis.

L. Intracellular Cytokine Staining of CD8$^+$ T cells.

$2 \times 10^5$ CD8$^+$ T cells were mixed with $2 \times 10^4$ HLA-matched BLCLs and peptide or PHA in the presence of anti-CD107a antibodies and BD Golgi Stop/Golgi Plug™ for 6hrs. Cells were washed in PBS and stained with 1μL of 1:80 dilution of the dead cell marker LIVE/DEAD® Green (Molecular Probes, Invitrogen Corp.). Cells were washed with FACS Buffer (PBS/2%FBS/0.1% sodium azide) and incubated with surface antibodies to
CD3, CD8, and CD19 and incubated at 4°C for 30 minutes (Table 2.3). The cells were washed with 2mL of FACS buffer and then fixed and permeabilized using BD Cytofix/CytoPerm™ for 20 minutes at 4°C. The cells were washed with 1mL of BD Perm Wash buffer™ in preparation for intracellular staining. The antibodies to IFN-γ, TNF-α and MIP-1β (Table 2.3) were added and incubated at 4°C for 30 minutes. Cells were then washed with 1mL BD Perm Wash Buffer™, fixed with 100µL of BD Stabilizing Fixative™ (BD Biosciences) diluted 1:3, and kept at 4°C until flow analysis. Data were collected on a BD FACSARia™ and analyzed using FlowJo version 10.

M. Assessment of Degranulation of KIR3DL1⁺ Versus KIR3DL1⁻ NK cells

1.5x10⁶ PBMC were mixed with 3x10⁵ 721.221 target cells in the presence of anti-CD107a antibodies and BD Golgi Stop™ for 6 hours. Cells were washed in PBS and stained with 1µL of 1:80 dilution of the dead cell marker LIVE/DEAD® Green (Molecular Probes, Invitrogen Corp.). Cells were washed with FACS Buffer and incubated with surface antibodies specific for CD3, CD16, CD56, KIR3DL1, CD14, and CD19 (Table 2.3) and incubated at 4°C for 30 minutes. Cells were then washed with 1mL FACS Buffer and fixed with 100µL of BD Stabilizing Fixative (1:3) and kept at 4°C until flow analysis. Data were collected on a BD FACSARia™ and analyzed using FlowJo version 10.
N. Binding of pMHC TETs to KIR3DL1 Transfected Cell lines

These analyses were performed by Dr. Geraldine O’Connor at National Cancer Institute, Bethesda, MD as reported elsewhere. Briefly, HEK 293 cells were transfected with FLAG-tagged constructs of KIR3DL*001, *005, or *015. Anti-FLAG antibody was used to verify KIR3DL1 expression. Transfected cells were pre-incubated with 10µg/µl blocking DX9 antibody or control IgG. Cells were then stained with 0.25µg of TET (B57-NS1<sub>26-34</sub> or B57-LF9).

O. KIR3DL1<sup>+</sup> NK Cell Depletion and B57-NS1<sub>26-34</sub> TET Staining

PBMC were isolated from blood drawn from KIR3DL1<sup>+</sup> healthy subjects. PBMC were depleted of KIR3DL1<sup>+</sup> cells by magnetic bead depletion (MACS, Miltenyi Biotec, Auburn, CA). KIR3DL1-depleted PBMC were washed with FACS Buffer and incubated with B57-NS1<sub>26-34</sub> TET for 50 minutes at 4°C. After incubation, the cells were washed with 1mL FACS Buffer, fixed with 100µL BD Cytofix (diluted 1:4) and kept at 4°C until flow analysis. Data were collected on a BD FACSaria™ and analyzed using FlowJo version 10.

P. Blocking of B57-NS1<sub>26-34</sub> TET Binding Using an Anti-KIR3DL1 Antibody

PBMC from a KIR3DL1<sup>+</sup> donor were washed in PBS and stained with 1µL of 1:80 dilution of the dead cell marker LIVE/DEAD® Green (Molecular Probes, Invitrogen Corp.). Cells were washed with FACS Buffer and stained with either the anti-KIR3DL1/KIR3DS1 antibody Z27 or B57-NS1<sub>26-34</sub> TET for 20 minutes at 4°C. Then
B57-NS126-34 TET or Z27 was added to the cells stained with Z27 or B57-NS126-34 TET respectively, was added and incubated for an additional 20 minutes at 4°C. The surface antibodies CD3, CD16, CD56, CD14 and CD19 were then added and incubated for 30 minutes at 4°C. The PBMC were washed with FACS buffer, resuspended in BD Stabilizing Fixative (1:3), and kept at 4°C until flow analysis. Data were collected on a BD FACSARia™ and analyzed using FlowJo version 10.

**Q. Flow Cytometry for the Identification of CD8⁺ T Cells in Thai Study Cohort**

**PBMC**

Cryopreserved PBMC were thawed and washed in RPMI before resting in RPMI-10 at 37°C for 2 hours. Cells were washed in PBS and stained with 1µL of 1:80 dilution of the dead cell marker LIVE/DEAD® Green (Molecular Probes, Invitrogen Corp.). Cells were then washed with FACS Buffer (PBS/2% FBS/0.1% sodium azide) and incubated with 0.5-2µL pMHC tetramer for 20 minutes at 4°C. Monoclonal antibodies specific for CD3 (UCHT1), CD8, CD45RA, CCR7, CD69, CD38, CD57, CD71, CD28 or CD56, CD19, and CD14 were then added to the cells and incubated at 4°C for an additional 30 minutes (Table 2.3). Cells were washed and fixed with BD Stabilizing Fixative™ (1:3) (BD Biosciences) and kept at 4°C until flow analysis. Data were collected on a BD FACSARia™ and analyzed using FlowJo version 10 (Tree Star) and Gemstone (Verity House, Topsham, ME).
R. Flow Cytometry for the Identification of NK Cells in Healthy Donor PBMC

Cryopreserved PBMC were thawed and washed in RPMI before resting in RPMI-10 at 37°C for 2 hours. After incubation, the PBMC were washed in PBS and stained with 1µL of 1:80 dilution of the dead cell marker LIVE/DEAD® Green (Molecular Probes, Invitrogen Corp.) at RT for 20 minutes. Cells were then washed with FACS Buffer (PBS/2% FBS/0.1% sodium azide) and incubated with 0.5-2µL pMHC tetramer for 20 minutes at 4°C. Monoclonal antibodies specific for CD3 (OKT3), CD16, CD56, KIR3DL1, CD161, NKp30, NKp46, NKG2D, CD19, and CD14 were then added to the cells and incubated at 4°C for an additional 30 minutes (Table 2.3). Cells were washed and fixed with BD Stabilizing Fixative™ (1:3) and stored at 4°C until flow analysis. Data were collected on a BD FACSARia™ and analyzed using FlowJo version 10.

S. Statistical Analysis

The Mann-Whitney rank sum test was used to compare two groups for variables that were not normally distributed. We used a cutoff of $p \leq 0.05$ for statistical significance. P values $\leq 0.1$ but $>0.5$ identified non-significant trends. All statistical analyses were performed using GraphPad Prism 6 (La Jolla, CA, USA).
### Table 2.3: Antibodies used for flow cytometry studies

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<th>Marker</th>
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<th>Manufacturer</th>
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<td>V500</td>
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<td>D21-1351</td>
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<tr>
<td>KIR3DL1/S1</td>
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<td>APC</td>
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<td>HLA-A,B,C</td>
<td>W6/32</td>
<td>BD</td>
<td>PE, FITC, APC</td>
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<td></td>
<td>BioLegend</td>
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<td>PE</td>
</tr>
<tr>
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<td>One Lambda</td>
<td>PE-NeutrAvidin (Life Technologies)</td>
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<td>3H5</td>
<td>Millipore</td>
<td>PE, FITC, Alexa647 (indirect staining or conjugated in lab,)</td>
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CHAPTER III

CD8+ T CELL RESPONSES TO A NOVEL DENGUE VIRUS EPITOPE DURING ACUTE PRIMARY AND SECONDARY DENGUE VIRUS INFECTION

Several studies have reported associations between specific HLA class I alleles and DENV disease severity; these epidemiological links support a role for CD8+ T cells in contributing to clinical outcome88-91. Extended human MHC haplotypes containing TNF-4 and LTA-3, together with HLA-B*48, HLA-B*57, and HLA-DPB1*0501, were detected only in patients with secondary DHF 90. HLA-B57 has also been associated with slow progression following HIV infection, the clearance of acute HCV infection292-294 and with a number of type 2 idiosyncratic adverse drug reactions295,296. The relative ability of HLA-B57 to control HIV infection correlated with unique peptide-binding characteristics that affect thymic development of CD8+ T cells297. A larger proportion of the naïve repertoire of T cells restricted by HLA-B57 recognized HIV viral epitopes compared to other HLA alleles. We identified a highly conserved HLA-B57 restricted DENV epitope and utilized PBMC from HLA-B*57 subjects who were undergoing an acute DENV infection to investigate the role of HLA-B57-restricted CD8+ T cells in contributing to the pathogenesis of dengue disease.

A. Identification of a Highly Conserved HLA-B57-Restricted Dengue Virus Epitope

We previously identified HLA-B57-restricted CD8+ T cell lines, which recognized the DENV NS1 or NS2a protein, using convalescent PBMC from a Thai
patient with DF. As shown in Figure 3.1A, two representative T cell lines, 3C11 and 3F2, lysed autologous B-LCLs infected with a recombinant vaccinia virus expressing the DENV-2 NS1/2a proteins. We used pools of overlapping peptides from the NS1 protein and identified a minimal 9mer epitope recognized by these T cell lines corresponding to aa 26-34 (HTWTEQYKF) (Figure 3.1 B, C). Restriction of this epitope by HLA-B57 was confirmed by cytotoxicity assays using partially HLA-matched B-LCLs (data not shown).

We determined the degree of conservation of NS126-34 using the FLAVIdB database (http://cvc.dfci.harvard.edu/flavi/); this epitope had >99% sequence identity across >2600 sequences from all four serotypes of DENV. Comparison to previously identified CD8+ DENV epitopes indicated that this was the only epitope with such a high degree of similarity (Table 3.1).

T cell lines lysed DENV-infected primary dendritic cells from an HLA-B57+ individual (one of four T cell lines shown) (Figure 3.1D) indicating that this epitope can be recognized by T cells in the context of DENV infection. Differences in percent specific target cell lysis likely reflect differences in the percentage of DCs that were infected with each serotype.

For ex vivo analysis of epitope-specific T cells, we obtained an HLA-B*5701/NS126-34 tetramer (B57-NS126-34 TET). We confirmed the specificity of this tetramer by showing binding to the DENV-specific T cell line 3C11, but not to an HLA-B57-restricted HIV-specific T cell line. The DENV-specific T cell line did not bind a previously described HIV-B57 tetramer (TW10-Gag; TSTLQEQIGW) (Figure 3.1E).
Figure 3.1. Identification of the HLA-B57-restricted DENV epitope. (A) Cell lines 3C11 and 3F2, generated from PBMC of donor KPP94-037, were used in a $^{51}$Cr release assay using B-LCLs infected with vaccinia virus recombinants expressing DENV-2 NS1/2a as target cells. (B) $^{51}$Cr release assay using B-LCLs pulsed with peptide pool 1A and individual 15 mer peptides covering pool 1A of NS1. (C) Identification of the minimal 9mer epitope NS1$_{26-34}$ recognized by cell line 3C11. (D) Lysis of DENV-infected DCs by B57-NS1$_{26-34}$-specific cell line 3F11. (E) Validation of B57-NS1$_{26-34}$ TET staining using a B57-NS1$_{26-34}$-specific T cell line and an HIV gag-specific HLA-B57-restricted T cell line.
### Table 3.1: Conservation of amino acid sequences among known CD8+ DENY-specific T cell epitopes

<table>
<thead>
<tr>
<th>Epitope</th>
<th>% Conservation</th>
<th>HLA Restriction</th>
<th>Sequence</th>
<th>CD8+ Epitopes</th>
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<td>95</td>
<td>B55</td>
<td>CD8</td>
<td>001</td>
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<tr>
<td>EPITOP2</td>
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<td>EPITOP5</td>
<td>65</td>
<td>B55</td>
<td>CD8</td>
<td>001</td>
</tr>
</tbody>
</table>

*Note: This table represents the conservation of amino acid sequences among known CD8+ DENY-specific T cell epitopes.*
B. Detection of B57-NS126-34 TET$^+$CD8$^+$ T Cells in PBMC Collected During Acute Dengue Virus Infection

We hypothesized that HLA-B57 restricted, NS126-34-specific CD8$^+$ T cells would be preferentially expanded during secondary infection since the epitope sequence would be identical to that seen in primary infection. To address this hypothesis multi-parameter flow cytometry was used to analyze CD8$^+$ T cell responses in PBMC samples from HLA-B*57$^+$ Thai children with primary or secondary DENV infection$^{289}$. We used this B57-NS126-34 TET together with activation and phenotypic markers and performed a longitudinal analysis of B57-NS126-34-specific T cells in PBMC from HLA-B*57$^+$ subjects. We tested samples obtained at multiple time points during and after acute DENV infection from eleven HLA-B*57$^+$ children, two with primary and nine with secondary DENV infection (Table 3.2).

Each experiment included PBMC from a healthy subject and PBMC from an HLA-B*57$^+$ DENV-naïve subject as a negative control (Figure 3.2 A). As a TET$^+$ control for each experiment, we also included healthy (DENV-naïve) donor PBMC spiked with a T cell line specific for the NS126-34, NS3133-142, or E213-221 epitope (Figure 3.2 B). Figure 3.3A shows our gating strategy. Figures 3.3B and 3.3C show tetramer frequencies for two subjects over time. Subject KPP94-037 had a very high frequency of B57-NS126-34-specific T cells reaching ~20% at fever day +7. Frequencies of B57-NS126-34-specific T cells in subject CHD06-029 were more representative of the staining observed in the remaining donors. Expansion of B57-NS126-34 TET$^+$ T cells during infection with contraction during convalescence was detected in PBMC from every dengue subject.
tested. Peak frequencies ranged from 0.5-20% (Figure 3.3D). Only subject KPP94-037 with secondary DENV infection had high B57-NS1_{26-34}-specific T cell frequencies (Figure 3.3D). Excluding this subject, frequencies of B57-NS1_{26-34} TET^{+} T cells were not higher in those with secondary infection compared to primary infection (Figure 3.3D).

We used tetramers for two other DENV CD8 T cell epitopes (A11-NS3_{133-142} TET or A2-E_{213-221} TET) to compare the frequencies of TET^{+} cells in subjects who were HLA-B*57^{+} and HLA*A11^{+} or HLA*A2^{+} (Figure 3.3E). T cell frequencies were similar for all epitopes in PBMC from the 7 subjects tested.
TABLE 3.2: Clinical, viral and immunogenetic profiles of the Thai study cohort subjects

<table>
<thead>
<tr>
<th>Donor</th>
<th>Serology</th>
<th>Serotype</th>
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<td>DF</td>
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<td>DHF-1</td>
<td>HLA-A33,34 HLA-B57,75</td>
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<td>DENV-2</td>
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<td>HLA-A1,11 HLA-B57,60</td>
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<td>DHF-3</td>
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<td>DHF</td>
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<td>HLA-A203 HLA-B46,57</td>
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<td>DF</td>
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<td>DENV-4</td>
<td>DHF-2</td>
<td>HLA-A1,33 HLA-B57,35</td>
</tr>
</tbody>
</table>

a Primary (P) versus secondary (S) infection as determined by IgM/IgG ratios
b Of current infection. Unknown=could not be determined
c According to WHO guidelines 1997; DF = dengue fever, DHF = dengue hemorrhagic fever grades 1-3
Figure 3.2. Tetramer staining controls. (A) PBMC from DENV naïve HLA-B57⁺, A2⁺, or A11⁺ individuals were stained with B57-NS¹26-34 TET, A2-E²13-221 TET, or A11-NS³133-142 TET. (B) PBMC spiked with the appropriate epitope-specific cell line was stained with B57-NS¹26-34 TET, A2-E²13-221 TET, or A11-NS³133-142 TET.
Figure 3.3. Expansion of DENV specific T cells during acute infection. (A) Gating strategy used to identify TET<sup>+</sup>CD8<sup>+</sup> T cells started by selecting cells within the lymphocyte gate as defined by forward and side scatter profiles followed by gating for singlet cells. Live CD14<sup>-</sup>CD19<sup>-</sup> cells were next selected by exclusion of the viability marker LIVE/DEAD<sup>®</sup> Green along with αCD14-FITC and αCD19-FITC. CD8<sup>+</sup> T cells were identified by CD8 expression. (B) Kinetics of B57-NS1<sub>26-34</sub> TET<sup>+</sup> frequencies in PBMC from donor KPP94-037 and (C) donor CHD06-029 over the course of acute illness and convalescence. (D) B57-NS1<sub>26-34</sub> TET<sup>+</sup>CD8<sup>+</sup> T cell frequencies versus fever day in PBMC from study subjects. Symbols distinguish subjects with primary (n=2, grey symbols) versus secondary (n=9, black symbols) DENV infections and lines distinguish those with DF (n=6, black line) versus DHF (n=5, dashed line). (E) PBMC from subjects who were also HLA*A2- or HLA*A11-positive (n=6) were stained with A2-E<sub>213-221</sub> TET or A11-NS3<sub>133-142</sub> TET. Two of these subjects had primary infections (grey symbols) and one subject had DHF (dashed line). Fever Day is defined from the day of defervescence (Fever Day 0).
C. Antigen-Specific CD8+ T Cells Are Activated during Acute Dengue Virus Infection

Using antibodies to CD69 and CD38, we analyzed CD8+ T cell activation over the course of acute dengue illness. CD69+CD8+ T cells were present early in acute illness with the peak frequencies (10.7%-46.3% of CD8+ T cells) occurring at or before fever day –4 (Figure 3.4A, B). Peak frequencies of B57-NS126-34 TET+CD69+ cells (Figure 3.4C) and A2-E213-221 TET+CD69+ or A11-NS3133-142 TET+CD69+ cells (Figure 3.4D) were 10.5%-48.5% and 15.4-50.3% of TET+ T cells, respectively. CD38 expression peaked later than CD69 expression, on fever days –1 and 0 (Figure 3.4E). Frequencies of CD38+ cells in the total CD8+ population were between 2.45%-57.3%. Peak frequencies of B57-NS126-34 TET+CD38+ cells (Figure 3.4F) and A2-E213-221 TET+CD38+ or A11-NS3133-142 TET+CD38+ cells (Figure 3.4G) were 15.8%-92.4% and 10%-77.8% of TET+ T cells, respectively. The pattern of CD38 and CD69 expression on all TET+ T cells followed the same pattern as the expression on the total CD8+ population.
Figure 3.4. Antigen-specific T cells are highly activated during acute DENV infection and early convalescence. (A) Representative staining of CD69 and CD38 on total CD8+ T cells during acute infection and in convalescence from 1 subject. (B and E) Staining of CD69 and CD38 on total CD8 cells, (C and F) B57-NS126-34 TET+ T cells and A11-NS3133-142 TET+ or (D and G) A2-E213-221 TET+ T cells over the course of acute DENV infection and convalescence, respectively. PBMC from 11 subjects with primary (grey symbols) or secondary (black symbols) infection and DF (black lines) or DHF (dashed lines) were tested.
D. Assessment of CD57-Expression during Acute Dengue Virus Infection

We assessed CD57 expression, a marker of cell exhaustion and cytokine dysregulation, on total CD8 T cells and DENV-specific T cells. Figure 3.5A shows representative staining of CD57 on PBMC from a subject 6 months following infection. On average 15.6% of CD8 T cells expressed CD57 in PBMC from these donors. This expression varied only slightly over the course of DENV infection and was similar during acute infection and at six months and one year post-infection (Figure 3.5B). The mean frequency of B57-NS1\textsubscript{26-34} TET\textsuperscript{+} T cells expressing CD57 was 24.0% (Figure 3.5C). In 3 of 11 donors the frequency of CD57\textsuperscript{+}B57-NS1\textsubscript{26-34} TET\textsuperscript{+} cells was higher during acute infection than at the six months and/or one year time point. The mean frequency of CD57\textsuperscript{+} A11-NS3\textsubscript{133-147} TET\textsuperscript{+} or A2-E2\textsubscript{213-221} TET\textsuperscript{+} T cells was 29.5% of TET\textsuperscript{+} T cells (Figure 3.5D).
Figure 3.5. CD57 expression varies only slightly during DENV infection. (A) Representative staining of CD57 on total CD8⁺ T cells from one donor. (B) Staining of CD57 on total CD8⁺ T cells, (c) B57-NS₁₂₆₋₃₄ TET⁺ cells and (D) A₁₁-NS₃₁₃₋₁₄₂ TET⁺ or A₂-E₂₁₃₋₂₂₁ TET⁺ T cells over the course of acute DENV infection and convalescence. PBMC from 11 subjects with primary (grey symbols) or secondary (black symbols) infection and DF (black lines) or DHF (dashed lines) were tested.
E. Increased Frequencies of CD71-Expressing Cells in the Dengue Virus-Specific B57-NS1\textsubscript{26-34} TET\textsuperscript{+}, A11-NS3\textsubscript{133-147} TET\textsuperscript{+} and A2-E\textsubscript{213-221} TET\textsuperscript{+} CD8\textsuperscript{+} T Cell Populations

We assessed CD71 expression, a marker associated with cell cycle activity\textsuperscript{298}, on total CD8 T cells and DENV-specific T cells. Figure 3.6G shows representative staining of CD71 on PBMC from a subject during acute infection. CD71 expression was low on total CD8\textsuperscript{+} T cells with a mean frequency of 2.1\% during acute illness (fever day -4 through fever day +3) (Figure 3.6A). In contrast, the mean frequency of B57-NS1\textsubscript{26-34} TET\textsuperscript{+} T cells expressing CD71 was 18.4\% and of A11-NS3\textsubscript{133-147} TET\textsuperscript{+} or A2-E\textsubscript{213-221} TET\textsuperscript{+} T cells was 12.2\% during acute illness (Figure 3.6B, C). The mean frequencies of CD71-expressing cells during acute illness were statistically significantly higher in the CD8\textsuperscript{+} DENV-specific T cells compared to the total CD8\textsuperscript{+} population (\(p < 0.0001\), Table 3.3). There were no statistically significant differences in CD71 expression between the B57-NS1\textsubscript{26-34} TET\textsuperscript{+} and the A11-NS3\textsubscript{133-147}/A2-E\textsubscript{213-221}-specific T cell populations.

The peak frequency, as determined for each donor during acute illness, of CD71\textsuperscript{+} DENV-specific CD8 T cells was also significantly higher than that of the total CD8\textsuperscript{+} T cells (\(p < 0.005\)). Frequencies of CD71\textsuperscript{+} DENV-specific T cells remained higher compared to the total CD8 T cell population 1 year following infection (Figure 3.6A, B, C) (\(p < 0.0001\)). While frequencies of CD71\textsuperscript{+} DENV-specific CD8 T cells were high at days 180 and 365, frequencies were lower than the peak CD71 frequencies during acute infection in most donors. Interestingly, mean and peak frequencies of CD38 expression...
during acute illness were significantly higher than during convalescence in B57-NS1\textsubscript{26-34} TET\textsuperscript{+}, but not A11-NS3\textsubscript{133-147} TET\textsuperscript{+} / A2-E\textsubscript{213-221} TET\textsuperscript{+}, T cells. CD69 expression was minimally increased only in A11-NS3\textsubscript{133-147} TET\textsuperscript{+} T cells (Figure 3.4 and Table 3.3). We also compared the geometric mean fluorescence intensity (gMFI) of CD71 expression between populations (Figure 3.6D, E, F) and again found statistically significant differences in the intensity of CD71 staining on the CD71\textsuperscript{+} cells during acute illness between the DENV-specific populations and total CD8\textsuperscript{+} T cells (p<0.05).

Due to the variations in CD71 expression between populations in the Thai study cohort we wanted to know more about the kinetics of CD71 expression in response to the NS1\textsubscript{26-34} epitope. We stimulated the B57-NS1\textsubscript{26-34}-specific T cell line, 3C11, with different concentrations of the NS1\textsubscript{26-34} peptide and measured the intensity of CD71 expression. Figure 3.6H shows representative staining of CD71 expression on cell line 3C11 at 24 hours after stimulation with peptide. We detected CD71 upregulation as early as 1 hr post stimulation with the peptide and the MFI of CD71 expression depended both on the concentration of peptide and the duration of incubation (Figure 3.6I).
Figure 3.6. **CD71 expression on total CD8\(^+\) and DENV-specific CD8\(^+\) T cells.** Frequency of CD71\(^+\) cells in (A) total CD8\(^+\) cells, (B) B57-NS1\(_{26-34}\) TET\(^+\) T cells and (C) A11-NS3\(_{133-142}\) TET\(^+\) or A2-E\(_{213-221}\) TET\(^+\) T cells over the course of acute DENV infection and convalescence. MFI of CD71 expressed on CD71\(^+\) (D) CD8\(^+\) cells, (E) B57-NS1\(_{26-34}\) TET\(^+\) T cells and (F) A11-NS3\(_{133-142}\) TET\(^+\) or A2-E\(_{213-221}\) TET\(^+\) T cells over the course of acute DENV infection and convalescence. (G) Representative staining of CD71 on CD8\(^+\) T cells at fever day -2 from a subject with primary infection. (H) Representative staining of CD71 on a CD8\(^+\) T cell line 24 hours after stimulation with (black) or without (NS, grey) peptide stimulation. (I) CD71 expression of a B57-NS1\(_{26-34}\)-specific cell line following stimulation with 10, 1, 0.1 and 0.01\(\mu\)g/mL NS1\(_{26-34}\) peptide HTWTEQYKF.
Table 3.3: **Statistical analysis of activation markers on CD8+ T cells**

<table>
<thead>
<tr>
<th>Populations compared</th>
<th>Mean frequency</th>
<th>Peak frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD69*</td>
<td>CD38*</td>
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<tr>
<td>Total CD8+ vs.</td>
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</tr>
<tr>
<td>B57-NS126-34 TET*</td>
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<tr>
<td>Total CD8+ vs.</td>
<td>N.S.</td>
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</tr>
<tr>
<td>B57-NS126-34 TET*</td>
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<td></td>
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<tr>
<td>B57-NS126-34 TET* vs.</td>
<td>0.04</td>
<td>N.S.</td>
</tr>
<tr>
<td>A11-NS3133-142 TET+/A2-E213-221 TET*</td>
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</tr>
<tr>
<td>B57-NS126-34 TET* vs.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>A11-NS3133-142 TET+/A2-E213-221 TET*</td>
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</tbody>
</table>

*Mean frequency = average frequency of CD69, CD38 and CD71 positive cells for all times points between fever day -4 to fever day +3.*

*Peak frequency = average of the peak frequency of CD69, CD38 and CD71 between fever day -4 to fever day +3.*

*N.S. = not significant*

*p-values determined using Mann-Whitney*
F. CD71, CD69, CD107a and Cytokine Expression in Epitope-Specific T Cell Lines

Since the NS126-34 epitope is highly conserved with only rare variants, we next assessed CD71 expression on other DENV-specific cell lines where epitope variants are more common. We used a well characterized A11-NS3133-147 epitope-specific cell line 10C11, which was cross-reactive for the pD1 and pD3/4 variant peptides but did not recognize the pD2 variant in tetramer staining and ICS assays. We stimulated 10C11 with three variant peptides for 6 hrs and evaluated the expression of CD107a, CD69, and CD71 (Figure 3.7A). We detected similar CD69 upregulation following stimulation with the pD1 and pD3/4 variant peptides. CD107a staining was more uniform following stimulation with the pD3/4 variant compared to the pD1 variant. A higher frequency of the 10C11 cell line upregulated CD71 following stimulation with the pD3/4 variant compared to the pD1 variant peptide (Figure 3.7A). We did not detect CD69, CD107a or CD71 upregulation after stimulation with the pD2 variant of the A11-NS3133-147 epitope.

We also stimulated the A2-E213-221 epitope-specific cell line P1A07, generated from an HLA-A*207 subject with secondary DENV-2, with four peptide variants. Cell line P1A07 had similar upregulation of CD69 following stimulation with all four peptide variants (Figure 3.7B). In contrast, there was stronger upregulation of CD71 and CD107a with the pD1 and pD2 variants compared to the pD3 and pD4 variant peptides (Figure 3.7B). We found the largest production of TNF-α and IFN-γ following stimulation with pD1 and pD2 variants and significant production following stimulation with the pD4 variant (Figure 3.7C) which mirrored CD71 and CD107a expression patterns. MIP-1β production was upregulated with pD4 ≥ pD1 > pD2 variant peptide stimulation. The pD3
variant peptide did not induce cytokine production (Figure 3.7C). Together, our data using cell lines suggest that CD71 expression was differentially sensitive to stimulation by homologous and heterologous variant peptides.
CD71 expression and effector functions on epitope-specific T cell lines.

CD107a, CD69 and CD71 expression after in vitro stimulation of cell line (A) 10C11 for 6 hrs with 10µg/mL A11-NS3133-142 variant peptides pD1, pD2, and pD3/4 and cell line (B) P1A07 for 6 hrs with 10µg/mL A2-E213-221 variant peptides pD1, pD2, pD3, and pD4. NS= no peptide stimulation.

C) Intracellular cytokine staining (ICS) of cell line P1A07 with variant peptides pD1, pD2, pD3, and pD4 at 10µg/mL. NS= no peptide as the negative control. Data are displayed as histograms with the gMFI of each parameter listed.

Figure 3.7.
G. Probability State Modeling of CD69, CD38, and CD71 Expression during Acute Dengue Virus Infection

We used Gemstone probability state modeling software designed for automated analysis of high dimensional flow cytometry data (see Appendix A), to assess the expression and progression of phenotypic markers on total CD8 and B57-NS126-34 TET$^+$ T cells. As with traditional gating of data, singlet, live CD14$^-$CD19$^-$, CD8$^+$ T cells were selected for modeling. Modeling of CD8$^+$ T cells was initiated based on the known expression patterns of CD8, CCR7 and CD45RA. Data events were ordered into 3 populations: Naïve T cells (CCR7$^+$, CD45RA$^+$), Central Memory & Effector Memory T cells (CM/EM$^-$CCR7$^+$, CD45RA$^+/CCR7^+$/CD45RA$^-$), and Effector T cells (EF$^-$CCR7$^+$, CD45RA$^+$). Expression patterns of co-related markers on these T cell subsets were revealed as colored ribbons of relative fluorescence intensity on the y-axis versus linear progression on the x-axis where the frequency of each subcategory can be read. The width of the ribbon represents the coefficient of variation (cv) of staining of that marker and is determined for every 1% along the x-axis. Figure 3.8 shows a representative Gemstone analysis of CD8$^+$ T cells from subject KPP94-037. Naïve T cells had high uniform expression of CD8 (thin green band), while memory T cells had a wider, generally lower range of CD8 expression (broader green band) (panel A). We observed a higher MFI of CD69 (dark blue band) and CD38 (mustard band) staining on memory cells with the highest expression on CM/EM cells (panel B). Consistent with other publication Gemstone analysis highlighted the presence of a CD57$^{high}$ population exclusive to the EF subset in all subjects (data not shown)$^{299}$. 
Black boxes drawn at the same point along the x-axis in panels A, B and C at fever day 0 identified a subset of cells that were CD45RA⁻, CCR7\textsuperscript{low/-} (panel A), CD69\textsuperscript{low}, CD38\textsuperscript{+/high}, CD71\textsuperscript{+}, CD57⁻ (panel B) and show that B57-NS1\textsubscript{126-34} TET\textsuperscript{+} T cells on this day fall into this phenotype (panel C). Panel C shows the B57-NS1\textsubscript{126-34} TET\textsuperscript{+} T cells aligned along the x-axis. B57-NS1\textsubscript{126-34} TET\textsuperscript{+} T cells were predominantly associated with a CM/EM phenotype (red) as early as fever day 0 and were divided between the CM/EM (red) and EF (yellow) populations during convalescence (Figure 3.8C).
Figure 3.8 Probability state modeling reveals novel phenotypes of CD8+ T cells during acute DENV infection. (A) The progression of CD8 (green), CD45RA (dark blue) and CCR7 (light blue), (B) the progression of CD69 (blue), CD38 (orange), CD57 (pink), and CD71 (purple) expression on the total CD8+ T cells in PBMC obtained from subject KPP94-037 over the course of acute DENV infection and convalescence. (C) B57-NS126-34 TET+ populations can be visualized along this progression over the course of infection. The cells highlighted within the black boxes (fever day 0) in Panel A are the same cells highlighted by this box in Panels B and C.
H. Discussion

We analyzed the frequency, kinetics, and phenotype of T cells specific for a novel HLA-B57-restricted epitope, B57-NS1\textsubscript{26-34}, over the course of acute DENV infection. Alignment of over 2610 strains of DENV from all four serotypes revealed >99% sequence identity in the epitope. This conservation led us to hypothesize that it might be an important target for DENV control in HLA-B*57-positive individuals. Variation in the sequence of T cell epitopes between DENV serotypes has been shown to influence the effector functions of DENV-specific memory T cells\textsuperscript{120,300}. Since the sequence of this epitope in a secondary DENV infection would be identical to the sequence from an earlier primary DENV infection, we predicted that PBMC from donors with secondary DENV infection would have particularly strong secondary CD8\textsuperscript{+} T cell responses to the B57-NS1\textsubscript{26-34} epitope. While we detected B57-NS1\textsubscript{26-34} TET\textsuperscript{+} T cells in all subjects tested, their frequencies in subjects with secondary infections were not higher than in subjects with primary infections, with one exception. Frequencies of B57-NS1\textsubscript{26-34} TET\textsuperscript{+} T cells were similar to those of A11-NS3\textsubscript{133-142} TET\textsuperscript{+} and A2-E2\textsubscript{13-221} TET\textsuperscript{+} T cells in the same subjects and to the frequencies of A11-NS3\textsubscript{133-142} TET\textsuperscript{+} T cells reported elsewhere\textsuperscript{114,134}. One donor had a peak frequency of B57-NS1\textsubscript{26-34} TET\textsuperscript{+}CD8\textsuperscript{+} T cells at day 180. While we may have missed the peak frequency during acute illness a second subclinical infection at the 6 month time point cannot be ruled out.

One possible explanation for the lower-than-expected frequency of TET\textsuperscript{+} cells could be differential processing and presentation of this epitope between the four DENV serotypes. Differential processing of HIV epitopes has been shown to result in striking...
differences in cytolytic (CTL) recognition. We demonstrated that B57-NS126-34-specific cell lines were able to lyse cells infected with any of the four DENV serotypes in vitro. Whether there is differential processing of the four serotypes for this epitope in vivo is unknown. Alternatively, a yet unidentified factor may dampen the activation of B57-NS126-34 TET+ T cells during a second infection.

Previous studies have used a number of cell surface markers to phenotype CD8+ T cells in DENV infection. We included a diverse panel of surface markers including CD57 and CD71 which have not previously been studied in CD8+ T cell responses to DENV infection. The timing of expression of CD69 in this cohort was consistent with previous reports. While Akondy et al. reported that CD38, HLA-DR, and Ki-67 are specific markers of activation when present in combination, there were a significant proportion of cells that expressed only CD38. Friberg et al. found a lower intensity of CD38 expression on influenza TET+ cells compared to A11-NS3133-142 TET+ cells during DENV infection. The findings of Akondy et al. and Friberg et al., suggest that the intensity of CD38 staining correlates with the specificity of activation and that bystander cells which are activated become CD38+, but not CD38high. The high frequency of CD38 expression in our T cell population is consistent with the finding that CD38 is expressed on bystander T cells.

Our study is the first to assess CD71 (transferrin receptor) expression on CD8+ T cells in the context of an acute viral illness. Over the course of DENV infection we observed upregulation of CD71 predominantly on DENV-specific CD8+ T cells and not on total CD8+ T cells. This was in contrast to CD69 and CD38 expression, which was
similar between B57-NS1_{26-34} TET^{+} T cells, A2-E213-221 TET^{+} or A11-NS3_{133-142} TET^{+} T cells and total CD8^{+} T cells during acute DENV infection. Gemstone analysis reveals that the CD71 was expressed on the total CD8^{+} T cell population and was concentrated on naïve and EF memory cells. The subtle variations in staining intensity seen in the Gemstone ribbon plots suggest a low base level of expression on these populations. These differences cannot be resolved using traditional dot plots. CD71 is required for DNA synthesis and cell division and is upregulated on dividing cells^{171, 298, 303}. Upon cell activation, CD71 is recruited to the immunological synapse coincident with upregulation of surface CD71^{172}. Salmeron et al. demonstrated that CD71 plays a role in the phosphorylation of TCRζ chain following CD3 and CD28 stimulation^{304}, and anti-CD71 mAb abrogates CTL responses to alloantigens^{305}. Upregulation of CD71 on DENV-specific T cells may therefore indicate that these cells had a more productive activation and are more cytolytic. Our data suggest that CD71^{hi} expression more accurately identifies DENV-specific T cells compared to expression of CD69 and/or CD38, with significant differences in both frequency and MFI of CD71 expression between the total CD8^{+} T cell population and the DENV-specific populations. Previous in vitro work showed upregulation of CD71 following αCD3 or mitogen stimulation^{306, 307}. We are the first to show robust expression of CD71 on T cell lines after peptide stimulation in vitro. Unlike CD69, the extent of CD71 upregulation was dependent on the peptide variant used and for the most part matched CD107a expression. These in vitro experiments showing that CD71 expression varies in parallel to CD107a expression based on the peptide sequence used to trigger activation support our ex vivo observation that CD71 is a more
specific marker of activation and suggest that CD71 expression may reflect qualitatively different signaling in the T cell response to DENV infection.

We noted high levels of CD71 in B57-NS1_{26-34} TET^{+} and A11-NS3_{133-147} TET^{+}/A2-E_{213-221} TET^{+} T cell populations in many donors at days 180 and 365 after the acute infection. We have similarly found that antigen-specific cell lines have marked levels of CD71 2 to 3 weeks after in vitro culture (data not shown). Gemstone analysis suggests that the EF subset of memory cells have slightly higher baseline levels of CD71 but further studies are needed to confirm these findings.

Our study population, although small, included subjects with primary and secondary DENV infections, DF and DHF, and each of the four DENV serotypes. This small sample size precluded comparing the magnitude of B57-NS1_{26-34}-specific T cells during primary and secondary infections. Previous work has provided conflicting data on the role of CD8^{+} T cells in the development of severe dengue disease and has focused heavily on responses to the HLA-A11-restricted NS3_{133-142} epitope \textsuperscript{114,117}. The number of consecutive blood draws at early time points during illness and consistency of patient care during acute illness are important strengths of this cohort. Additionally, our data suggest that even within 72hrs of fever onset immune responses are well underway, and therefore potentially important early events may not have been captured.

We found modestly increased frequencies of HLA-B57-restricted NS1-specific T cells in PBMC from the majority of Thai donors with secondary DENV infection. The absence of a stronger B57-NS1_{26-34}-specific response leads us to believe that other factors may be involved in influencing the magnitude of the response to this highly conserved
epitope. The finding of a novel and distinct phenotype (CD71+\textsuperscript{+}) in these epitope-specific T cells suggests that many of the CD69\textsuperscript{+} and CD38\textsuperscript{+} CD8\textsuperscript{+} T cells are in fact bystander cells that were not activated by direct interaction with their antigen and merits further investigation.

I. Chapter Summary

We identified a highly conserved 9aa epitope on the NS1 protein recognized by HLA-B57-restricted T cells. We hypothesized that B57-NS1\textsubscript{26-34}-specific CD8\textsuperscript{+} T cells would be preferentially expanded during secondary DENV infection since the epitope sequence would be identical to that seen in primary infection. Using PBMC samples from Thai children with primary or secondary DENV infection\textsuperscript{289}, we found that frequencies of B57-NS1\textsubscript{26-34} TET\textsuperscript{+} T cells were elevated during acute DENV infection but only one subject out of nine with secondary DENV infection had particularly high frequencies of B57-NS1\textsubscript{26-34} TET\textsuperscript{+} T cells (~20% of CD8\textsuperscript{+} T cells). Consistent with previous studies, expression of the activation markers CD69 and CD38 was upregulated on the total CD8\textsuperscript{+} T cell population as well as on DENV-specific T cells. In contrast, the expression of the transferrin receptor CD71 was significantly upregulated on B57-NS1\textsubscript{26-34} TET\textsuperscript{+}, A2-E\textsubscript{213-221} TET\textsuperscript{+} and A11-NS3\textsubscript{133-142} TET\textsuperscript{+} CD8\textsuperscript{+} T cells, but not on total CD8\textsuperscript{+} T cells. In vitro studies demonstrated that, while stimulation with homologous and heterologous peptides induced similar levels of CD69 expression, the intensity of CD71 expression was differentially sensitive to variant peptide stimulation. This suggests that CD71 may be a more specific marker of activation than CD69 or CD38.
The lack of preferential expansion of B57-NS1_{26-34}-specific T cells, despite the conservation of this epitope across all four DENV serotypes, suggests that as yet unidentified factors may be involved in shaping the T cell responses to DENV.
CHAPTER IV

THE B57-NS1_{26-34} TETRAMER INTERACTS WITH THE INHIBITORY RECEPTOR KIR3DL1 ON NK CELLS

KIR3DL1 is an inhibitory receptor on NK cells present in >90% of the world’s human population\(^{308}\). KIR3DL1 has three extracellular domains and a long cytoplasmic tail with an immunoreceptor tyrosine-based inhibition motif (ITIM). KIRs are stochastically expressed. The percentage of NK cells carrying KIR3DL1 varies between individuals, ranging from approximately 5-40\%\(^{309}\), and KIR3DL1 is expressed primarily on the CD56\textsuperscript{dim} subset of NK cells\(^{310}\). There are currently 92 identified alleles of KIR3DL1 which code for 62 allotypes\(^{311-313}\) that can be divided into three categories: those that are retained intracellularly (*004), those that are expressed at low levels, and those that are expressed at high levels\(^{250,252}\) which can be measured via flow cytometry using the monoclonal antibody DX9 by the shift in MFI of the positive NK cells. The ligands for KIR3DL1 are MHC class I molecules containing the HLA-Bw4 motif, which include HLA-B27, HLA-B57, and some of the HLA-A allotypes such as HLA-A24\(^{247,314}\). The interaction between KIR3DL1 and HLA-B57 has been extensively explored for other viral infections, and this has given us some insight into the possible role the interaction may have on NK cell responses during DENV infections.

In epidemiological studies, the presence of both KIR3DL1 and HLA-B57 has been associated with slower progression to AIDS in HIV patients. Individuals with KIR3DL1\textsuperscript{hi} expressing alleles are even less likely to progress from HIV to AIDS,
possibly due to a stronger inhibitory signal during development of NK cells\textsuperscript{315}. KIR3DL1 and HLA-B57 interact at position 80 of HLA-B57 along with position 8 or 9 of the presented peptide and the D2 area of KIR3DL1\textsuperscript{247}. Because of the involvement of peptide in the binding interaction Fadda et al proposed that the peptide could alter binding affinity\textsuperscript{250}. Studies that have examined the binding of KIR3DL1 to HLA-B57 loaded with a variety of peptides have shown that single amino acid changes in the peptide can completely abolish KIR3DL1 binding to the HLA-B57-peptide complex\textsuperscript{250, 291}. Peptide specificity has been reported for KIR3DL1 binding to other HLA-Bw4 alleles\textsuperscript{316} as well as for other KIR/HLA pairs including HLA-A11 and KIR3DL2\textsuperscript{260, 317, 318}. To date the effects of different HLA-B57-bound peptides on the function of NK cells have not been reported for KIR3DL1\textsuperscript{+} NK cells although such functional differences have been reported for other KIR/HLA interactions\textsuperscript{319}. Recently, O’Connor et al., used PBMC from KIR3DL1\textsuperscript{+} individuals to evaluate how peptide sequence affects B57 TET binding to primary human NK cells\textsuperscript{291}. They observed great variation in TET binding based on single aa changes consistent with what has been observed using KIR3DL1 transfectants\textsuperscript{250, 291}.

The absence of MHC binding to NK cells is thought to trigger a loss of NK cell inhibition resulting in the activation of NK cells. This is known as the ‘missing self’ hypothesis\textsuperscript{320}. As our understanding of NK cells has evolved it has become clear that the absence of MHC by itself is not sufficient to trigger activation of NK cells but the presence of activating ligands is also required\textsuperscript{233, 234} (Figure 1.1). Inhibitory receptors are thought to be particularly important in the development of NK cells by “licensing” NK
cells to respond to activating signals received later on\textsuperscript{235}. Licensed NK cells have increased sensitivity to these activating stimuli though most activating stimuli are still insufficient to overcome inhibitory signals, when these are also present. The interaction between HLA-B57 and KIR3DL1 is thought to protect HIV patients by increasing NK cell activity against virally infected cells that have down-regulated MHC class I expression, thereby more quickly controlling viral replication and giving the adaptive immune system time to develop protective responses\textsuperscript{252, 253, 321}. While the KIR3DL1\textsuperscript{+} subset of NK cells is expanded during acute HIV infection\textsuperscript{253}, the incubation of HIV-infected CD4\textsuperscript{+} T cells with KIR3DL1\textsuperscript{+} NK cells inhibited NK cell function\textsuperscript{322}. The exact means by which the presence of KIR3DL1 is protective in HIV infection thus has yet to be fully elucidated.

Rhesus macaques have also been used to study the role of KIRs in SIV infection. Colatonio et al published the first report of TET binding to NK cells. They described binding of a Mamu-A1*00201 TET to NK cells in rhesus macaques. They demonstrated that a Mamu-A1*00201 TET was likely binding to NK cells via KIR3DL05 by showing that incubating lymphocytes from KIR3DL05\textsuperscript{+} rhesus macaques with NK target cells expressing Mamu-A1*00201 suppressed the degranulation only of Mamu-A1*00201 TET\textsuperscript{+} NK cells\textsuperscript{323}.

The role of KIR3DL1 in shaping the NK cell response in HLA-Bw4\textsuperscript{+} individuals has yet to be investigated in flaviviral infections. Since the importance of NK cells in shaping the development of the adaptive immune response to viral infections is becoming better understood, we were interested in exploring the effect that genetics may play in
shaping NK cell responses and subsequently what their effect was on the adaptive immune response to DENV infection.

During our investigation of human CD8+ T cell responses to a highly conserved HLA-B57-restricted DENV epitope (Chapter III), we observed substantial binding of the B57-NS126-34 TET to an NK enriched population. We hypothesized that the B57-NS126-34 TET was binding to NK cells via the known HLA-B57 binding partner KIR3DL1. Staining of a KIR3DL1 transfectant cell line confirmed that B57-NS126-34 TET bound KIR3DL1. Consistent with the function of an inhibitory KIR, incubation of healthy donor PBMC with HLA-B57-expressing, NS126-34-pulsed target cells suppressed the degranulation of only the KIR3DL1+ NK cells. Both self and viral peptides have been shown to modify recognition of target cells by NK cells and modify NK cell function324-326. Furthermore, staining of PBMC from our cohort of Thai children with acute DENV infection revealed marked activation of NK-enriched cells only in HLA-B57+ patients who developed DHF (6 DHF of 11 total subjects Table 3.2). The differences in NK cell activation between patients with DF and those with DHF implicate NK cells in the pathogenesis of severe dengue disease.

A. Binding of the B57-NS126-34 TET to CD8- Cells in PBMC from Dengue Patients

While studying the responses of CD8+ T cells to the HLA-B57-restricted epitope NS126-34 (HTWTEQYKFF), discussed in Chapter III, we observed binding of the B57-NS126-34 TET to CD8- cells (Figure 4.1A). In order to evaluate if this binding was specific to B57-NS126-34 we stained PBMC from a convalescent time point in two subjects from
our study cohort with a known tetramer of HLA-B57 complexed with a known HIV epitope TW10n (TSNLQEQIGW) reported not to bind KIR3DL1 \textit{in vitro}. We saw minimal binding of the B57-TW10n TET to CD8\(^+\) cells (Figure 4.1B), indicating that our DENV TET, B57-NS1\(_{26-34}\), was likely binding via KIR3DL1 in a peptide dependent manner. We next looked at the frequency of B57-NS1\(_{26-34}\) TET staining in CD8\(^-\) cells over the course of acute DENV infection in our Thai study cohort (Figure 4.1C). Since our staining panel on clinical samples was developed to phenotype CD8\(^+\) T cells and did not include NK cell-specific markers, we first confirmed that the live CD3\(^-\)CD8\(^-\)CD14\(^-\)CD19\(^-\) population predominantly comprised NK cells. Using convalescent samples from 9 study subjects with sufficient cells available (Figure 4.2), we found that on average 75% of CD3\(^-\)CD8\(^-\)CD14\(^-\)CD19\(^-\) cells were CD56\(^+\), hereafter referred to as “NK-enriched” cells. The frequency of B57-NS1\(_{26-34}\) TET staining in the NK-enriched cells varied over the course of DENV infection in PBMC from HLA-B57\(^+\) individuals, and for a number of subjects the frequency was the lowest around Fever Day 0 (Figure 4.1C).
Figure 4.1. Binding of the B57-NS1_{26-34} TET to non-CD8 cells in Thai study cohort PBMC. (A) Binding of B57-NS1_{26-34} TET or (B) B57-Tw10n TET to CD3^− CD8^− CD14^− CD19^− “NK-enriched” cells at the 1 year time point from two HLA-B57^+ subjects. (C) Frequency of B57-NS1_{26-34} TET^+ in the NK-enriched population (CD3^− CD8^− CD14^− CD19^−) over the course of acute DENV illness and at convalescent time points from the HLA-B57^+ Thai study cohort.
Figure 4.2. **CD3^− CD8^− CD14^− CD19^−** cells are predominantly NK cells. (A) Gating strategy used in the identification of CD3^− CD8^− CD14^− CD19^− cells. (B) Percentage of CD3^− CD8^− CD14^− CD19^− cells which are CD56 and/or CD16 positive.
B. Binding of the B57-NS1_{26-34} TET to KIR3DL1

We speculated that binding of B57-NS1_{26-34} TET to NK cells in PBMC of Thai donors was mediated through KIR3DL1. To test this hypothesis, we first tested whether the B57-NS1_{26-34} TET binding to NK cells could be blocked by pre-incubating PBMC with an anti-KIR3DL1/S1 antibody. In a representative experiment pre-incubation with KIR3DL1/S1 antibody reduced B57-NS1_{26-34} TET binding from 1.07% to 0.064% on total PBMC, although binding was not completely eliminated (Figure 4.3A,B- totals for two top quadrants). Depletion of KIR3DL1^+ cells from PBMC also reduced the binding of B57-NS1_{26-34} TET to PBMC, in the representative experiment shown from 2.14% to 0.7% (Figure 4.4A,B-totals for two top quadrants). The results suggest that KIR3DL1 interacts with the B57-NS1_{26-34} TET resulting in the binding observed in all HLA-B57^+ Thai study subjects.

We next used KIR3DL1 transfectant cell lines to confirm the interaction between B57-NS1_{26-34} and KIR3DL1. An HLA-B57 TET loaded with a well-described self peptide LF9 (LSSPVTKSF) (grey line) was used as a positive control. We found robust binding of B57-NS1_{26-34} to KIR3DL1 transfectant cell lines (black line). Both the B57-NS1_{26-34} and B57-LF9 TETs bound all three alleles of KIR3DL1 that were tested: *001,005,015 (Figure 4.5). Pretreatment of the cells with anti-KIR3DL1 antibody, DX9, blocked binding of both tetramers to all three alleles of KIR3DL1 (dashed lines) (Figure 4.5). The data indicate that the DENV NS1 B57-NS1_{26-34} TET binds KIR3DL1, a known inhibitory receptor on NK cells.
Figure 4.3. **Anti-KIR3DL1 antibody blocks binding of B57-NS1\textsubscript{26-34} TET.** (A) PBMC pre-incubated with anti-KIR3DL1 antibody for 30 min at 4°C then with B57-NS1\textsubscript{26-34} TET for 50 min at 4°C. (B) PBMC pre-incubated with B57-NS1\textsubscript{26-34} TET for 50 min at 4°C then anti-KIR3DL1 antibody for 30 min at 4°C. One representative experiment of five experiments is shown.

Figure 4.4. **Depletion of KIR3DL1+ cells decreases B57-NS1\textsubscript{26-34} TET binding.** (A) PBMC stained with B57-NS1\textsubscript{26-34} TET. (B) PBMC depleted of KIR3DL1\textsuperscript{+} cells by MACS and then stained with the B57-NS1\textsubscript{26-34} TET. One representative experiment of three experiments is shown.
Figure 4.5. **B57-NS1<sub>26-34</sub> TET staining on KIR3DL1 transfectants.** Histograms showing B57-NS1<sub>26-34</sub> TET (black) as well as B57-LF9 TET (grey) binding (solid lines) to (A) an untransfected cell line, (B) KIR3DL1*001, (C) KIR3DL1*005, and (D) KIR3DL1*015 transfected cell lines. Binding of B57-NS1<sub>26-34</sub> TET and B57-LF9 TET in the presence of a KIR3DL1 blocking antibody DX9 is shown (dashed lines).
C. Expression of KIR3DL1 on NK Cells in the HLA-B57+ Thai Study Cohort and Healthy Donor PBMC.

Because we did not have KIR typing data available for all subjects we next sought to assess KIR3DL1 expression on peripheral blood NK cells in PBMC from our Thai study cohort as well as healthy individuals. Due to the limited availability of PBMC from acute illness, we used PBMC collected at a convalescent time point to determine the expression of KIR3DL1 in these subjects using the DX9 antibody. We detected KIR3DL1+ CD56+ NK cells in most subjects at frequencies that were consistent with frequencies reported elsewhere309 (3.95% to 16% of CD56+ NK cells, Figure 4.6A). Two subjects tested had no detectable KIR3DL1 staining (Figure 4.6C). The intensity of KIR3DL1 staining varied between subjects. Based upon this staining pattern we found that seven donors were homozygous KIR3DL1hi and two donors were homozygous KIR3DL1low (CHD02-073, KPP94-041).

In order to ascertain if B57-NS126-34 TET bound was bound to NK cells and assess if KIR3DL1 staining intensity or frequency affected B57-NS126-34 TET binding we obtained PBMC from twelve healthy donors, we confirmed were KIR3DL1+ or KIR3DL1– by staining (Figure 4.6B), and stained the PBMC with either anti-KIR3DL1 or B57-NS126-34 TET (Figure 4.6C, D, data not shown). We found that the frequency of TET+ cells varied between donors; there were no obvious associations between the frequency or intensity of staining of the TET+ cells and the frequency or brightness of KIR3DL1 staining. Of these twelve healthy UMMS donors, two individuals were
KIR3DL1 negative by DX9 staining; we saw very low frequencies of B57-NS126-34 TET⁺ NK cells (Figure 4.6C).

Extended phenotyping of the B57-NS126-34 TET⁺ NK cells was performed for PBMC from multiple donors to confirm that this population was consistent with NK cells and evaluate whether they could be associated with a particular phenotype. Figure 4.7 shows representative staining from one donor with the B57-NS126-34 TET⁺ NK cells (red dots) overlaid on the total NK cell population. Extended phenotyping of B57-NS126-34 TET⁺ NK cells in these healthy donors revealed no differences in the expression of the NK cell receptors CD161, NKp30, NKp46, and NKG2D between B57-NS126-34 TET⁺ NK cells and the rest of the CD56dim NK cells. B57-NS126-34 TET⁺ NK cells are phenotypically similar to total NK cells, but do not appear to occupy a subset identified by these markers.
Figure 4.6. **KIR3DL1 staining on PBMC from healthy donors and Thai study cohort subjects.** (A) Gating strategy for the identification of CD56+ and/or CD16+ NK cells. (B) Frequency of KIR3DL1+ cells in the NK cell population of Thai Donors. (C) Frequency of KIR3DL1+ cells in the NK cell population of healthy donors. (D) Staining with anti-KIR3DL1 and B57-NS126-34 TET of PBMC from a KIR3DL1+ donor. (E) Binding of anti-KIR3DL1 or B57-NS126-34 TET to NK cells from a KIR3DL1+ donor.
Figure 4.7. Expression of NK cell markers on B57-NS1\textsubscript{26-34} TET\textsuperscript{+} cells. The expression of CD161, NKp30, NKp46, NKG2D in the total NK cell population (zebra plot), with B57-NS1\textsubscript{26-34} TET\textsuperscript{+} NK cells overlaid (red dots). The expression pattern of CD161, NKp30, NKp46, NKG2D is consistent between the B57-NS1\textsubscript{26-34} TET\textsuperscript{+} NK cells and the total NK cell population.
D. Binding of HLA-B57-NS1_{26-34} to KIR3DL1 Results in Functional Inhibition of KIR3DL1\(^+\) NK Cells

Having demonstrated that B57-NS1\(_{26-34}\) TET bound KIR3DL1 on NK cells, we next wanted to determine whether this interaction resulted in functional inhibition of KIR3DL1\(^+\) NK cells. In order to answer this question we used the NK-sensitive target cell line 721.221 (221), 221 cells stably transfected with HLA-A2 (221-A2), or with HLA-B57 pulsed with NS1\(_{26-34}\) peptide (221-B57-NS1\(_{26-34}\)) to assess activation of NK cells. PBMC from KIR3DL1\(^+\) HLA-B57\(^+\) healthy subjects were mixed with these target cells at an E:T of 5:1, and degranulation of NK cells assessed by CD107a expression was used to measure activation (Figure 4.8). CD107a expression was detected predominantly on the CD56\(^{\text{dim}}\) NK cells, but some CD56\(^{\text{bright}}\) NK cells also expressed CD107a. Figure 4.8A shows the response of KIR3DL1\(^+\) NK cells in a representative experiment, where stimulation with 221 or 221-A2 resulted in 33.73\% and 32.57\% of KIR3DL1\(^+\) NK cells expressed CD107a (right top and bottom quadrants), respectively, but stimulation with 221-B57-NS1\(_{26-34}\) resulted in only 12.74\% of KIR3DL1\(^+\) NK cells expressing CD107a. Approximately 18\% of KIR3DL1\(^-\) NK cells expressed CD107a when stimulated with 221, 221-A2, or 221-B57-NS1\(_{26-34}\). This experiment was performed 5 times resulting in an average of 46\% lower frequency of CD107a expression on KIR3DL1\(^+\) NK cells when stimulated by 221-B57-NS1\(_{26-34}\), the same pattern was observed following stimulation with 221-B57 (data not shown). As expected the “licensed” or KIR3DL1\(^+\) NK cells responded more robustly to stimulation with 221 than the KIR3DL1\(^-\) NK cells, of which
only a portion are licensed by other KIRs, and the activation of only the KIR3DL1\(^+\) NK cells was inhibited by the presence of B57-NS1\(_{26-34}\). A CTL assay using a CD8\(^+\) T cell line, 3C11, specific for NS1\(_{26-34}\) confirmed that, following peptide pulsing, NS1\(_{26-34}\) was presented by 221-B57 cells (data not shown).

Since our antibody panel used for staining of PBMC from the Thai study cohort did not include CD107a, we also examined other markers of activation on NK cells (CD69, CD71, and CD38) included in the staining of Thai study cohort PBMC. In an attempt to relate the expression of these activation markers to the in vivo stimuli the NK cells may receive during dengue we incubated PBMC from healthy KIR3DL1\(^+\)B57\(^+\) subjects with stimuli known to activate NK cells via multiple pathways: K562 cells, 721.221 cells, P815 cells with anti-CD16, or IL-12 and IL-18. We examined the expression of CD69, CD71 (Figure 4.9), and CD38 (data not shown) on NK cells 24 hours later. We found that CD38 was highly expressed on unstimulated NK cells and therefore was not a useful marker of activation in these experiments. Upregulation of CD69 and CD71 was observed on the KIR3DL1\(^-\) NK cells following stimulation with K562, 221, 221-B57-NS1\(_{26-34}\), and IL12/18 (Figure 4.9A, B), but only CD69 was upregulated following activation via the CD16 receptor (Figure 4.9A, B). A similar pattern of activation in response to the different stimuli was observed on the KIR3DL1\(^+\) NK cells though the response of these licensed NK cells was more robust than that of the KIR3DL1\(^-\) NK cell population resulting in higher MFIs of CD69 and CD71 expression with two exceptions (Figure 4.9C, D). However, KIR3DL1\(^+\) NK cells were inhibited in the presence of 221-B57-NS1\(_{26-34}\) when compared with their response to 221.
Figure 4.8. Inhibition of KIR3DL1⁺ NK cells by HLA-B57-NS1<sub>26-34</sub>. PBMC were incubated for six hours with anti-CD107a antibody either alone (N.S.) or in the presence of NK cell target lines 721.221 (221), 221-B57 pulsed with NS1<sub>26-34</sub> (221-B57-NS1<sub>26-34</sub>), or 221-A2. Degranulation of (A) KIR3DL1⁺ NK cells was compared to that of (B) KIR3DL1⁻ NK cells. Only degranulation of KIR3DL1⁺ NK cells was inhibited in the presence of 221-B57-NS1<sub>26-34</sub>. No inhibition of KIR3DL1⁺ NK cells was observed when HLA-B57 was replaced with HLA-A2 (221-A2). One representative experiment of five experiments is shown.
Figure 4.9. **Activation of NK cells through multiple pathways.** Expression of CD69 and CD71 on the KIR3DL1⁻ (A and B) and KIR3DL1⁺ (C and D) NK cells respectively. PBMC were either (1) unstimulated or incubated with target cell lines K562 or 221 (2, 3), target cell line 221 transfected with HLA-B5701 (4), anti-CD16 (5), or IL-12 and IL-18 (6). CD69 and CD71 expression was assessed 24 hours later.
(Figure 4.9C, D) and they had less CD71 expression in response to IL12/18 (Figure 4.9D). The expression of CD69, but not the expression of CD71, paralleled expression of CD107a.

**E. Activation of NK Cells by Autologous DCs Infected with Dengue Virus**

Flaviviruses have long been reported to increase class I expression on infected cells\(^{208-210}\); therefore, we expected an abundance of NS1\(_{26-34}\) peptide to be presented on virally infected cells during DENV infection. We wanted to determine specifically whether HLA-B57 was upregulated by DENV infection. We infected primary monocyte-derived (mo) DCs from two HLA-B57\(^+\) individuals with DENV-2 16681 (MOI=10) and forty-eight hours later, we examined HLA-B57 expression. We found increased levels of HLA-B57 expression in the infected culture (Figure 4.10A). Based on intracellular staining with antibody to DENV E, we found that the upregulation of HLA-B57 expression occurred predominantly on bystander (uninfected) cells in the infected culture (Figure 4.10C). There was no change in the expression of HLA-B57 on the DENV-infected DCs (Figure 4.10C). Additionally, we demonstrated earlier that DENV infection of DCs resulted in presentation of the NS1\(_{26-34}\) peptide in the context of HLA-B57, as a CD8\(^+\) T cell line specific for B57-NS1\(_{26-34}\) lysed virus-infected DCs (Figure 3.1D).

Our previous data indicated that 221-B57 cells were able to inhibit the activation of KIR3DL1\(^+\) cells and that the peptide NS1\(_{26-34}\) did not disrupt this inhibition (Figure 4.8 and 4.9); however, we wanted to determine whether the level of HLA-B57 expressed following DENV infection of DCs was able to maintain inhibition in the midst of the
other signals being received. As mentioned in Chapter I.I., many of the NK cell activating ligands have yet to be identified and the ligands responsible for NK recognition of DENV-infected cells are not known. Therefore, our ability to assess expression of activating ligands following DENV-infected was limited. We decided to focus on the activation profile of NK cells by measuring the expression of CD107a, CD69, and CD71. We used DENV-infected DCs as a model for what NK cells might encounter early after DENV infection327.

We incubated PBMC from a healthy KIR3DL1+/HLA-B57+ subject for 24 hrs with autologous DCs that were uninfected (Figure 4.10B) or infected with DENV-2 16681 48 hrs before (Figure 4.10C). To investigate the effect of DC derived cytokines on NK cell functional responses, we included tubes where PBMC were added to DCs without replacing the media (i.e., with conditioned media) or where the media was replaced with fresh media (i.e., without conditioned media). NK cells, including KIR3DL1+ NK cells, were strongly activated in the presence of DENV-infected DCs as assessed by the increase in CD107a, and CD69 expression (Figure 4.11). NK cell activation was lower in DENV-infected cultures with conditioned media than in DENV-infected cultures without conditioned medium (Figure 4.11-4,5), but the conditioned media did not alter the pattern of the response. The data suggest that cytokines have an affect on all NK cell responses to DENV and that the cytokine milieu produced by DENV-infected moDCs serve to dampen NK cell responses.

It has been shown that variations in the quantity of matching MHC class I seen by KIR+ NK cells affects their functional set point328. To test whether the increase in
expression of HLA-B57 seen on DCs in DENV infected cultures affects the responsiveness of KIR3DL1⁺ NK cells we transferred PBMC, by gentle pipetting, from culture with infected DCs to culture with uninfected DCs for the last 6 hours of co-culture (Figure 4.11-6). We found increased expression of CD107a, CD69, CD38, and CD71 on KIR3DL1⁺ (Figure 4.11G-I), KIR3DL1⁻ (Figure 4.11D-F) and total NK cells (Figure 4.11A-C) compared with the levels of expression after co-culture only with DENV infected DCs. Only minimal expression of CD71 was observed on the total NK cell population, the KIR3DL1⁻ NK cells and the KIR3DL1⁺ NK cells following incubation with DENV-infected DCs. The data suggest that NK cells can adjust to increased levels of MHC class I expression as a new set point, even in a short time (18hrs), and subsequently see lower levels of expression as the release of the inhibitory signal. The consistency of the increase in response across all NK cells also highlight the extent to which other inhibitory receptor/MHC class I pairs may play a role in NK cell responses to DENV infection.
Figure 4.10. Upregulation of MHC-I on DCs following DENV infection for 48 hours. (A) Histogram showing expression of HLA-B57 on all DCs in DENV infected culture versus an uninfected culture. (B,C) HLA-B57 expression versus DENV-2 infection showing DCs in (B) uninfected culture or (C) infected culture.
PBMC cultured with:
6 DENV-infected DCs then uninfected DCs with conditioned media
5 DENV-infected DCs with conditioned media
4 Uninfected DCs with conditioned media
3 DENV-infected DCs without conditioned media
2 Uninfected DCs without conditioned media
1 No Stimulation
Figure 4.11. **Activation of NK cells by DENV-infected DCs.** Expression of CD107a, CD69, and CD71 on the total NK cell populations (A, B, C) KIR3DL1− NK cell populations (D, E, F) or KIR3DL1+ NK cell populations (G, H, I) respectively. PBMC were (1) unstimulated or stimulated with (2) uninfected DCs or (3) DENV-infected DCs without the DC conditioned media, (4) uninfected DCs or (5) infected DCs with the DC conditioned media for 24hrs, (6) DENV-infected DCs with the conditioned media for 18hrs then incubated for 6hrs with uninfected DCs. One representative experiment of three experiments is shown.
F. Activation of NK-Enriched Cells Correlates with Disease Severity

Now that we had explored the activation of NK cells \textit{in vitro}, we were interested in looking further at the activation of NK cells during and after acute dengue in PBMC from the HLA-B57\(^+\) Thai study cohort. Due to limited sample availability we were unable to use an NK cell-specific panel to assess NK cell activation in PBMC from the Thai study cohort. Therefore, we evaluated activation profiles by assessing levels of CD69, CD38 and CD71 on NK-enriched populations using the data generated from staining of the HLA-B57\(^+\) Thai study cohort PBMC with the CD8\(^+\) T cell panel described in chapter III. CD69, an early marker of NK cell activation, was elevated early in disease but remained relatively high at convalescent time points (Figure 4.12A). We observed no difference in the level of CD69 on the B57-NS1\(_{26-34}\) TET\(^+\) NK-enriched cells (black lines) or the total NK-enriched populations (red lines) from donors with DF (solid lines) versus DHF (dashed lines) (Figure 4.12A). CD71 expression was elevated around fever day 0, the day of defervescence, with expression predominately on B57-NS1\(_{26-34}\) TET\(^+\) NK-enriched cells (Figure 4.12B). CD71 expression was slightly elevated in subjects with DHF (Figure 4.12B). While CD71 expression did not segregate based on clinical diagnosis of DF or DHF, it was statistically significantly higher (p<0.01, Mann-Whitney) in the B57-NS1\(_{26-34}\) TET\(^+\) NK-enriched cells compared to total NK-enriched cells. CD57, a marker of NK cell maturity, remained consistent during and after acute dengue with no apparent differences between the B57-NS1\(_{26-34}\) TET\(^+\) or total NK-enriched cells, nor between NK-enriched cells from subjects with DF or DHF (Figure 4.12C).
CD38 expression was elevated on NK cells in PBMC during acute illness, decreased during early convalescence, and remained expressed on up to 40% of NK-enriched cells 1 year after infection (Figure 4.12E). However, when we stratified CD38 expression into CD38\(^{\text{hi}}\) and CD38\(^{\text{low}}\) we saw a very different pattern emerge (Figure 4.12F,G). Figure 4.10D is a representative flow cytometry plot of PBMC from Fever Day +1 and Fever Day +180 in one donor to demonstrate the distinction between CD38\(^{\text{hi}}\) and CD38\(^{\text{low}}\) expression. CD38\(^{\text{low}}\) expression followed the same pattern as CD69 expression on NK cells. In contrast, CD38\(^{\text{hi}}\) expression peaked at fever day +1 and returned to baseline at 1 year post-infection. The peak of CD38\(^{\text{hi}}\) expression was between fever day 0 and +1 on total NK-enriched cells as well as on B57-NS1\(_{26-34}\) TET\(^{+}\) NK cells in most subjects, both those with DF and those with DHF. Some subjects whose disease was classified as DF did not have appreciable frequencies of CD38\(^{\text{hi}}\) NK cells. Higher frequencies of CD38\(^{\text{hi}}\)-expressing cells were seen in subjects who had DHF compared to those who had DF (p=0.0571, Mann-Whitney).

We found that peak expression of CD71\(^{+}\) on B57-NS1\(_{26-34}\) TET\(^{+}\) NK-enriched cells coincided with peak CD38\(^{\text{hi}}\) expression at fever day 0. This suggests that NK cells, especially in donors who developed DHF, are activated coincident with the clearance of viremia and therefore may play a role in dengue pathogenesis. The decreasing expression of CD69 suggests that we catch only the end of the early phase of NK cell activation following DENV infection in these subjects. The difference in expression of surface activation markers on the NK-enriched population between the first phase of activation seen in these donors, prior to fever day 0, and the second phase of activation, around
Figure 4.12. **Expression of surface activation markers over the course of acute DENV illness.** (A) The expression of CD69 (B) the expression of CD71 (C) the expression of CD57. The B57-NS1\textsuperscript{26-34} TET\textsuperscript{+} NK-enriched cells from each donor at each time point are in grey for donors undergoing a primary infection and black for donors undergoing a secondary infection. Patients are also denoted as having been diagnosed with DF (solid lines) or DHF (dashed lines). The average surface expression of these markers on the total NK-enriched population for donors diagnosed with DF are shown using a solid red line and for those diagnosed with DHF using a dashed red line.
Figure 4.12 continued. **Expression of surface activation markers over the course of acute DENV illness.** (D) Representative CD38 staining showing the gating of CD38$^{hi}$ versus CD38$^{low}$ on the NK cell population. (E) The expression of CD38 (F) CD38$^{hi}$ and (G) CD38$^{low}$. The B57-NS1$_{26-34}$ TET$^+$ NK-enriched cells from each donor at each time point are in grey for donors undergoing a primary infection and black for donors undergoing a secondary infection. Patients are also denoted as having been diagnosed with DF (solid lines) or DHF (dashed lines). The average surface expression of these markers on the total NK-enriched population for donors diagnosed with DF are shown using a solid red line and for those diagnosed with DHF using a dashed red line.
fever day 0, suggest there is something mechanistically or qualitatively different in how these NK cells are being activated at these two time points.

G. Discussion

In addition to innate immune control of virus infections, variations in early NK cell responses may have profound effects on the subsequent development of the adaptive immune response. The interplay between NK cells and dendritic cells is dynamic and can shape adaptive immune responses to an infection. Alternatively, a very rapid NK cell response which quickly eliminates a pathogen may leave only low levels of antigen available for presentation to CD8+ T cells, therefore decreasing the likelihood of developing a strong adaptive immune memory response. NK cells have been implicated in the regulation of T cell responses during viral infections, potentially acting to prevent pathological responses to high viral loads by attenuating T cell activation. These effects may be particularly relevant for DENV infection if NK cell responses affect the quality of T cell memory which develops during a primary infection, because of the strong epidemiological link between secondary infection and increased risk of DHF.

In this chapter, we identified an interaction between a DENV-specific B57-NS126-34 TET and KIR3DL1, an inhibitory receptor on NK cells. We found that B57-NS126-34 TET bound to NK cells in PBMC from all Thai study cohort subjects and from every KIR3DL1+ healthy donor. We were particularly interested in investigating the possible role of KIR3DL1+ NK cells in DENV pathogenesis due to the many associations of Class I MHCs and KIRs with both beneficial and detrimental outcomes during various viral
infections and the development of autoimmune diseases. The interaction between HLA-B57 and KIR3DL1 has been extensively studied in the context of HIV. Following the initial recognition that HLA-B57+ individuals were more likely to be long term non-progressors, it was additionally recognized that HLA-B57+ individuals who were also KIR3DL1+ or KIR3DS1+ were even more likely to be long term non-progressors than individuals who are only HLA-B57+. We saw a striking activation phenotype of B57-NS126-34 TET+ NK-enriched cells in our ex vivo analysis of Thai PBMC. Our in vitro data suggest that these TET+ cells represent a subset of KIR3DL1+ NK cells in these donors. Differences between the total NK-enriched population and B57-NS126-34 TET+ NK-enriched cells may reflect a role for the interaction between KIR3DL1 and B57-NS126-34 in modulating NK cell responses. Peak frequencies of CD38hi and CD71+ NK cells were detected around defervescence (fever day 0), coincident with the peak activation of CD8+ T cells and the critical period when patients are at increased risk of plasma leakage. In support of a role in disease pathogenesis, CD38hi NK-enriched cells were present in higher frequencies in PBMC from donors whose disease was classified as DHF than those classified as having DF. This difference approached statistical significance (p=0.0571) but the small cohort limited the power of the study. Although NK cells are usually considered to be activated early in acute viral infections, our data suggest that a subset of NK cells may be activated late in DENV infection in patients who develop DHF.
Our study of NK cell responses during DENV infection in this cohort was limited by sample availability, which prevented us from using an NK cell-specific panel to further investigate our observations. Additionally, the investigation of innate immune responses in clinical samples is limited by the delay between the mosquito bite that initiates viral infection in the study subject and their presentation to the hospital likely after many early immune responses are already underway. Many studies in mice with acute viral infections have shown that initial NK cell activation occurs in the first 3 days\textsuperscript{339, 340}. Patients with DENV typically present to the clinic more than a week following the mosquito bite that initiated the infection\textsuperscript{53, 341}.

The complexity of the NK cell receptor repertoire, the number of unknown ligands, and the timing of sample collection in children undergoing acute DENV infection, made it challenging to dissect the NK cell interaction with DENV-infected cells \textit{ex vivo}. We therefore designed a series of \textit{in vitro} experiments to complement our \textit{ex vivo} studies and provide further insight into the interaction between DENV and inhibitory receptors on NK cells.

NK cells can be activated by a variety of stimuli; we used target cell lines devoid of MHC class I expression (K562, 721.221), signaling via the CD16 receptor (P815 with anti-CD16 antibody), and stimulation with cytokines (IL-12 plus IL-18) to examine the activation of NK cells \textit{in vitro} via multiple pathways. Activation of NK cells by each of these pathways resulted in degranulation, as detected by an increase in CD107a, but we found subtle differences in the expression of surface activation markers especially CD71. CD71 was highly expressed on the B57-NS1\textsubscript{26-34} TET\textsuperscript{+} NK cells in PBMC of Thai
children with dengue at Fever Day 0 (the day of Defervescence). The in vitro data suggest that only certain stimuli can trigger CD71 expression and that the B57-NS126-34 TET⁺ NK-enriched cells may have been activated by exposure to cells with reduced MHC class I expression or by exposure to IL-12 and IL-18, or by a combination of these stimuli near the time of defervescence. Both IL-12 and IL-18 have been measured in patients during acute DENV infection. Higher levels of IL-18 have been reported in patients with dengue with warning signs compared to patients with dengue without warning signs. IL-18 levels have also been shown to be increased in patients with DHF. IL-12 levels, on the other hand, have been reported to be lower in patients with dengue compared to healthy controls, with very little IL-12 production in patients with DHF. The findings of a CD38⁺ population, while very clear as a distinct population in ex vivo staining, was not observed after any in vitro stimulation. The expression of CD38⁺ on in vitro stimulated cells may require more time or a more complex combination of activation signals.

The functional response of KIR3DL1⁺ NK cells to the target cell line 721.221 was inhibited by transfecting the target cell line with HLA-B57 and pulsing with NS126-34 peptide did not disrupt this inhibition. In contrast, KIR3DL1⁻ NK cells remained unaffected by the presence of B57-NS126-34. Self-peptides binding to HLA-B57 are also known to mediate inhibition of KIR3DL1⁺ NK cells, making it challenging to conclusively demonstrate the role of the DENV NS126-34 peptide in mediating the inhibition. However, we demonstrated that the NS1 peptide was presented on the surface
of 221-B57 cells as a T cell line lysed only peptide-pulsed target cells. Our experiments suggest that the NS1_{26-34} peptide does not disrupt inhibition of KIR3DL1^{+} NK cells.

We did not observe early activation of NK cells during acute DENV infection in our Thai study cohort. However, stimulation for 24hrs by DENV-infected DCs did activate NK cells. The data suggest that early in DENV infection activating signals on target cells may overwhelm any inhibitory signals the NK cells is receiving. As mentioned previously, we likely missed this phase of NK cell activation in the PBMC collected for our analysis.

Consistent with other reports about effects of DENV infection on MHC class I expression^{208, 210, 214}, we found that HLA-B57 was upregulated on DCs in response to infection with DENV; however, this upregulation appeared to be limited to bystander DCs (DENV antigen-negative DCs in the infected cell culture). This is in contrast to the work by Hershkovitz et al. showing that the NS proteins are sufficient to upregulate MHC class I^{208}. It is possible that the mechanism of upregulation of MHC class I during DENV infection is cell type specific; however work by Libraty et al. showing upregulation of DENV infected DCs and a greater extent of upregulation on bystander DCs^{212} suggests that something other than cells type is responsible for our observations. It is possible that the burden of DENV per cell affects the level of MHC on infected cells.

High levels of HLA-B57 expression during DENV infection could create a strong inhibitory environment for KIR3DL1^{+} NK cells. Even though upregulation of HLA-B57 appears not to occur on DENV infected cells circulating NS1 may be taken up by bystander cells and NS1_{26-34} may be cross presented on MHC class I. While we were
unable to look exclusively at the effect of NS126-34 peptide on the function of KIR3DL1
NK cells, our data demonstrate that the presentation of NS126-34 by HLA-B57 molecules
does not disrupt the inhibition of KIR3DL1 NK cells. KIR3DL1 NK cell activation is
controlled by a balance of signals which appear to be skewed in favor of activation 48hrs
after DENV infection of DCs despite the increased expression of HLA-B57. To
determine whether the environment of DENV-infected DCs alters NK cell responses
toward healthy DCs, we transferred NK cells from culture with DENV-infected DCs to
culture with uninfected DCs. Under these conditions, we observed an increase in the
expression of the activation markers CD69 and CD71. Perhaps the in vitro data give
some insight into the factors affecting activation of NK cells in children in vivo at Fever
Day 0 when viremia has been cleared. NK cells which have been in a DENV-induced
environment for a week or more now see APCs returning to a healthy state. We predict
that NK cells are more susceptible to activation as MHC class I levels return to normal
following resolution of DENV viremia.

H. Chapter Summary

B57-NS126-34 TET NK cells were identified in HLA-B57 donors during acute
DENV infection and at convalescent time points. We showed that HLA-B57 complexed
with the DENV NS126-34 peptide (B57-NS126-34) interacts with KIR3DL1 and that this
interaction resulted in inhibition of KIR3DL1 NK cells. While NK cells are typically
considered to be activated early in acute infection in response to virally infected cells we
observed peak activation of a subset of NK cells coincident with viral clearance. The
frequency of this activated population correlated with the development of DHF. This is the first study to examine the role KIRs play in the pathogenesis of DENV infection and suggests that late activation of NK cells may contribute to the development of DHF.

The data set a framework for future research which should aim to take a fresh look at the role of NK cells in DENV infection. Phenotyping studies using an NK cell-specific antibody panel that include other KIR/HLA interactions, more prevalent in the Thai population, should be pursued. As animal models of dengue improve, it may be possible to investigate the role that NK cell responses to DENV play in shaping CD8$^+$ T cell responses.
A. Thesis Summary

We identified a HLA-B57 DENV epitope (NS126-34) conserved across all four serotypes of DENV. We hypothesized that CD8$^+$ T cell responses to the NS126-34 epitope would be more robust in secondary infection compared to responses to the more commonly encountered non-conserved epitopes since the CD8$^+$ T cells would encounter the identical sequence during a second DENV infection for the NS126-34 epitope (Table 2.1). We used PBMC obtained during acute dengue and convalescence from a cohort of hospitalized children in Thailand to evaluate CD8$^+$ T cell responses to the highly conserved HLA-B57 restricted epitope (NS126-34) and when possible compare NS126-34-specific responses to other DENV epitope-specific CD8$^+$ T cell responses. We expected to find increased frequencies of B57-NS126-34 TET$^+$ CD8$^+$ T cells compared to frequencies of A11-NS3133-142 TET$^+$ CD8$^+$ T cells or A2-E213-221 TET$^+$ CD8$^+$ T cells in all subjects undergoing secondary dengue. We also expected that activation of B57-NS126-34-specific T cells during secondary DENV-infection would be more consistent and occur more rapidly than activation of A11-NS3133-142 or A2-E213-221-specific T cells. However, we observed high frequencies of B57-NS126-34 TET$^+$ CD8$^+$ T cells in only one of the nine donors in our cohort undergoing a secondary DENV infection.

Despite the lack of selective-expansion of B57-NS126-34-specific CD8$^+$ T cells during secondary infection we did observe activation of CD8$^+$ T cells in all donors. We
found peak expression of CD38 on CD8$^+$ T cells in PBMC from all donors at fever day 0 when patients are at risk for developing plasma leakage. The CD8$^+$ T cells in PBMC from subjects undergoing a secondary DENV infection showed similar expression of the activation markers CD69, CD38, and CD71 on the B57-NS1$_{26-34}$ TET$^+$ and the A11-NS3$_{133-142}$ TET$^+$ /A2-E$_{213-221}$ TET$^+$ (epitopes which vary between serotypes, Table 2.1) populations.

Unlike CD69 and CD38, CD71 was mainly expressed on DENV-specific CD8$^+$ T cells, suggesting that CD71 may be a more reliable marker of specific T cell activation. Follow-up studies in vitro highlighted the specificity of CD71 with expression varying only slightly even with very low peptide concentrations when CD8$^+$ T cells were stimulated with homologous peptides, but revealing greatly diminished expression when CD8$^+$ T cells were stimulated with heterologous peptides. We observed no clear measure which differentiated T cell responses in HLA-B57$^+$ patients with DF apart from those with DHF. This could be, in part, due to the low number of HLA-B57$^+$ study subjects available. Alternatively, it is possible that CD8 T cell responses, while contributing to the overall response to DENV infection were not responsible for the development of dengue pathology in this cohort. As with all human studies which predominantly rely on PBMC it is also possible that the CD8$^+$ T cells of interest migrate into tissues during acute illness and we thus are unable to reveal any important difference between CD8$^+$ T cell responses in subjects with DF and DHF.
We observed binding of the B57-NS126-34 TET to CD8<sup>-</sup> cells in all HLA-B57<sup>+</sup> subjects from our Thai cohort and all healthy donors who were HLA-B57<sup>+</sup>KIR3DL1<sup>+</sup> and showed that this binding was to the inhibitory receptor KIR3DL1. We found a distinct difference in the response of the NK-enriched cells in patients with DF versus DHF, with CD38<sup>hi</sup> NK-enriched cells more frequent in subjects with DHF (p=0.057). Peak expression of CD38<sup>hi</sup> on NK-enriched cells was coincident with peak expression of CD38 on T cells, occurring at fever day 0. The data suggest that the activation of subsets of NK cells may contribute to disease severity in HLA-B*57 individuals. We found little activation of NK-enriched cells early during acute infection (prior to fever day -1) as assessed by CD38<sup>hi</sup> and CD71 expression. We found upregulation of CD69 on NK-enriched cells early during acute infection, but high frequencies of CD69 seen at six months and one year after infection made it difficult to draw solid conclusions from this data.

We used a series of in vitro experiments to investigate whether the interaction between HLA-B57 and KIR3DL1 might contribute to the delay in NK cell activation detected in our ex vivo studies. We found increased expression of HLA-B57 on DCs in DENV-infected cultures. Exposure of NK cells from healthy adult donors to DENV-infected autologous DCs resulted in activation of NK cells as assessed by the expression of CD107a and CD69. We also found CD71 upregulation on KIR3DL1<sup>+</sup> NK cells following stimulation with an NK-sensitive target cell 221, and inhibition of KIR3DL1<sup>+</sup> NK cells in the presence of 221 target cells transfected with HLA-B57 and pulsed with the NS1<sub>26-34</sub> peptide. Our in vitro data suggest that the interaction between B57-NS1<sub>26-34</sub>
and KIR3DL1 could shape responses of the KIR3DL1⁺ subset of NK cells during DENV infection to make these cells more susceptible to activating signals received around fever day 0. These activated NK cells could produce cytokines that weaken the endothelial barrier and contribute to plasma leakage.

**B. Proposed Model**

Based on our *in vitro* and *ex vivo* data we propose the following model of NK and T cell activation in HLA-B57⁺ patients who are infected with DENV. We speculate that increased levels of MHC class I expression inhibit the development of NK cell responses by keeping “licensed” NK cells, such as KIR3DL1⁺ NK cells, from responding robustly early during DENV infection (Figure 5.1 viremic phase). Presentation of the NS126-34 peptide by HLA-B57 molecules serves to maintain inhibition of KIR3DL1⁺ NK cells in HLA-B57⁺/KIR3DL1⁺ subjects during the viremic phase. While DENV-infected APCs may not upregulate MHC class I during DENV, soluble NS1 can be taken up by uninfected APCs and the NS126-34 peptide can be cross-presented on MHC class I. Since NS1 is the only DENV protein known to be secreted from DENV-infected cells, cross-presentation of DENV peptide epitopes is likely restricted to those present on the NS1 protein. Following the clearance of DENV viremia (around fever day 0), we expect MHC class I levels to return to baseline. This change in MHC class I expression releases the inhibitory signal received by KIR3DL1⁺ NK cells in HLA-B*57⁺ individuals (Figure 5.1 critical phase). Our findings of peak activation of a subset of NK cells in patients with severe disease, DHF, around fever day 0 support the model. While we are unable to
further classify this subset due to limited amount of acute PBMC collected from the Thai study cohort, we hypothesize that these cells are, at least in part, KIR3DL1⁺ NK cells.

NK cell activation is important for the development of CD8⁺ T cell responses. Damned NK cell responses during DENV infection may therefore lead to qualitatively poor CD8⁺ T cell responses, resulting in poor development of memory T cells, and subsequently contribute to the lack of robust proliferation of B57-NS1₂₆-₃₄ TET⁺CD8⁺ T cells during secondary DENV infection. Additionally, the activation of NK cells at Fever Day 0, coincident with peak activation of CD8⁺ T cells, in subjects who develop DHF contributes to the production of cytokines, such as TNF-α, which can lead to loss of endothelial barrier integrity.

The activation of “licensed” NK cells around Fever Day 0 is likely triggered by the upregulation of an NK cell-activating ligand. The abundance of NK cell activating receptors for which ligands remain to be identified makes it challenging to define how NK cells were activated. The activating ligands MICA/MICB have been linked epidemiologically to the development of symptomatic but not severe dengue in a Cuban study cohort and a genome-wide association study identified MICB with susceptibility for DSS (Table 1.2). These epidemiological relationships suggest that NK cells can contribute to dengue pathology.
Figure 5.1. Proposed model of the interaction between HLA-B57 and KIR3DL1 during DENV infection. (A) DENV infection results in the upregulation of HLA-B57 expression on uninfected bystander APCs during the viremic phase (yellow). During the critical phase (red), when viremia has resolved, HLA-B57 levels return to baseline. (B) KIR3DL1⁺ NK cells may be activated by interaction with virally infected APCs, in subjects undergoing a secondary infection, anti-DENV antibodies can engage the CD16 receptor on NK cells leading to activation. (C) KIR3DL1⁺ NK cells receive a strong inhibitory signal during viremia (yellow) due to the upregulation of HLA-B57. Presentation of the NS1_{26-34} peptide on HLA-B57 maintains the interaction between HLA-B57 and KIR3DL1. Following the clearance of viremia HLA-B57 levels return to normal which decreases the inhibitory signal received by KIR3DL1⁺ NK cells. This release of inhibition allows KIR3DL1⁺ NK cells to be activated at fever day 0, coincident with the activation of CD8⁺ T cells.
C. Final Conclusions and Future Implications

Even with over 60 years of research much is still unknown about the pathogenesis of dengue, treatment is still supportive, and effective tetravalent vaccines are still elusive. The prevalence of DENV infections has increased appreciably in the last decade and represents a major global disease burden\textsuperscript{53, 54}. DENV is even beginning to re-emerge in the United States\textsuperscript{344}. The geographical spread and increase in incidence of severe disease has raised awareness of dengue and interest in the production of a vaccine. DENV presents a unique challenge to vaccine manufactures due to the need to simultaneously elicit strong protective responses to all four serotypes.

An improved understanding of protective and pathologic responses to DENV should help to direct development and evaluation of candidate vaccines. It is likely that many factors contribute to DENV disease severity for any one patient. The adaptive immune system has been the focus of most research to date due to the strong association between secondary infection and DHF\textsuperscript{58, 69-76}. NK cells have been shown to be important for the development of the adaptive responses to viral infections\textsuperscript{275, 277, 330, 343} and could shape the adaptive responses to DENV. The innate immune response to DENV thus deserves more attention.

In chapter 4, we showed late activation of NK cells in HLA-B57\textsuperscript{+} KIR3DL1\textsuperscript{+} subjects during DENV infection. This delay in NK cell activation could hamper the development of protective memory CD8\textsuperscript{+} T cell responses to DENV through decreased production of cytokines early in infection and delayed maturation of DCs. NK cells appear to play a direct role in the development of mature DCs, particularly in the
presence of IL-12\textsuperscript{345}. Low levels of IL-12 have been reported in patients with DHF compared to healthy control or patients with DF\textsuperscript{342}. Thus NK cell responses may explain why secondary CD8\textsuperscript{+} T cell responses to this highly conserved epitope were no greater than primary responses in our Thai study cohort\textsuperscript{346}. Additionally, NK cells are important for T cell development via the production of IFN-\(\gamma\), IL-15, and IL-18\textsuperscript{347}. However, it is difficult to specifically measure early NK cell responses in DENV patients since they present at the hospital several days following infection\textsuperscript{53,341}. Our data indicate that the innate immune response to DENV is more complex and variable between subjects than previously appreciated and may have a profound effect on the subsequent development of the adaptive immune response.

We speculated that increased levels of MHC class I during DENV infection allow the inhibitory NS1 epitope to be presented to KIR3DL1\textsuperscript{+} NK cells maintaining inhibition of this subset of NK cells during the viremic phase. We hypothesized that the subsequent decrease in MHC class I expression to normal levels results in the removal of this major inhibitory signal for KIR3DL1\textsuperscript{+} NK cells, making these NK cells easier to activate near the resolution of DENV viremia. Not all B57\textsuperscript{+}KIR3DL1\textsuperscript{+} individuals go on to develop DHF. Therefore, additional signals must be involved in the activation of NK cells at Fever Day 0. Identifying these signals should be an important focus although it will be challenging since the function of many human NK cells receptors and their ligands are unknown.

The expression of ligands for the activating NK cell receptor NKG2D, include MICA and MICB which was associated with symptomatic but not severe cases of DENV.
in two epidemiological studies\textsuperscript{92,93} and MICB which was associated with DSS in a genome-wide association study\textsuperscript{94} have yet to be studied in dengue patients. Studies which look at the timing of MIC expression and the subsequent NK cell responses during DENV infection should be undertaken. Especially since the close physical association of immune genes on Chromosome 6 makes it difficult to use epidemiological and human studies to identify the important ligands, receptors, and/or cytokines involved in disease pathogenesis and to investigate the relationship between these ligands and receptors. This is because many of these genes are located on Chromosome 6 near the MHC I genes and are therefore likely in linkage disequilibrium with HLA genes\textsuperscript{348}. A number of cytokines are also part of this extended haplotype. We would expect that the expression of NKG2D ligands peaks shortly prior to fever day 0 and contributes substantially to NK cell activation at fever day 0 in subjects with DHF, but not in subjects with DF.

Epidemiological links provide the most logical starting place for future work. Differential expression of MIC alleles\textsuperscript{349} raises the possibility that other NK cell ligands may be differentially expressed and that these variations may be important in affecting dengue disease severity. Despite these challenges attempts should be made to study the expression of potential activating ligands on APCs in PBMC from subjects with acute DENV infection. The study of known NK cell ligands, especially those identified epidemiologically as important, needs to be undertaken for DENV. DENV E protein has been reported to bind an activating receptor NKp44\textsuperscript{216}. MICA/MICB and DENV E would make reasonable targets for initial analysis of possible activating ligands.
The ongoing DENV vaccine trials could potentially be leveraged to study the expression of NK cell ligands and early NK cell frequency and activation status in response to DENV infection. Punch biopsies of the injection site would allow the evaluation of known ligands on APCs. Vaccine studies also offer the benefit of easy access to pre-infection PBMC, and have the advantage of knowing exactly when the subject was infected.

Certain strains of DENV have been linked to the widespread development of severe disease after introduction to a new region, such as occurred in the Americas in 1981\textsuperscript{81,82}. NK cell interaction with DENV could help to explain some differences seen between strains through direct interaction with NK cell receptors. More recently the concept of NK cell memory has been widely debated. Data suggesting clonal expansion of MCMV-specific NK cells and the presence of NK cells capable of memory responses to viral antigens in mice have been reported\textsuperscript{350,351}. While it is unclear to what extent NK cell memory exists in humans and how this memory might be shaped, the high level of sequence identity between DENV serotypes suggests the possibility that NK cell memory could play a role in secondary responses to DENV infection. This may be difficult to evaluate in human subjects. As humanized mouse models improve, it may become more feasible to attempt to study NK cell responses to DENV in a humanized mouse system. These models could be particularly useful for investigating the possibility of NK cell memory responses to DENV infection.

There is mounting evidence that NK cells may be able to modulate responses to chronic infection not only at early time points but even weeks into the infection\textsuperscript{275,276,333,339}. 
Our data suggest that NK cells may play a more long-term role in acute infections as well. In B57$^+$ KIR3DL1$^+$ individuals, the association between late NK cell activation and DHF suggests a role for NK cells in dengue pathogenesis. It is not known if a similar phenomenon may be occurring in individuals with other MHC/KIR combinations. Recent work by Beltram et al. supports our finding that KIR/HLA interactions affect responses to DENV infection. This study identified an epidemiological relationship between a number of KIRs and the outcome of DENV infection in a cohort of DENV patients in Brazil$^{139}$. In particular, they determined that individuals who had symptomatic DENV infection were more likely to be HLA-Bw4$^+$ KIR3DL1$^+$ compared to healthy controls.

Our work is the first, we are aware of, to assess CD71 expression on CD8$^+$ T cells during an acute viral illness. CD71 appears to be a better marker of antigen-specific activation compared to CD69 or CD38 since we detected expression primarily on TET$^+$ cells. CD71 has also been recognized as an activation marker for CD4$^+$ T cells and B cells$^{352-354}$. While CD71 is occasionally included in activation panels our work suggests that CD71 should be considered a more specific marker of activation for T cell studies.

This is also the first study to identify tetramer binding to NK cells during DENV infection. Based on ex vivo phenotyping of an NK-enriched population, our studies reveal an underappreciated role for NK cells in DENV pathogenesis. Future research should focus on phenotyping NK cell subsets, in response to natural DENV infection and DENV vaccination and consider other HLA/KIR interactions. If the delayed NK cell activation we observed in our cohort occurs in all DHF patients regardless of the KIR/MHC partnerships present, this would suggest that NK cell responses are an important
component in all DHF cases. In contrast, if this delayed activation is only present in HLA-B57$^+$ KIR3DL1$^+$ donors the data will serve to highlight how variable and complex the development of DHF may be. Our work strongly suggests that NK cells play a role in the development of DHF and highlights gaps in our understanding of innate immune responses to DENV infections. NK cells are a highly dynamic and complex population of cells which likely play a larger role in immune responses than once thought. Revisiting the role of NK cells during DENV may provide insights into DENV pathogenesis which can help shape vaccine and drug development.
A. The Challenges of Multiparameter Flow Cytometry Analysis

Flow cytometry analysis has been one of the great advances in immunological studies. The ability to examine individual cells has dramatically increased our appreciation for immune cell heterogeneity and has allowed us to ask targeted questions about the functions of these diverse populations. However, as the technology advances and allows for more and more markers to be studied, the challenge of analyzing this data, and presenting the data in an understandable way to others, has become increasingly complicated. The use of flow cytometry to study expression of markers also raises questions of the extent to which biologically relevant divisions exist. Should markers with a broad range of fluorescence intensity be divided into multiple populations? How many divisions should be made and how should we decide where the divisions are?

By the simplest mathematical reduction, 12 color parameter flow yields 144 possible two-dimensional plots. In practice, manual gating will begin with the disregard of those plots which duplicate another plot or display a parameter versus itself, and the process of selecting populations of interest by defining those characteristics for further data analysis begins to reduce the number of plots, but still leaves a large amount of data to analyze. Practical concerns also abound. The computing power required for these studies can be high and new analysis paradigms and new “flow” systems such as CyTOF,
mass cytometry, have required creative expansion of computing power and inventive programming \(^{355}\).

Many options for flow data analysis exist, but they fall basically into two types of systems: 1) manual gating of two-dimensional plots as described above or 2) computer aided automated analysis that is able to cluster or order individual events for multiple parameters simultaneously.

A number of well known manual analysis software programs exist including Diva (BD Biosciences) and FlowJo (Tree Star). Gating is the process by which the researcher chooses the population(s) of interest by manually defining the boundaries of each marker included in the study. These programs put no restrictions on how the user defines a population of cells. Gating of samples is very subjective and as such the quality of analysis is heavily dependent on the experience and opinion of the researcher. Templates for analysis help to introduce consistency between researchers \(^{356}\).

There are also a number of computer automated analysis programs for high dimensional data \(^{357}\). One such platform is Gemstone produced by Verity Software House. Gemstone is modeling software that generates, what they term, a probability state model (PSM). The idea is, rather than define an event as strictly positive or negative, consider the dynamic history of a cell for a given marker. The usefulness of this type of analysis paradigm becomes evident when considering the histogram for any marker where multiple populations exist but overlap (Figure 6.1 E). Simply dividing this histogram in two is unlikely to accurately reflect the biological division. Gemstone utilizes information from other surface markers to aid in correctly identifying cells as belonging to the
positive or negative state, and those cells which are in the process of transitioning from one state to the other.

Gemstone can consider as many parameters as required by the researcher. For a single parameter the simplest state can be thought of as either positive or negative. Then more complex parameters with transition between negative and positive can be added to build the model. User input then organizes the data into biologically useful groupings. The more biologically relevant data the program is given the more useful the model will be. Once you have selected your population of interest, for example CD8$^+$ T cells, Gemstone allows the simultaneous viewing of all parameters. However, this data may not yet provide much insight into how the parameters are coordinated (Figure 6.1A). The model can be further refined by adding known relationships to the model. For instance, arranging cells along the x-axis with progression from naïve (CCR7$^+$/CD45RA$^+$) cells at the left hand side of the axis to the more terminal CM/EM cells (CCR7$^/$/CD45RA$^-$) and TE (CCR7$^-$CD45RA$^+$) cells, one is able to see how the expression of other surface markers such as CD69 and CD38 (Figure 6.1 B) or CD57 (Figure 6.1 C) varies between these populations. Such plots are referred to as ribbon plots. It also becomes apparent at what stage rare populations such as TET$^+$ T cells emerge (Figure 6.1 C). Once the data has been organized by Gemstone, arrows show how markers move through the progression from naïve to memory cells on two-dimensional plots (Figure 6.1 D).

Inokuma et al recently published on the use of PSM for analysis of memory CD8$^+$ T-cell differentiation allowing the simultaneous viewing of expression levels of seven markers$^{299}$. This paper highlights the usefulness of PSM for modeling kinetic processes.
While Gemstone is automated it is important to remember that the software still requires input from the user and that fitting data to a bad model will result in bad data. This is not unique to Gemstone, and indeed the robustness of the model developed can be determined by standard statistical means and rejected when insufficient number of events are classified. Gemstone builds in a statistical readout providing the reduced chi-square (RCS) value of data correlation to the generated model.

As stated earlier, manual gate creation and placement in flow-cytometric data analysis is very subjective\textsuperscript{356}. The use of automated analysis correlates well with expert manual gating, but provides for inter-laboratory consistency. This is particularly useful in clinical laboratories to improve speed and consistency of analysis\textsuperscript{358}. As the number of flow-based diagnostic tests in clinical settings expands, and in the research setting the number of markers studied simultaneously increases, the need for automated analysis will also increase.

The studies presented in this thesis are additionally complicated by the added requirement for multiple samples from one individual over the course of disease. This makes finding helpful ways of presenting the data for others to quickly and easily visualize even more challenging. The apparent ease of visualizing all parameters simultaneously, even as ribbon plots, becomes more daunting when confronted with such a plot for every time point from every donor. Hence other graphical displays, which compile the data, such as graphs of frequency or MFI, are still likely the best endpoint for sharing the data with others once an important variable emerges from the data.
Figure 6.1. **Gemstone Analysis.** (A) Expression of CCR7, CD45RA, CD69, CD38, CD71 on CD8⁺ T cells before any modeling. (B) Expression of CD69, CD38, and CD71 on CD8⁺ T cells after modeling of CCR7 and CD45RA to identify naïve (CCR7⁺, CD45RA⁺ left) and memory (CCR7⁻ right) populations across the x-axis. This highlights that CCR7⁻CD45RA⁻ cells are those with the highest expression of CD69 and CD38 in this donor at this time point. (C) Modeling of CD8⁺ T cells as in B shows that CD57 is expressed mostly on CCR7⁻CD45RA⁺ terminal effector memory cells. This also highlights that CD8⁺ T cells which bind B57-NS1₂₅-₃₄ TET are memory cells in this donor at this time point. (D) Two-dimensional plots in Gemstone show the progression of the markers along the x-axis using arrows overlaid on the dot plot. (E) Gemstone, rather than dividing the distribution into negative and positive events, uses probability state modeling to address the overlap region of the positive and negative peaks to accurately assign cells in this overlap region.
CHAPTER VII
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