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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

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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

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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, $NS1_{26\text{-}34}$. We predicted higher frequencies of $NS1_{26\text{-}34}$-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-$NS1_{26\text{-}34}$ 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-$NS1_{26\text{-}34}$-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 (FLDLPLPWT, FLDLPLPWL, FFDLPLPWT, FFDLPLPWL)
A11-$NS3_{133-142}$ 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-$NS1_{26-34}$ TET	B57 tetramer loaded with the NS1 peptide position 26-34 (HTWTEQYKF)
B57-TW10 TET	B57 tetramer loaded with the HIV-Gag peptide TW10 (TSTLQEQIGW)
B57-TW10n 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

DC	dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing non-intergrin
DENV	dengue virus
DF	dengue fever
DHF	dengue hemorrhagic fever
DSS	dengue shock syndrome
E	envelope protein
ER	endoplasmic reticulum
FBS	fetal bovine serum
FITC	fluorescein
gMFI	geometric mean fluorescence intensity
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP	inducible protein
ITIM	immunoreceptor tyrosine-based inhibition motif
JEV	Japanese encephalitis virus
KIR	killer cell immunoglobulin-like receptor

LCMV	lymphocytic choriomeningitis virus
L-SIGN	liver/lymph node specific ICAM-3 grabbing non-integrin
LTA	lymphotoxin alpha
LTB	lymphotoxin beta
MDA5	melanoma differentiation-associated protein 5
MHC	major histocompatibility complex
MICA/B	major histocompatibility complex class I-related chains
MIP-1β	macrophage inflammatory protein 1β
NC	nano crystal
NFκB	nuclear factor-κB
NIAID	National Institute of Allergy and Infectious Disease
NK	natural killer
NS	no stimulation
NS[#]	non-structural protein [#]
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
pMHC	peptide-MHC complex
prM	precursor form of M protein
Qdot	quantum dot
RCS	reduced chi square
rh	recombinant human

RNA	ribonucleic acid
RPMI	Rosewell Park Memorial Institute cell culture medium
SS	single stranded
TAP	transporter associated with antigen processing
TCR	T cell receptor
TLR	Toll-like receptor
TNF-α	tumor necrosis factor
UMMS	University of Massachusetts Medical School
WHO	World Health Organization
WNV	West Nile virus
WRAIR	Walter Reed Army Institute of Research
YFV	yellow fever virus

PREFACE

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

Townsley E, Woda M, Thomas SJ, Kalayanarooj S, Gibbons RV, Nisalak A, Srikiatkhachorn A, Green S, Stephens HAF, Rothman AL and Mathew A. Distinct Activation Phenotype of a Highly Conserved Novel HLA-B57-Restricted Epitope during Dengue Virus Infection. *Immunology* 2014;141(1) PMID:23941420

Chapter IV: The B57-$NS1_{26\text{-}34}$ TET Interacts with the Inhibitory Receptor KIR3DL1 on NK cells

Townsley E, O'Connor G, Cosgrove C, Woda M, Co M, Thomas SJ, Kalayanarooj S, Gibbons RV, Nisalak A, Srikiatkhachorn A, Green S, Stephens HAAF, McVicar D, Alter G, Rothman AL, Mathew A (2014). Interaction of a dengue-specific CD8+ T cell NS1 epitope with KIR3DL1 on NK cells reveals an underappreciated role for NK cells in impacting dengue disease severity. Manuscript in preparation

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

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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-intergrin [DC-SIGN] and liver/lymph node specific ICAM-3 grabbing non-integrin [L-SIGN])[17, 18] and

aminoglycan-type adhesion molecules[19] 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[20, 21]. 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[22].

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[23, 24]. 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[25]. 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[26].

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[27]. NS3, in addition to participating as a serine proteinase with NS2B, functions as an RNA helicase and RNA triphosphatase[28, 29]. NS5 stimulates and modulates the enzymatic activities of NS3[30]. NS2A, NS4A, NS4B,

and NS5 are involved in suppression of the innate immune response by suppressing type I interferon (IFN) production or signaling[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[35]. NS1 is secreted from infected cells, based on its glycosylation status[36], and its presence in serum has been used to diagnose DENV infection[37-39]. During DENV infection NS1 can exist in monomeric, dimeric or hexameric forms[40]. Levels of NS1 in plasma correlate with disease severity[41-43], and soluble and cell-associated NS1 have been reported to activate the complement cascade[44]. Avirutnan et al. found high levels of NS1 and the complement protein C5a in pleural fluids of patients with dengue shock syndrome (DSS)[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[45-48].

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

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[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[49]. A fifth serotype was recently identified which does

not yet appear to have sustained transmission in humans.[50] The first reports of dengue may have been as early as 1635 when a dengue-like epidemic was recorded in Martinique[51], and in 1780 there was a well described outbreak of "bilious fever" in Philadelphia which was likely caused by DENV[52].

DENV is transmitted to humans through mosquitoes, primarily via the vector *Aedes aegypti* but it can also be transmitted by *Aedes albopictus*[53]. *Aedes aegypti* are found primarily in residential areas, breed commonly in water that has accumulated in man-made containers, and primarily feed on humans[53]. 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*[51]. In the last decade, the territory of *Aedes aegypti* has continued to spread with the appearance of DENV in many South American countries[51].

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)[53]. In 2013, Bhatt et al. estimated that 390 million people become infected each year with DENV of which 96 million are clinically apparent

cases[54]. 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[55]. 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[49]. The benefits of this new system have yet to be demonstrated and many research groups still use the old system for classifying patients[56]. 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[57].

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[53]. DHF is characterized by high fever, plasma

leakage, thrombocytopenia, and bleeding tendency, which are coincident with the resolution of fever and viral clearance[58-60]. Hepatomegaly is also present in over 90% of Thai children with DHF [57]. The presence and extent of pleural effusion, an indication of plasma leakage, can be measured using chest X-ray[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.

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[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[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[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[62]. One chimeric flavivirus vaccine, for Japanese encephalitis virus (JEV), has been licensed and is currently in use[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[64]. A number of other candidate dengue vaccines built off various platforms are also in clinical trials[65-67]. 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[68].

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[69-71]. 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[58, 72-76]. Additionally, it has been suggested that the sequence of infecting serotypes modulates the risk of developing DHF[77-80].

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[81, 82]. 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[50].

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[83]. 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[84-86]. 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[87].

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[88-91]. 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[92-94]. These proteins are upregulated in cells under stress and are ligands for an activating receptor on natural killer (NK) cells[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[96]. Moreover, patients undergoing a second infection with a different serotype are at increased risk for developing DHF[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[98].

i. Role of Antibodies in Dengue Pathogenesis

Antibodies to DENV can mediate a number of activities *in vitro*[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[99, 100]. This more efficient viral entry mediated by viral antibodies is referred to as antibody dependent enhancement (ADE)[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[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[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[69, 104]. Follow-up studies suggested that non-neutralizing antibodies, via ADE, may enhance viral load and immune

activation during DENV infection[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[108].

ADE may facilitate virus entry and initiate intracellular antiviral responses[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[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[97].

While ADE can be observed *in vitro*, demonstrating ADE *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 FcγR binding eliminated these effects[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[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[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 [87, 114-116]. 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[117]. Increased levels of cytokines, which are secreted *in vitro* by DENV-specific $CD8^+$ T cells[118-120], 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[121-128].

Table 1.1. **Dengue CD8$^+$ T cell association studies**

Study Location[a]	**# of Subjects**[b]	**Timing of Enrollment**[c]	**DF vs. DHF**[d]**; P vs. S**[e]	**Timing of Acute Samples**[f]	**Timing of Convalescent Samples**[g]	**HLA-Typing Done?**
Thailand 1999[122]	51 children	within 72hrs of fever onset	29 DF 22 DHF 22 P 29 S	study day 2 and 1 day after defervescence	NONE	NO
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						
Thailand 2002[129]	10 children	within 72hrs of fever onset	5 DF 5 DHF 1 P 9 S	during illness & 8 to 11 days after study entry	6 months 1 year 2 year 3 year	YES
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						
Thailand 2003[114]	19 children		5 DF 14 DHF 19 S	4 times during acute illness Fever day -2, -1,0,+1	14 days after defervescence	YES
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						
Vietnam 2005[130]	48 adults		48 S	day of admission, study day 3, study day 5	2 weeks, 1 month post admission	
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.						
Thailand 2006[131]	13 children		13 DF 10 DHF 1 P 12 S	during illness & 8 to 11 days after study entry	6 months	YES
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[116]	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 $HLA\text{-}A11^+$ subjects $NS3_{133\text{-}142}$ FINDINGS: $CD69^+CD8^+$ T cells were significantly, but transiently, increased in DHF. Found measurable frequencies of $NS3_{133\text{-}142}$ TET^+CD8^+ T cells only in convalescence.						
Vietnam 2008[117]	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 ($NS3_{133\text{-}142}$) FINDINGS: Peak TET^+ $CD8^+$ T cell frequencies after plasma leakage commenced.						
Thailand 2010[132]	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[133]	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.						

Thailand 2011[134]	44 children		25 DF 19 DHF 17 P 27 S	daily during acute illness and 1 week after defervescence	6 month 1 year 2 years	YES
Evaluated $CD8^+$ T cell responses to the HLA-A11 restricted epitope $NS3_{133-142}$ FINDINGS: no correlation between frequency of TET^+CD8^+ T cells and disease severity						
Sri Lanka 2012[135]	24 adults 5 controls		24 healthy seropositive			
Evaluated *ex-vivo* responses to peptides from each of the four DENV serotypes to determine serotype specific T cells . FINDINGS: 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						

[a] *Study location and reference*

[b] *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.*

[c] *When patients were enrolled in the study*

[d] *Number of subjects with DF versus DHF(or DSS as noted),*

[e] *Number of subjects with Primary (P) versus secondary (S) DENV infection*

[f] *Timing of collections of samples taken during acute dengue illness*

[g] *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**

Study Location[a]	# of Subjects[b]	DF vs. DHF[c]; P vs. S[d]
Thailand 2002[89]	263 children	149 DF 54 P 114 DHF 209 S
FINDINGS: Associated HLA-A*203 and HLA-B*52 with DF, HLA-A*207 and HLA-B*51 with DHF, HLA-B*44, B*62, B*76, and B*77 with protection from developing clinical disease		
India 2007[136]	197 pts 100 controls	90 DF 109 P 75 DHF 78 S 32 DSS
FINDINGS: Associated TAP1 333 ILE/VAL and HPA1a/a1 and HPA2a/2b with DHF.		
Thailand 2009[90]	435 children	65 s.c 69 P 229 DF 301 S 142 DHF
FINDINGS: Associated TNF-238A together with HLA-B*48 or B*57 with DHF		
Vietnam 2011[94]	2008 DSS 2018 controls	2008 DSS
FINDINGS: Found MICB and PLCE1 to be susceptibility loci for susceptibility to DSS		
Jamaica 2011[137]	50 dengue 177 healthy	45 DF 5 DHF
FINDINGS: Found that HLA-A*24 associated with DF		
Cuba 2011[92]	104 adults	68 DF 36 DHF
FINDINGS: Found that MICA*008 and MICB*008 associate with susceptibility to illness but greater likelihood of DF then DHF.		
Brazil 2012[138]	109 pts	67 DF 42 DHF
FINDINGS: Associated HLA-A*01 with DHF		
Brazil 2013[139]	104 pts 172 controls adults	
FINDINGS: Associated KIR2DS1, KIR2DS5, KIR2DL5,KIR3DS1-Bw4, KIR3DL1-Bw4, KIR2DL1-C2, KIR2DS1-C2, KIR2DL3-C1/C1 with dengue		
Brazil 2013[140]	187 pts with DENV-3	120 DF 66 P 67 DHF 121 S
FINDINGS: Associated HLA-B*44 with DHF in patients with DENV-3		

[a] *Study location and reference*

[b] *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.*

[c] *Number of subjects with DF vs DHF(or DSS as noted),*

[d] *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-4* 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**

Protein	Amino acids[a]	Sequence[b]	MHC[c]	Reference
C	22-31	RVSTVQQLTK	A03/11	[142]
	107-115	CLIPTAMAF	B15	[143]
	107-115	MLIPTAMAF	B35	[143]
prM	133-141	FTILAFLAH	B35	[126]
E	211-219	FFDLPLPWT	A02	[144]
	297-306	MSYSMCTGKF	B35	[143]
	414-422	ILGDTAWDF	B07	[130]
NS2a	198-206	ATGPILTLW	B58	[143]
NS2b	52-60	ELERAADVK	A03/11	[142]
	97-106	ILIRTGLLVI	A0201/24	[142]
NS3	25-32	RIKQKGIL	B08	[142]
	64-74	RIEPSWADVK	A03/11	[142]
	71-79	SVKKDLISY	B62	[145]
	112-120	AIKRGLRTL	A02/24	[142]
	130-144	GTSGSPIIDKK	A11.1	[114]
	176-184	NPEIEDDIF	B35	[143]
	194-203	HPGAGKTKRY	B35	[143]
	222-230	APTRVVAAE	B07	[146]
	235-243	AMKGLPIRY	B62	[145]
	291-300	DPASIAARGY	B35	[143]
	422-431	RVIDPRRCMK	A03/11	[142]
	500-508	TPEGIIPTL	B35	[147]
	521-530	GEFRLRGEQR	B40	[143]
	528-537	GEARKTFVEL	B40	[143]
	555-564	INYADRRWCF	A24	[130]
	606-614	MALKDFKEF	B35	[143]
NS4a	56-64	LLLGLMILL	A02	[144]
	55-64	LLLLTLLATV	A02/24	[142]
NS4b	6-13	LEKTKKDL	B08	[142]
	23-32	TETTILDVDL	B53	[143]
	40-48	TLYAVATTI	A02	[148]
	49-58	TPMLRHTIEN	B07	[143]
	69-77	IANQATVLM	B35	[143]
	92-100	VPLLAIGCY	B35	[143]
	111-119	VLLLVTHYA	A02	[144]
	119-128	AIIGPGLQAK	A03/11	[142]
	181-189	LLLMRTSWA	A02	[144]
	198-206	ATGPILTLW	B58	[143]

NS5	182-190	VLNPYMPSV	A02/24	[142]
	291-299	WHYDQDHPY	B35	[143]
	329-337	KPWDVIPMVT	B55	[118]
	343-351	DTTPFGQQR	A68	[143]
	373-382	VMGITAEWLW	B53	[143]
	375-383	KITAEWLWK	A03/11	[142]
	389-398	KPRICTREEF	B07	[143]
	393-402	TPRMCTREEF	B07/35	[143]
	563-571	KLAEAIFKL	A02/24	[142]

[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[131, 132] and 6 months[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 $CD8^+$ 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^+ T-cell frequencies in early convalescence rather than at the time of plasma leakage, suggest that the frequency of A11-$NS3_{133-142}$ TET^+ 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 $NS3_{222-230,}$ may contribute to disease severity to a greater extent than the response to $NS3_{133-142}$[134].

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[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-$NS3_{133-142}$-specific T cells and disease severity[134]. It appears that neither the quantity nor the quality of responses to $NS3_{133-142}$ associated with disease severity[117, 134].

Studies focused on DENV-specific T cells have found varying levels of cytokine production and cytotoxic activity in $CD8^+$ T cells. The production of IFN-γ, TNF-α, and macrophage inflammatory protein 1β (MIP-1β) by $CD8^+$ T cells was dependent on the peptide sequence of the stimulating epitope which typically varies between DENV serotypes[118, 120, 144]. Stimulation of $CD8^+$ 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[120]. Simmons et al. demonstrated significant IFN-γ 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[130]. Most recently, Duangchinda et al. showed higher frequencies of TNF-α and/or IFN-γ -producing $CD8^+$ T cells in response to DENV peptides in PBMC collected during acute dengue illness from patients with DHF versus those with DF[132]. These studies suggest that not only high levels of T cell activation but also the effector response may contribute to DENV pathogenesis.

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 $CD8^+$ 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-γ 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-α, IFN-γ, 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-γ 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-γ and TNF-α, 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 clearance[151, 152]. Additionally, depletion of $CD8^+$ T cells but not $CD4^+$ T cells in mice resulted in higher viral loads[151, 152] and negated protection against a lethal strain provided by prior immunization[153, 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 suboptimal[155]. 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 peptides[131, 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 CD8$^+$ T Cells by Flow Cytometry

A number of cell surface markers have been used to characterize the phenotype and function of CD8$^+$ 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[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[157]. HLA-DR is expressed on cycling cells[158]. CD69 and CD38 are upregulated on activated CD8$^+$ T cells[159-161]. CD69 may function as a costimulatory receptor to enhance proliferation of activated T cells[162, 163], though T cells were shown to proliferate normally in CD69$^{-/-}$ mice[164]. CD38 is thought to have many functional roles for T cells including transmission of activating signals leading to increased intracellular Ca^{2+} and cytokine production[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[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$^+$ CD8$^+$ T cells are capable of expansion[168], contain high levels of granzyme and perforin, and have the ability to produce high levels of cytokines[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 TCRζ[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[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[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[182], human immune responses need to be improved[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[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[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[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)[197]. However, as noted earlier, the DENV proteins NS2A, NS4A, NS4B, and NS5 can prevent the production and/or signaling of type I IFNs[31-34]. Cytokines associated with T cells, such as IFN-γ and TNF-α, can also be produced by innate cells, such as monocytes, DCs, and NK cells, during DENV infection[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[199-201]. The production of interleukin (IL)-6, IL-8, inducible protein (IP)-10, and TNF-α by DENV-infected monocytes has been reported to correlate with maximum virus production[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[202].

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[203]. They observed heterogeneity in the NK cell population, but the mechanism by which NK cells recognized DENV-infected cells was not identified[203]. Subsequent research identified $CD16^+$ NK cells as being responsible for antibody-mediated cytotoxicity[204]. Yoshida et al. transiently depleted tamarins of $CD16^+$ 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[205]. However, they conducted this experiment in animals undergoing a primary infection, and did not address whether $CD16^-$ 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[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[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[207].

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[208-210]. Which is in contrast to most other viral infections that downregulate MHC class I[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[212]. Warke et al. showed that DENV-infected muscle satellite cells decrease MHC class I expression while bystander cells increase MHC class I expression[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[209] peptide translocation into the endoplasmic reticulum (ER)[214] and increased HLA promoter activity[210]. Work with WNV has shown increased nuclear factor-κB (NFκB) activity leading to increased MHC transcription in infected cells[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[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[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[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 $CD3^-$ lymphocytes identified by CD56 and CD16 expression and can be $CD56^+$ and/or $CD16^+$ [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[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[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)[233, 234]. CD16 provides a strong activating signal to NK cells and can independently activate NK cells even in the presence of inhibitory signals[234]. Inhibitory receptors are thought to be particularly

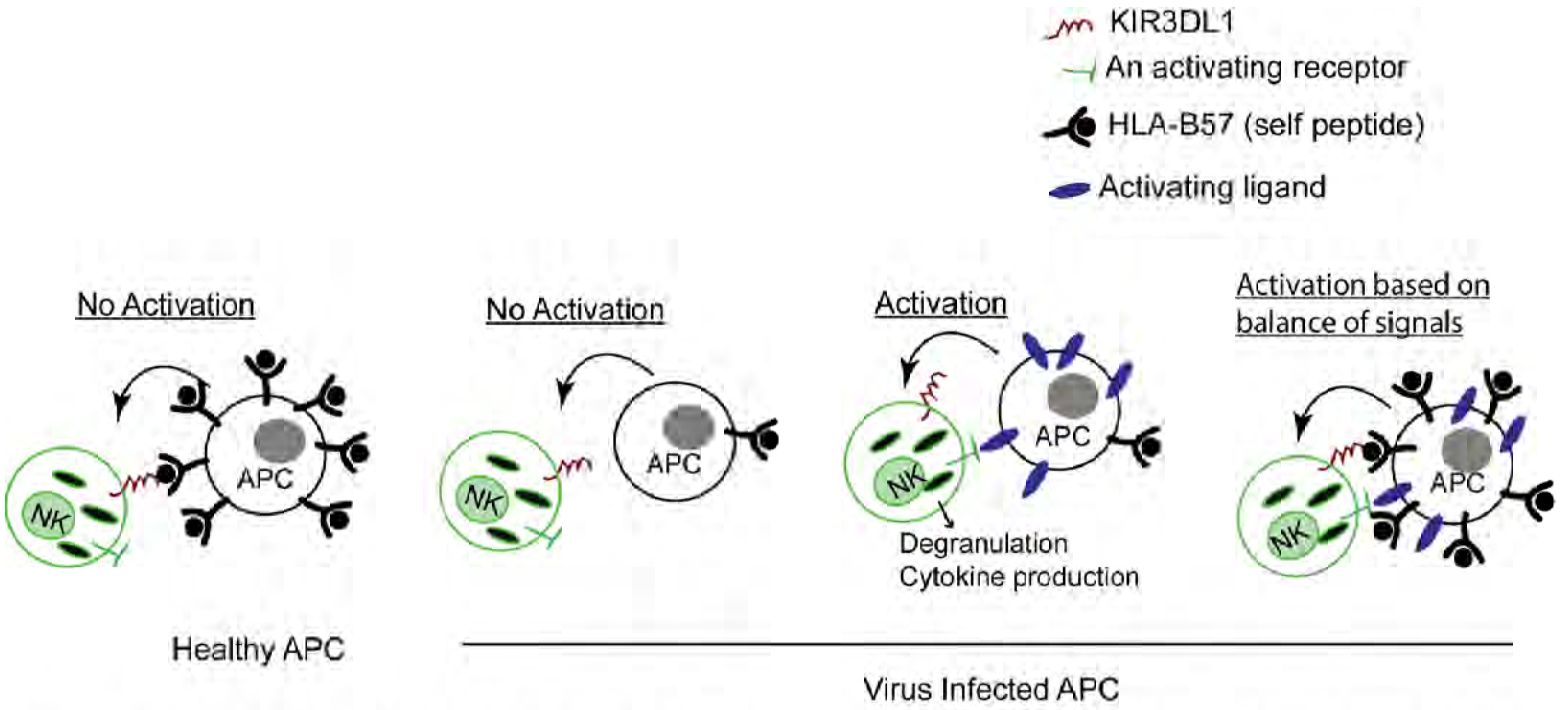


Figure 1.1 **Activation of NK cells.** NK cells which carry inhibitory receptors are licensed to respond and do so when the activating signals received overwhelm the inhibitory signals recieved.

Adapted from Lanier, 2005, Annu Rev Immunol

important in the development of NK cells by "licensing" NK cells to respond to activating signals received later on[235]. The strength of an inhibitory signal is proportional to the strength of the response to subsequent activating signals[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[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[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[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[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**

Receptor[a]	Ligand[b]	Function[c]	Reference
KIR2DL1	HLA-Cw4	Inhibitory	254, 255
KIR2DL2	HLA-Cw3	Inhibitory	254, 255
KIR2DL3	HLA-Cw3	Inhibitory	254, 255
KIR2DL4	HLA-G	Activating? Inhibitory?	256, 257
KIR2DL5A/B	unknown	Inhibitory	
KIR2DS1	HLA-C	Activating	258
KIR2DS2	HLA-C	Activating	258
KIR2DS3	HLA-C	Activating	
KIR2DS4	HLA-Cw4	Activating	259
KIR2DS5	unknown	Activating	
KIR3DL1	HLA-Bw4	Inhibitory	247
KIR3DL2	HLA-B27, HLA-A3, HLA-A11	Inhibitory	260, 261
KIR3DL3	HLA-Cw4	Inhibitory	
KIR3DS1	HLA-Bw4?	Activating	
CD94-NKG2A	HLA-E	Inhibitory	262
2B4	CD48	Activating or Inhibitory, coreceptor	263, 264
LILRB1	HLA class I	Inhibitory	265
CD16	IgG	Activating	266
CD94-NKG2C	HLA-E	Activating	262, 267
CD94-NKG2E	HLA-E	Activating	267
NKG2D	MICA, MICB, ULBP	Activating	268
NKp30	Unknown	Activating	269
NKp44	DENV protein E, viral hemagglutinins	Activating	216, 270
NKp46	unknown	Activating	271
CD161	LLT1	Activating and Inhibitory	272, 273
CD38	CD31	Activating	229, 230, 232

[a] *Receptor present on the surface of NK cells*
[b] *Ligand(s) bound by the NK cell receptor*
[c] *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[218]. Strikingly, this hypersensitivity was transferred to an unsensitized host through adoptive transfer of NK cells[220, 221]. Viral antigens can also induce recall responses by NK cells that can protect against subsequent viral challenge[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 $CD8^+$ T cells, and cytokine production[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.[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 $CD4^+$ and $CD8^+$ 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[275]. In the presence of NK cells and high levels of antigen $CD8^+$ T cells become exhausted minimizing their damage. This $CD8^+$ T cell exhaustion was governed indirectly by NK cell-mediated depletion of $CD4^+$ T cells. The authors later investigated how depletion of NK cells after the establishment of chronic infection affected $CD8^+$ T cell responses, immunopathology, and viral levels. They found that delayed NK cell depletion resulted in increased $CD8^+$ 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[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 $CD4^+$ and $CD8^+$ T cells through a perforin-mediated mechanism[277]. Lastly, NK cells are important contributors to the overall cytokine profile of the antiviral innate immune response, which subsequently

directs T cell responses[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[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[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[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[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[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[286]. Interestingly, poor NK cell activity leads to poor maturation of DCs and a reduced ability of NK cells to eliminate immature DCs[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[90]. 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 $NS1_{26\text{-}34}$ 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-$NS1_{26\text{-}34}$ TET to $CD8^-$ cells which lead us to additionally hypothesize that:

HLA-B57 molecules expressing the conserved epitope $NS1_{26\text{-}34}$ bind the inhibitory receptor KIR3DL1 on NK cells.

This Thesis assessed $CD8^+$ 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: $CD8^+$ 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 $CD8^+$ 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[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[57]. Serology and virus isolation were used to confirm acute DENV infections, and primary and secondary infections were distinguished based on serologic responses[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^{+} 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°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^{o}C

C. Generation of Peptides

Peptides were purchased from 21st 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[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^oC for 7 days as previously reported[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^oC, 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**

Epitope	Origin[a]	Sequence(s)	HLA[b]
$E_{213-221}$	DENV-1 DENV-2 DENV-3 DENV-4	FLDLPLPWT FLDLPLPWL FFDLPLPWT FFDLPLPWL	A2
$NS1_{26-34}$	DENV 1-4	HTWTEQYKF	B57
$NS3_{133-142}$	DENV-1 DENV-2 DENV-3,4	GTSGPIVNRE GTSGSPIVDR GTSGSPIIN	A11
LF9	Self	LSSPVTKSF	B57
TW10 TW10n	HIV	TSTLQEQIGW TNTLQEQIGW	B57 B57

[a] *Origin of the epitope listed*
[b] *HLA restriction of the epitope listed*

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

Serotype	**Strain**
DENV-1	BRP 04-00
DENV-2	16681
	S-16803
DENV-3	CH53489
DENV-4	341750

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 [146]. Briefly, HLA-$B57^+$ B-lymphoblastoid cell line (BLCLs) target cells were labeled with radio-labeled chromium (^{51}Cr) 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)x100.

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

At day 16 of culture approximately 2×10^5 CD8$^+$ T cells were cultured with 2×10^4 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^o C (Table 2.3). Finally, cells were washed and placed in BD Stabilizing Fixative™ (BD Biosciences) diluted 1:3 and kept at 4^oC for analysis.

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

$2x10^5$ CD8$^+$ T cells were mixed with $2x10^4$ 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

$1.5x10^{6}$ PBMC were mixed with $3x10^{5}$ 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[291]. 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-$NS1_{26-34}$ or B57-LF9).

O. $KIR3DL1^+$ NK Cell Depletion and B57-$NS1_{26-34}$ TET Staining

PBMC were isolated from blood drawn from $KIR3DL1^+$ healthy subjects. PBMC were depleted of $KIR3DL1^+$ cells by magnetic bead depletion (MACS, Miltenyi Biotec, Auburn, CA). KIR3DL1-depleted PBMC were washed with FACS Buffer and incubated with B57-$NS1_{26-34}$ TET for 50 minutes at 4^oC. After incubation, the cells were washed with 1mL FACS Buffer, fixed with 100µL BD Cytofix (diluted 1:4) and kept at 4^oC until flow analysis. Data were collected on a BD FACSAria™ and analyzed using FlowJo version 10.

P. Blocking of B57-$NS1_{26-34}$ TET Binding Using an Anti-KIR3DL1 Antibody

PBMC from a $KIR3DL1^+$ 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-$NS1_{26-34}$ TET for 20 minutes at 4^oC. Then

B57-$NS1_{26-34}$ TET or Z27 was added to the cells stained with Z27 or B57-$NS1_{26-34}$ 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 ≤ 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**

Marker	Clone	Manufacturer	Fluorochrome
CD3	UCHT1	BD Biosciences	V500
		Biolegend	BV510
	OKT3	Biolegend	BV510
CD8	SK1	Invitrogen	PE-alexafluor610
CD45RA	HI100	BD Pharmingen	APC-H7
CCR7	150503	BD Horizon	V450
CD69	CH/4, FN50	Invitrogen, BioLegend	PE-Cy5.5, BV650
CD38	HB7	eBioscience	eFluor®650NC
CD57	HCD57	BioLegend	PerCP/Cy5.5 (Lightening Link)
CD71	OKT9	eBioscience	PE-Cy7 (Lightening Link)
	CY1G4	Biolegend	APC
CD28	CD28.2	BioLegend	AlexaFluor700
CD56	B159	BD Biosciences	AlexaFluor700
CD19	HIB19	BD Biosciences	FITC
CD14	HCD14	BioLegend	FITC
CD107a	H4A3	BD Biosciences	FITC
	H4A3	Biolegend	BV421
MIP-1β	D21-1351	BD Biosciences	PE
TNF-α	MAb11	BD Biosciences	APC
IFN-γ	B27	BD Biosciences	AlexaFluor 700
CD56	HCD56	BioLegend	BV711
CD16	3G8	BD Horizon	APC-H7
NKp30	P30-15	BioLegend	APC
NKp46	9E2	BioLegend	BV421
CD161	HP-3G10	BioLegend	BV605
NKG2D	1D11	BD	PE-CF594
KIR3DL1	DX9	Beckman Coulter	PE
		Biolegend	PE
KIR3DL1/S1	Z27	Beckman Coulter	APC
HLA-A,B,C	W6/32	BD	PE, FITC, APC
		BioLegend	PE
HLA-B57	BIH0243	One Lambda	PE-NeutrAvidin (Life Technologies)
DENV E	3H5	Millipore	PE, FITC, Alexa647 (indirect staining or conjugated in lab,)
prM	2H2		

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 outcome[88-91]. 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 infection [292-294] and with a number of type 2 idiosyncratic adverse drug reactions [295, 296]. The relative ability of HLA-B57 to control HIV infection correlated with unique peptide-binding characteristics that affect thymic development of CD8$^+$ T cells [297]. 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 [146]. 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 $NS1_{26-34}$ 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/$NS1_{26-34}$ tetramer (B57-$NS1_{26-34}$ 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).

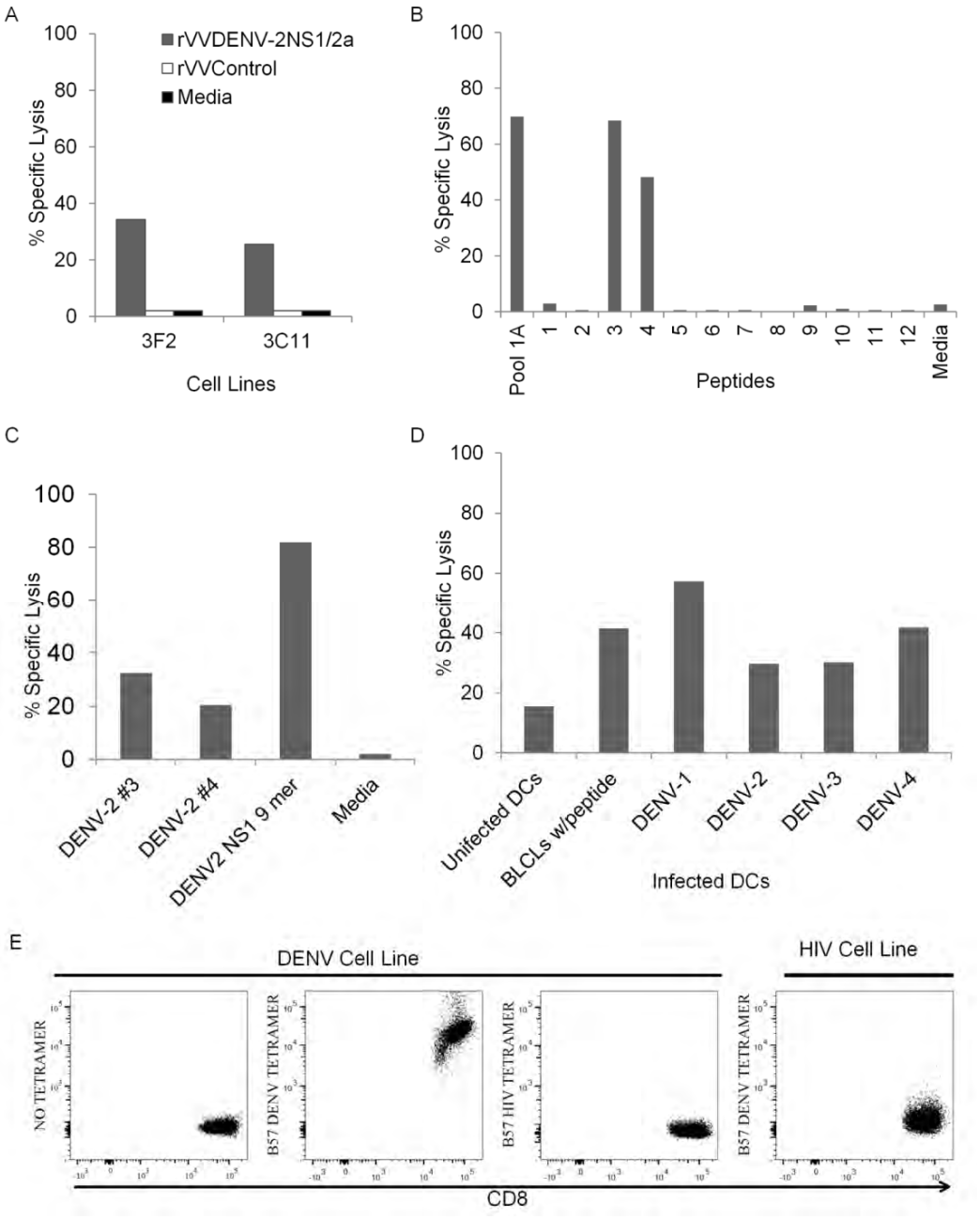


Figure 3.1. **Identification of the HLA-B57-restricted DENV epitope.** (A) Cell lines 3C11 and 3F2, generated from PBMC of donor KPP94-037, were used in a ^{51}Cr release assay using B-LCLs infected with vaccinia virus recombinants expressing DENV-2 NS1/2a as target cells. (B) ^{51}Cr release assay using 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⁺ DENV-specific T cell epitopes**

EPITOPE	SEQUENCE	HLA RESTRICTION	No. of SEQ	% CONSERVATION									
				P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
$E_{211-219}$	FFDLPLPWT	A02	1148	100	78	99	100	100	100	100	100	95	n/a
$NS1_{26-34}$	HTWTEQYKF	B57	2610	100	100	100	100	100	100	100	100	100	n/a
$NS3_{71-79}$	SVKKDLISY	B62	2554	66	100	96	97	100	97	100	100	100	n/a
$NS3_{133-142}$	GTSGSPIVNR	A11	2554	100	100	100	100	100	100	100	65	70	87
$NS3_{222-230}$	ILAPTRVVAA	B07	2554	99	100	100	100	100	100	100	100	100	55
$NS3_{501-509}$	TPEGIIPAL	B35	2554	100	100	100	100	100	100	100	66	70	n/a
$NS4a_{56-64}$	LLLALIAVL	A02	2554	51	100	97	49	100	45	76	45	66	n/a
$NS4b_{111-119}$	VLLLVAHYA	A02	2722	65	95	51	100	82	75	100	100	100	n/a
$NS4b_{181-189}$	ILLMRTTWA	A02	2772	45	100	69	100	100	100	79	100	100	n/a
$NS5_{329-337}$	KPWDVIPMV	B55	2715	100	100	100	100	100	51	100	89	100	n/a

Sequence conservation of a sample of known CD8⁺ T cell epitopes among 4 serotypes of human DENV strains calculated using sequence and variability analysis tool on flavidB database http://cvc.dfci.harvard.edu/flavi/index.php. A NCBI search revealed two strains with a variant B57 epitope. n/a indicated epitopes containing only 9aa.

B. Detection of B57-NS1$_{26-34}$ TET^{+}CD8^{+} T Cells in PBMC Collected During Acute Dengue Virus Infection

We hypothesized that HLA-B57 restricted, NS1$_{26-34}$-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-NS1$_{26-34}$ TET together with activation and phenotypic markers and performed a longitudinal analysis of B57-NS1$_{26-34}$-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 NS1$_{26-34}$, NS3$_{133-142}$, or E$_{213-221}$ 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-NS1$_{26-34}$-specific T cells reaching ~20% at fever day +7. Frequencies of B57-NS1$_{26-34}$-specific T cells in subject CHD06-029 were more representative of the staining observed in the remaining donors. Expansion of B57-NS1$_{26-34}$ 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**

Donor	Serology[a]	Serotype[b]	Diagnosis[c]	MHC- Class I
CHD95-039	P	DENV-1	DF	HLA-A1,11 HLA-B56,57
CHD06-029	P	DENV-3	DF	HLA-A2,11 HLA-B57,46
CHD01-058	S	DENV-2	DHF-1	HLA-A33,34 HLA-B57,75
CHD01-018	S	DENV-2	DF	HLA-A2,33 HLA-B57,46
CHD01-050	S	DENV-2	DHF-3	HLA-A1,11 HLA-B57,60
KPP94-037	S	DENV-2	DF	HLA-A1,11 HLA-B46,57
KPP94-041	S	DENV-1	DHF-3	HLA-A1,207 HLA-B54,57
CHD02-073	S	DENV-1	DHF	HLA-A1,11 HLA-B57,60
CHD00-054	S	unknown	DHF-2	HLA-A203 HLA-B46,57
CHD05-023	S	DENV-1	DF	HLA-A2,24 HLA-B46,57
CHD06-092	S	DENV-4	DHF-2	HLA-A1,33 HLA-B57,35

[a] *Primary (P) versus secondary (S) infection as determined by IgM/IgG ratios*[59]

[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*

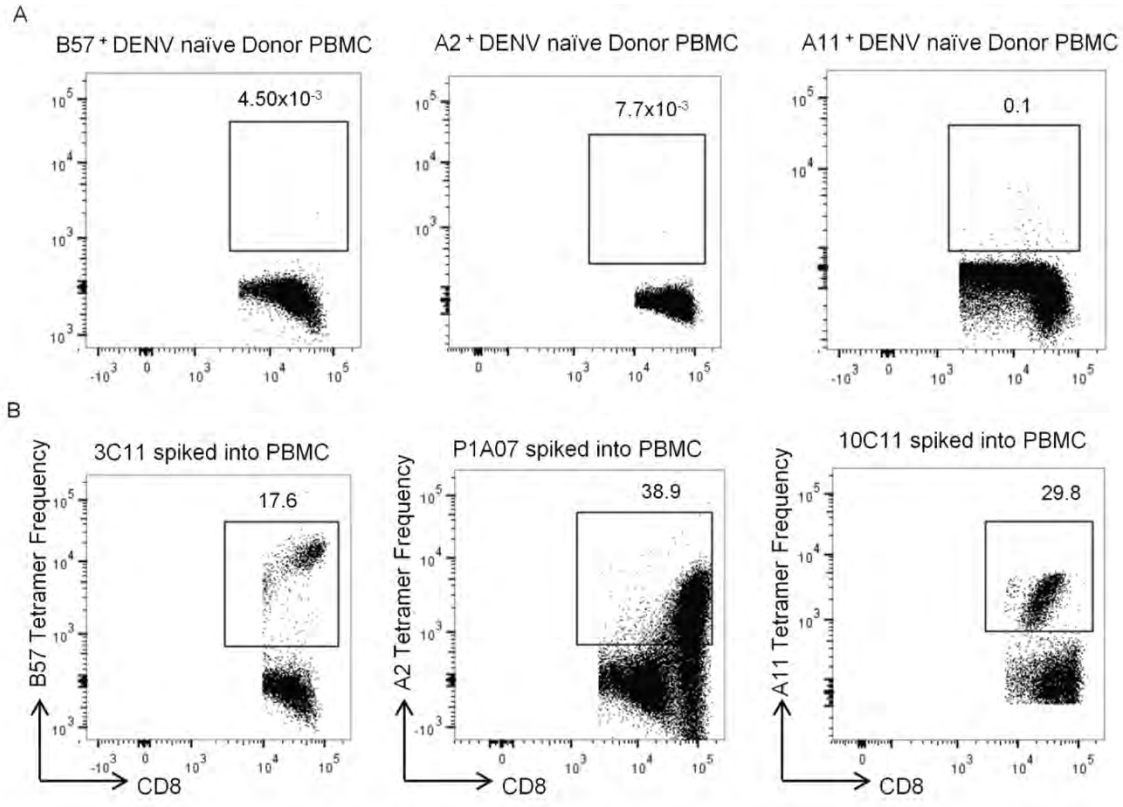


Figure 3.2. **Tetramer staining controls.** (A) PBMC from DENV naïve HLA-B57^{+}, A2^{+}, or A11^{+} individuals were stained with B57-NS1$_{26\text{-}34}$ TET, A2-E$_{213\text{-}221}$ TET, or A11-NS3$_{133\text{-}142}$ TET. (B) PBMC spiked with the appropriate epitope-specific cell line was stained with B57-NS1$_{26\text{-}34}$ TET, A2-E$_{213\text{-}221}$ TET, or A11-NS3$_{133\text{-}142}$ TET.

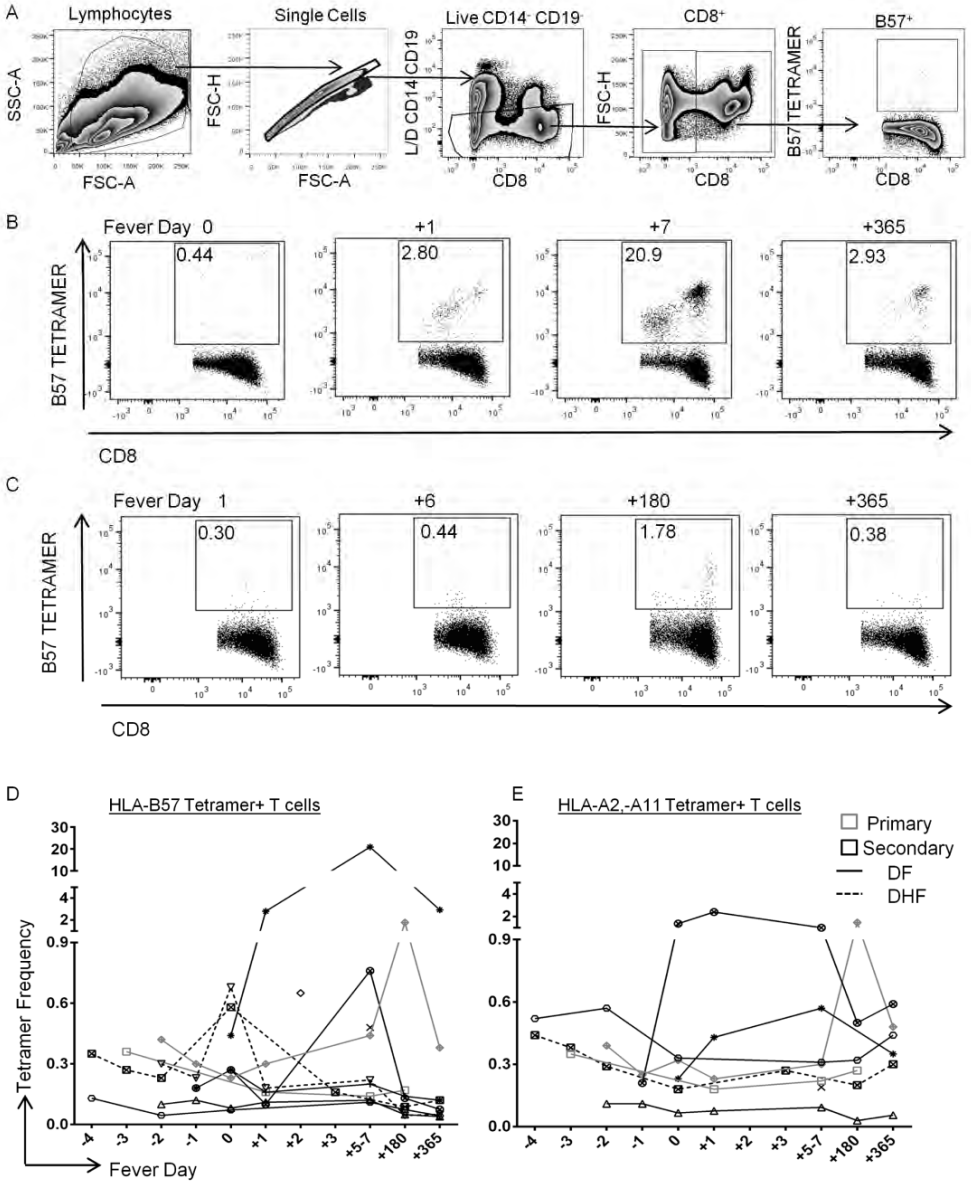


Figure 3.3. **Expansion of DENV specific T cells during acute infection.** (A) Gating strategy used to identify $TET^{+}CD8^{+}$ T cells started by selecting cells within the lymphocyte gate as defined by forward and side scatter profiles followed by gating for singlet cells. Live $CD14^{-}CD19^{-}$ cells were next selected by exclusion of the viability marker LIVE/DEAD® Green along with αCD14-FITC and αCD19-FITC. $CD8^{+}$ T cells were identified by CD8 expression. (B) Kinetics of B57-$NS1_{26\text{-}34}$ TET^{+} frequencies in PBMC from donor KPP94-037 and (C) donor CHD06-029 over the course of acute illness and convalescence. (D) B57-$NS1_{26\text{-}34}$ $TET^{+}CD8^{+}$ 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-$E_{213\text{-}221}$ TET or A11-$NS3_{133\text{-}142}$ 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-$NS1_{26\text{-}34}$ TET^+CD69^+ cells (Figure 3.4C) and A2-$E_{213\text{-}221}$ TET^+CD69^+ or A11-$NS3_{133\text{-}142}$ 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-$NS1_{26\text{-}34}$ TET^+CD38^+ cells (Figure 3.4F) and A2-$E_{213\text{-}221}$ TET^+CD38^+ or A11-$NS3_{133\text{-}142}$ 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.

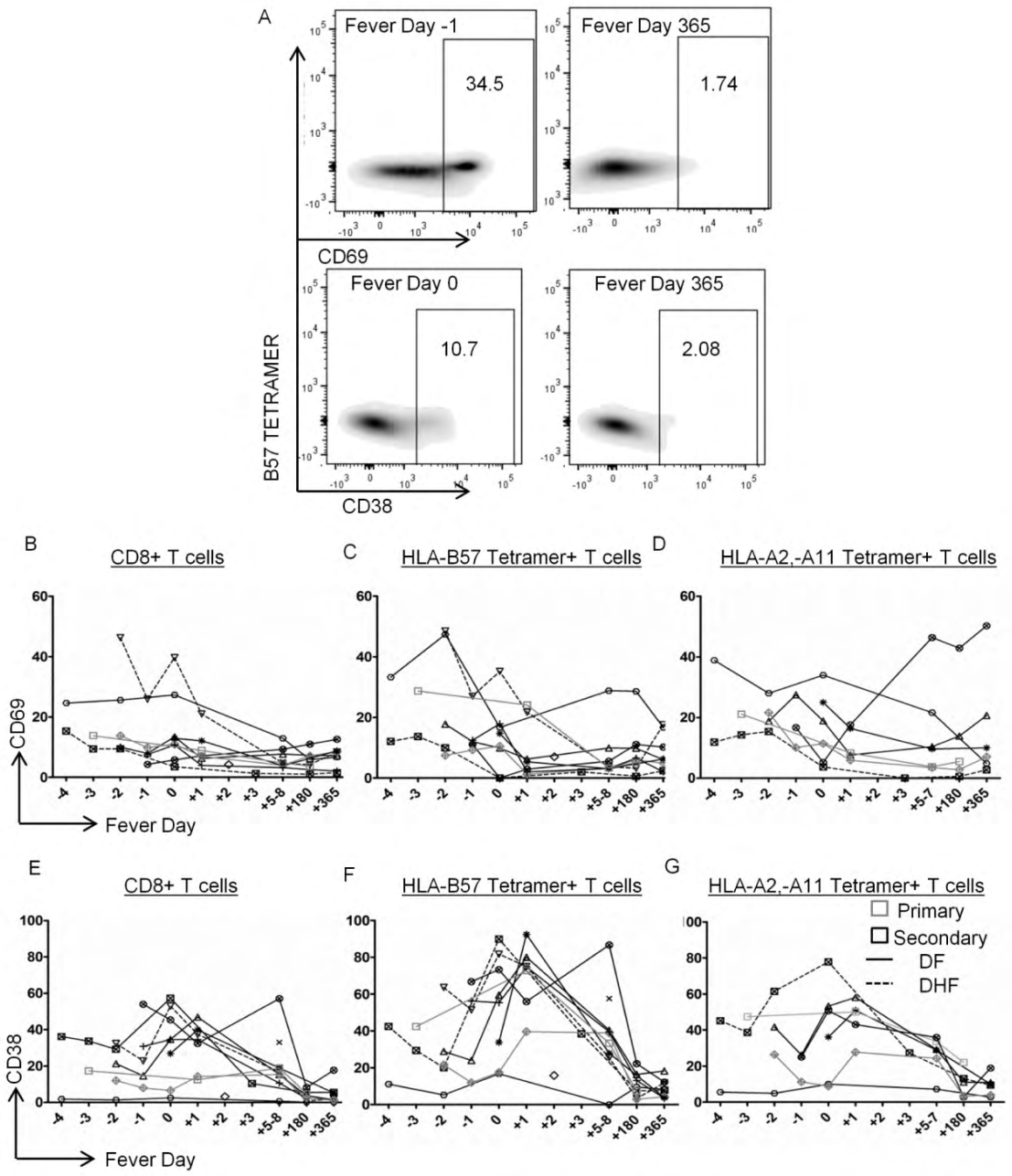


Figure 3.4. **Antigen-specific T cells are highly activated during acute DENV infection and early convalescence.** (A) Representative staining of CD69 and CD38 on total $CD8^+$ T cells during acute infection and in convalescence from 1 subject. (B and E) Staining of CD69 and CD38 on total CD8 cells, (C and F) B57-$NS1_{26-34}$ TET^+ T cells and A11-$NS3_{133-142}$ TET^+ or (D and G) A2-$E_{213-221}$ 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-$NS1_{26\text{-}34}$ TET^{+} T cells expressing CD57 was 24.0% (Figure 3.5C). In 3 of 11 donors the frequency of $CD57^{+}$B57-$NS1_{26\text{-}34}$ TET^{+} cells was higher during acute infection than at the six months and/or one year time point. The mean frequency of $CD57^{+}$ A11-$NS3_{133\text{-}147}$ TET^{+} or A2-$E_{213\text{-}221}$ TET^{+} T cells was 29.5% of TET^{+} T cells (Figure 3.5D).

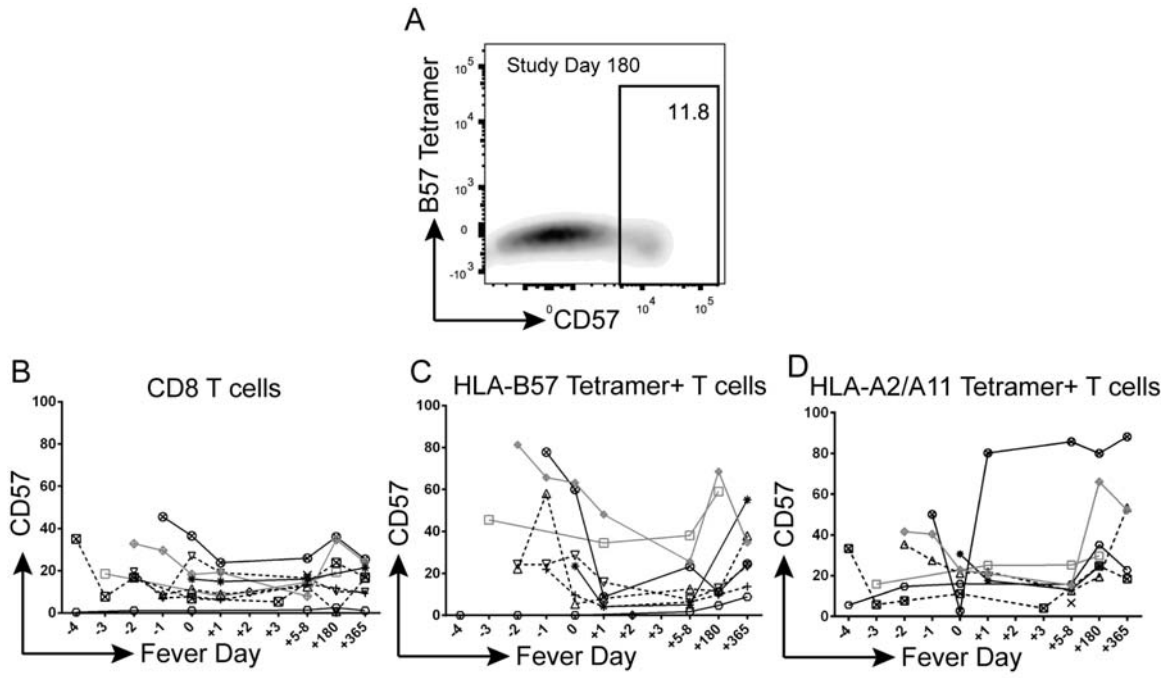


Figure 3.5. **CD57 expression varies only slightly during DENV infection.** (A) Representative staining of CD57 on total $CD8^+$ T cells from one donor. (B) Staining of CD57 on total $CD8^+$ T cells, (c) B57-$NS1_{26\text{-}34}$ TET^+ cells and (D) A11-$NS3_{133\text{-}142}$ TET^+ or A2-$E_{213\text{-}221}$ 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$_{26-34}$ TET$^+$, A11-NS3$_{133-147}$ TET$^+$ and A2-E$_{213-221}$ TET$^+$ CD8$^+$ T Cell Populations

We assessed CD71 expression, a marker associated with cell cycle activity [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$^+$ 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$_{26-34}$ TET$^+$ T cells expressing CD71 was 18.4% and of A11-NS3$_{133-147}$ TET$^+$ or A2-E$_{213-221}$ TET$^+$ 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$^+$ DENV-specific T cells compared to the total CD8$^+$ population ($p < 0.0001$, Table 3.3). There were no statistically significant differences in CD71 expression between the B57-NS1$_{26-34}$ TET$^+$ and the A11-NS3$_{133-147}$/A2-E$_{213-221}$-specific T cell populations.

The peak frequency, as determined for each donor during acute illness, of CD71$^+$ DENV-specific CD8 T cells was also significantly higher than that of the total CD8$^+$ T cells ($p < 0.005$). Frequencies of CD71$^+$ 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$^+$ 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-$NS1_{26-34}$ TET^+, but not A11-$NS3_{133-147}$ TET^+/ A2-$E_{213-221}$ TET^+, T cells. CD69 expression was minimally increased only in A11-$NS3_{133-147}$ TET^+ 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 $CD71^+$ cells during acute illness between the DENV-specific populations and total $CD8^+$ 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 $NS1_{26-34}$ epitope. We stimulated the B57-$NS1_{26-34}$-specific T cell line, 3C11, with different concentrations of the $NS1_{26-34}$ 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).

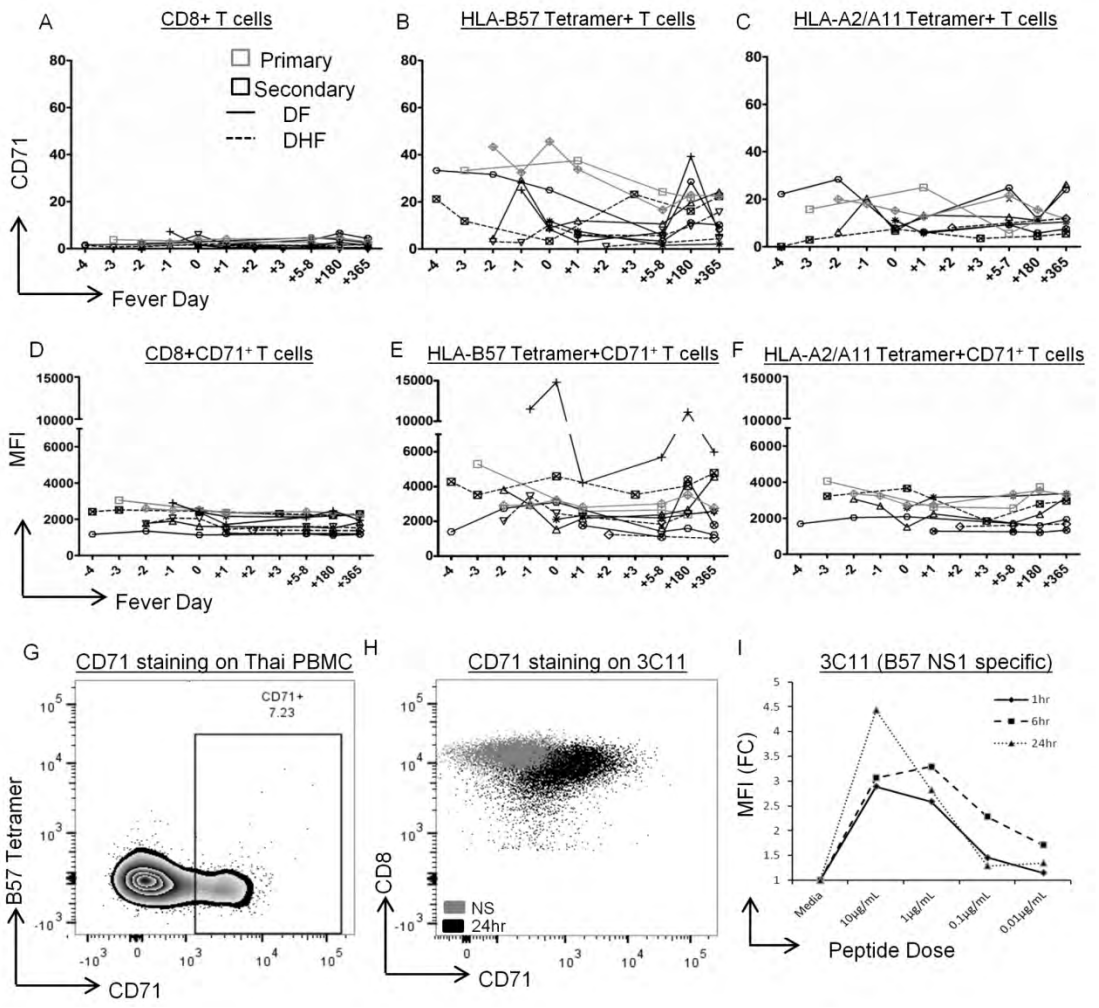


Figure 3.6. **CD71 expression on total CD8$^+$ and DENV-specific CD8$^+$ T cells.** Frequency of CD71$^+$ cells in (A) total CD8$^+$ cells, (B) B57-NS1$_{26\text{-}34}$ TET$^+$ T cells and (C) A11-NS3$_{133\text{-}142}$ TET$^+$ or A2-E$_{213\text{-}221}$ TET$^+$ T cells over the course of acute DENV infection and convalescence. MFI of CD71 expressed on CD71$^+$ (D) CD8$^+$ cells, (E) B57-NS1$_{26\text{-}34}$ TET$^+$ T cells and (F) A11-NS3$_{133\text{-}142}$ TET$^+$ or A2-E$_{213\text{-}221}$ 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$_{26\text{-}34}$-specific cell line following stimulation with 10, 1, 0.1 and 0.01µg/mL NS1$_{26\text{-}34}$ peptide HTWTEQYKF.

Table 3.3: **Statistical analysis of activation markers on $CD8^+$ T cells**

	Populations compared	**CD69**[a]	**CD38**[a]	**CD71**[a]
Mean frequency	Total $CD8^+$ vs. B57-$NS1_{26\text{-}34}$ TET^+	N.S.	0.0017	<0.0001
	Total $CD8^+$ vs. A11-$NS3_{133\text{-}142}$ TET^+/A2-$E_{213\text{-}221}$ TET^+	N.S.	N.S.	<0.0001
	B57-$NS1_{26\text{-}34}$ TET^+ vs. A11-$NS3_{133\text{-}142}$ TET^+/A2-$E_{213\text{-}221}$ TET^+	N.S.	N.S.	N.S.
Peak frequency	Total $CD8^+$ vs. B57-$NS1_{26\text{-}34}$ TET^+	N.S.	0.0115	0.0021
	Total $CD8^+$ vs. A11-$NS3_{133\text{-}142}$ TET^+/A2-$E_{213\text{-}221}$ TET^+	0.04	N.S.	0.0005
	B57-$NS1_{26\text{-}34}$ TET^+ vs. A11-$NS3_{133\text{-}142}$ TET^+/A2-$E_{213\text{-}221}$ TET^+	N.S.	N.S.	N.S.

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

[a] *p-values determined using Mann-Whitney*

F. CD71, CD69, CD107a and Cytokine Expression in Epitope-Specific T Cell Lines

Since the $NS1_{26-34}$ 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-$NS3_{133-147}$ 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 [120]. 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-$NS3_{133-147}$ epitope.

We also stimulated the A2-$E_{213-221}$ 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.

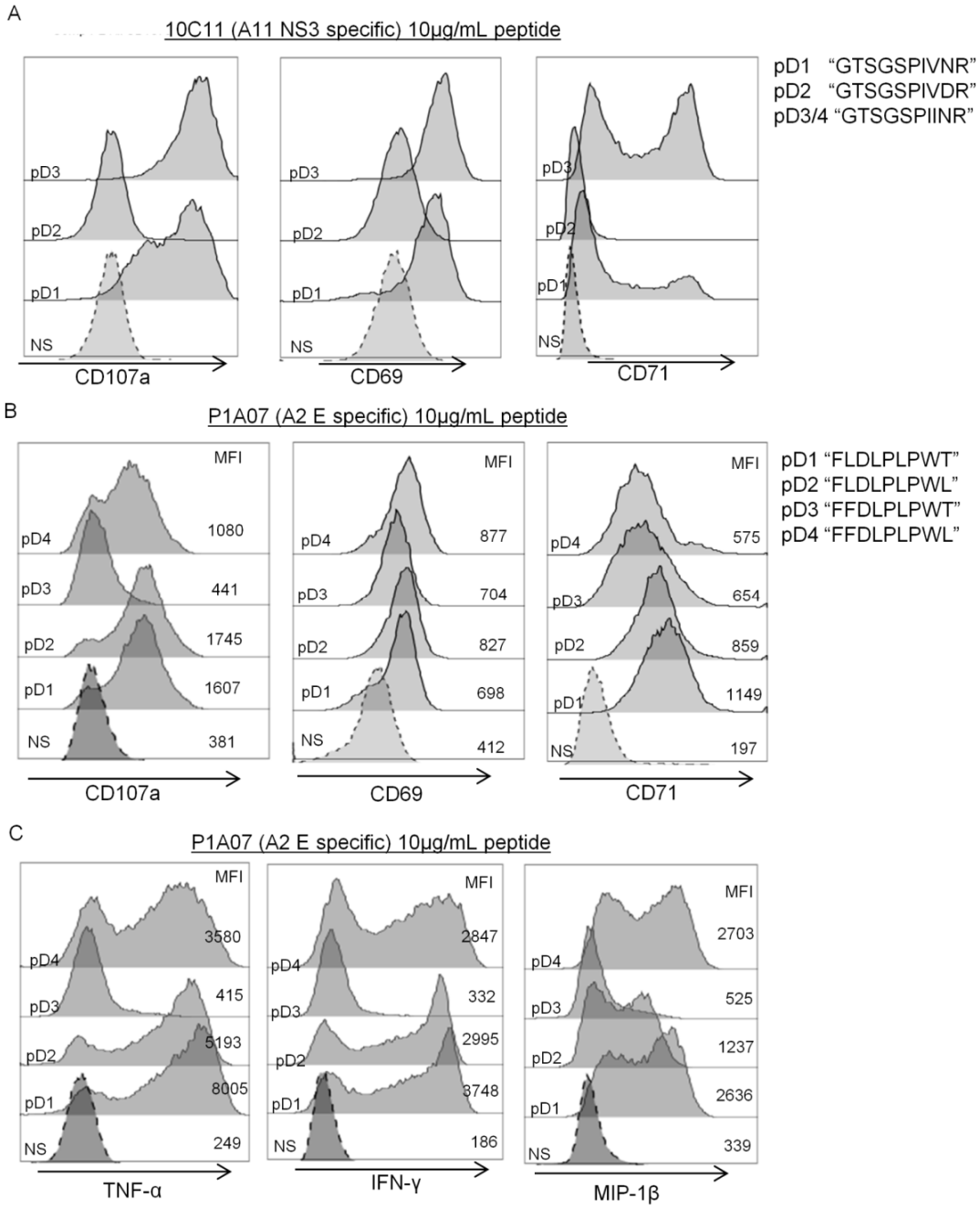


Figure 3.7. **CD71 expression and effector functions on epitope-specific T cell lines.** CD107a, CD69 and CD71 expression after in vitro stimulation of cell line (A) 10C11 for 6 hrs with 10µg/mL A11-NS3$_{133-142}$ variant peptides pD1, pD2, and pD3/4 and cell line (B) P1A07 for 6 hrs with 10µg/mL A2-E$_{213-221}$ 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.

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-$NS1_{26-34}$ 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 $CD45RA^{-}$, $CCR7^{low/-}$ (panel A), $CD69^{low}$, $CD38^{+/high}$, $CD71^{+}$, $CD57^{-}$ (panel B) and show that B57-$NS1_{26-34}$ TET^{+} T cells on this day fall into this phenotype (panel C). Panel C shows the B57-$NS1_{26-34}$ TET^{+} T cells aligned along the x-axis. B57-$NS1_{26-34}$ TET^{+} 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).

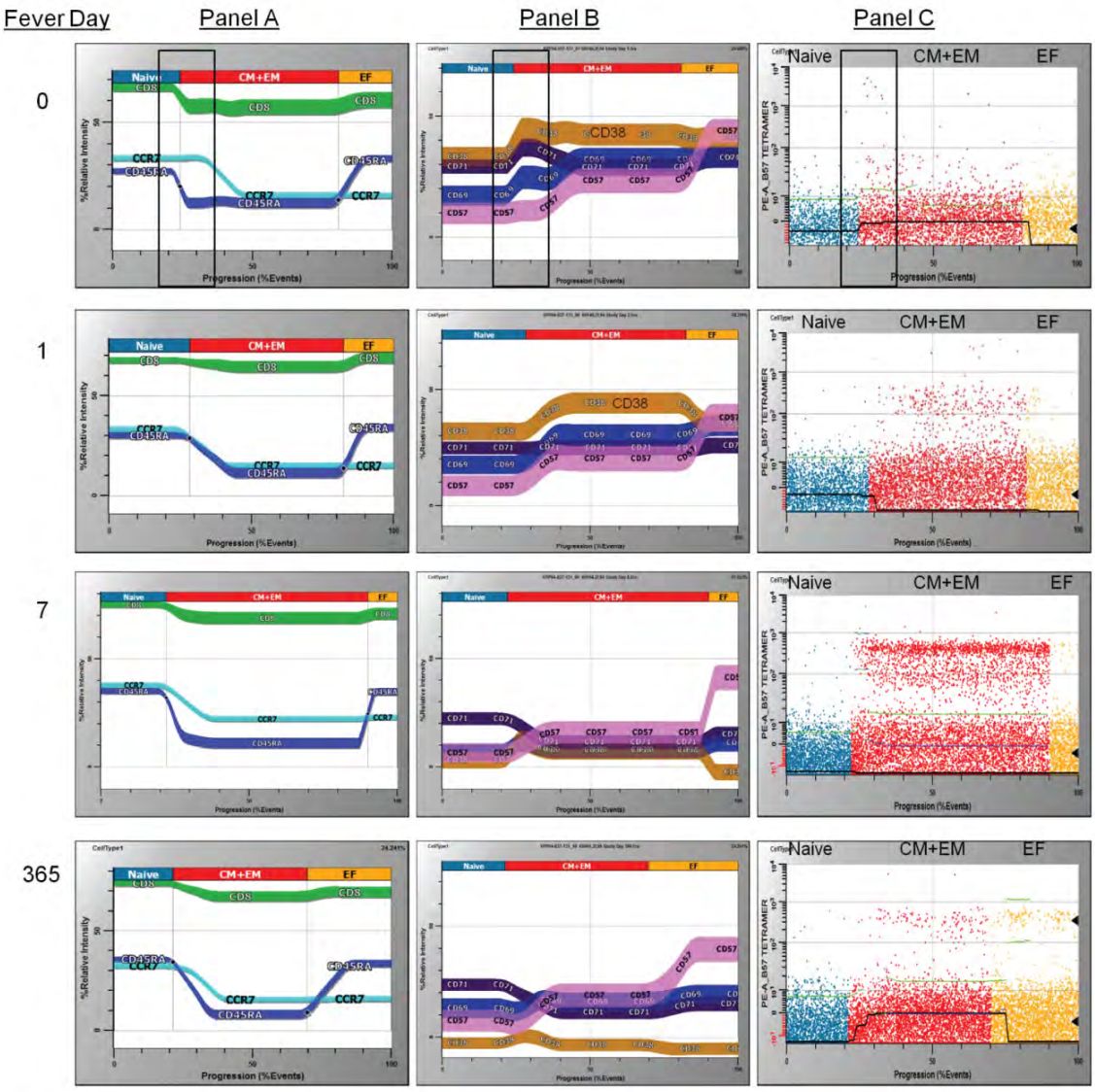


Figure 3.8 **Probability state modeling reveals novel phenotypes of CD8$^+$ T cells during acute DENV infection.** (A) The progression of CD8 (green), CD45RA (dark blue) and CCR7 (light blue), (B) the progression of CD69 (blue), CD38 (orange), CD57 (pink), and CD71 (purple) expression on the total CD8$^+$ T cells in PBMC obtained from subject KPP94-037 over the course of acute DENV infection and convalescence. (C) B57-NS1$_{26\text{-}34}$ 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-$NS1_{26-34,}$ 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[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 $CD8^+$ T cell responses to the B57-$NS1_{26-34}$ epitope. While we detected B57-$NS1_{26-34}$ TET^+ 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-$NS1_{26-34}$ TET^+ T cells were similar to those of A11-$NS3_{133-142}$ TET^+ and A2-$E_{213-221}$ TET^+ T cells in the same subjects and to the frequencies of A11-$NS3_{133-142}$ TET^+ T cells reported elsewhere[114, 134]. One donor had a peak frequency of B57-$NS1_{26-34}$ TET^+CD8^+ 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^+ 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[301]. We demonstrated that B57-NS1$_{26-34}$-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-NS1$_{26-34}$ 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 [114, 115, 117, 129, 134]. 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 [115]. 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 [302]. Friberg et al. found a lower intensity of CD38 expression on influenza TET^{+} cells compared to A11-NS3$_{133-142}$ TET^{+} cells during DENV infection [134]. 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 CD38^{high}. 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-$E_{213-221}$ 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 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 [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 ($CD71^+$) in these epitope-specific T cells suggests that many of the $CD69^+$ and $CD38^+$ $CD8^+$ 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-$NS1_{26-34}$-specific $CD8^+$ 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 [289], we found that frequencies of B57-$NS1_{26-34}$ TET^+ 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-$NS1_{26-34}$ TET^+ T cells (~20% of $CD8^+$ T cells). Consistent with previous studies, expression of the activation markers CD69 and CD38 was upregulated on the total $CD8^+$ 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-$NS1_{26-34}$ TET^+, A2-$E_{213-221}$ TET^+ and A11-$NS3_{133-142}$ TET^+ $CD8^+$ T cells, but not on total $CD8^+$ 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$_{26\text{-}34}$-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^{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^{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[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[247]. Because of the involvement of peptide in the binding interaction Fadda et al proposed that the peptide could alter binding affinity[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[250, 291]. Peptide specificity has been reported for KIR3DL1 binding to other HLA-Bw4 alleles[316] as well as for other KIR/HLA pairs including HLA-A11 and KIR3DL2[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^{+} NK cells although such functional differences have been reported for other KIR/HLA interactions[319]. Recently, O'Connor et al., used PBMC from KIR3DL1^{+} individuals to evaluate how peptide sequence affects B57 TET binding to primary human NK cells[291]. They observed great variation in TET binding based on single aa changes consistent with what has been observed using KIR3DL1 transfectants[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[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[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[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[252, 253, 321]. While the KIR3DL1^{+} subset of NK cells is expanded during acute HIV infection[253], the incubation of HIV-infected CD4^{+} T cells with KIR3DL1^{+} NK cells inhibited NK cell function[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^{+} rhesus macaques with NK target cells expressing Mamu-A1*00201 suppressed the degranulation only of Mamu-A1*00201 TET^{+} NK cells[323].

The role of KIR3DL1 in shaping the NK cell response in HLA-Bw4^{+} 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-$NS1_{26-34}$ TET to an NK enriched population. We hypothesized that the B57-$NS1_{26-34}$ TET was binding to NK cells via the known HLA-B57 binding partner KIR3DL1. Staining of a KIR3DL1 transfectant cell line confirmed that B57-$NS1_{26-34}$ TET bound KIR3DL1. Consistent with the function of an inhibitory KIR, incubation of healthy donor PBMC with HLA-B57-expressing, $NS1_{26-34}$-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 function[324-326]. 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-$NS1_{26-34}$ TET to $CD8^-$ Cells in PBMC from Dengue Patients

While studying the responses of $CD8^+$ T cells to the HLA-B57-restricted epitope $NS1_{26-34}$ (HTWTEQYKF), discussed in Chapter III, we observed binding of the B57-$NS1_{26-34}$ TET to $CD8^-$ cells (Figure 4.1A). In order to evaluate if this binding was specific to B57-$NS1_{26-34}$ 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 *in vitro*[250]. We saw minimal binding of the B57-TW10n TET to $CD8^-$ cells (Figure 4.1B), indicating that our DENV TET, B57-$NS1_{26\text{-}34}$, was likely binding via KIR3DL1 in a peptide dependent manner. We next looked at the frequency of B57-$NS1_{26\text{-}34}$ 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-$NS1_{26\text{-}34}$ TET staining in the NK-enriched cells varied over the course of DENV infection in PBMC from $HLA\text{-}B57^+$ individuals, and for a number of subjects the frequency was the lowest around Fever Day 0 (Figure 4.1C).

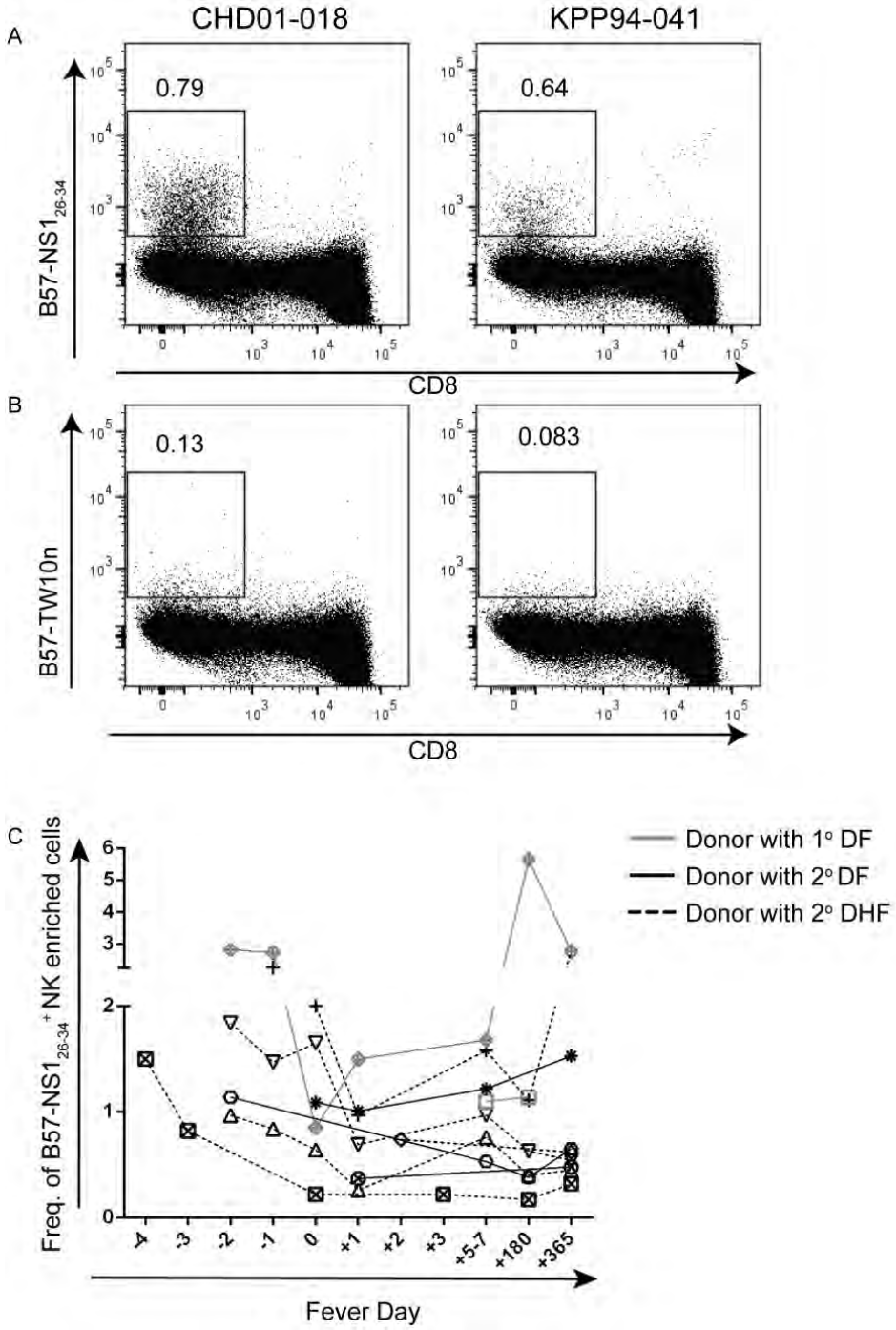


Figure 4.1. **Binding of the B57-NS1$_{26-34}$ TET to non-CD8 cells in Thai study cohort PBMC.** (A) Binding of B57-NS1$_{26-34}$ TET or (B) B57-Tw10n TET to CD3$^-$CD8$^-$CD14$^-$CD19$^-$ "NK-enriched" cells at the 1 year time point from two HLA-B57$^+$ subjects. (C) Frequency of B57-NS1$_{26-34}$ TET$^+$ 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.

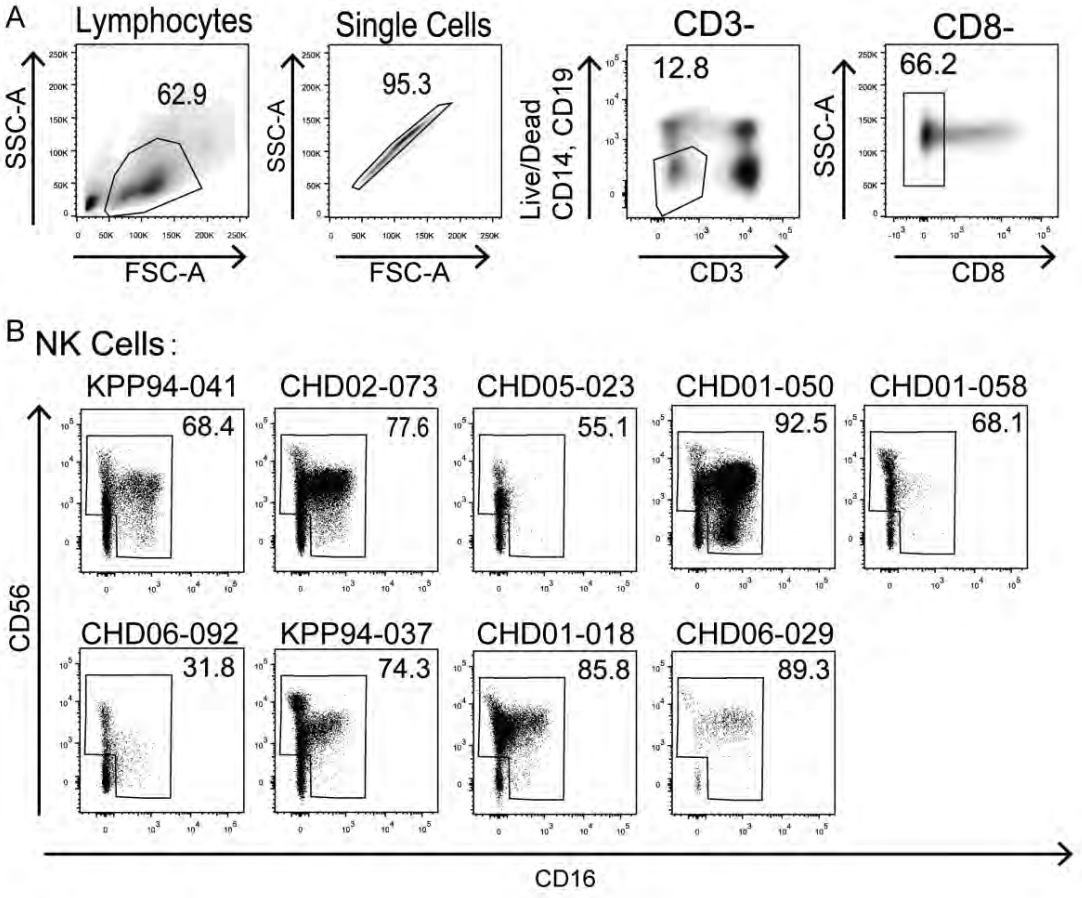


Figure 4.2. **CD3$^-$CD8$^-$CD14$^-$CD19$^-$ cells are predominantly NK cells.** (A) Gating strategy used in the identification of CD3$^-$CD8$^-$CD14$^-$CD19$^-$ cells. (B) Percentage of CD3$^-$CD8$^-$CD14$^-$CD19$^-$ cells which are CD56 and/or CD16 positive.

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.

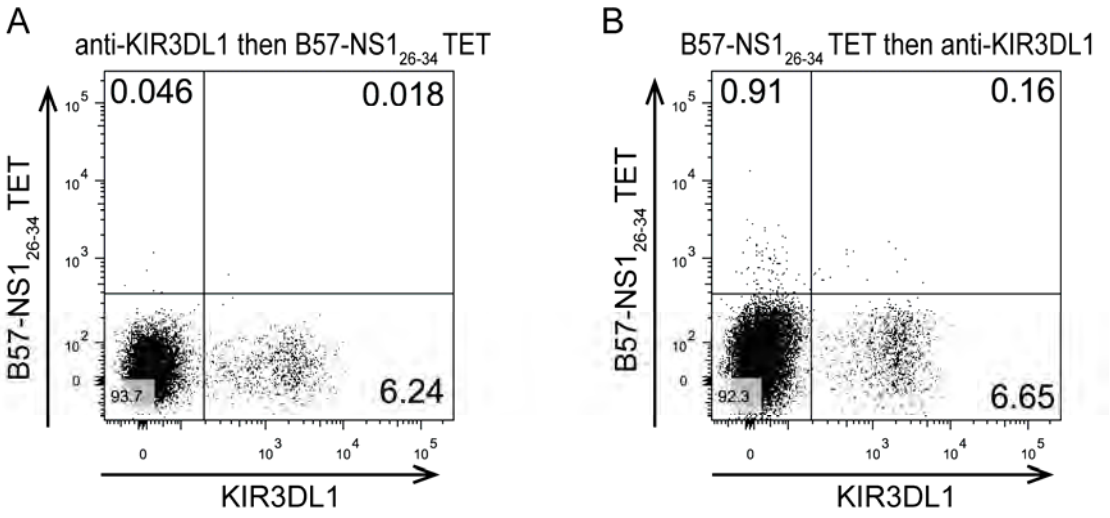


Figure 4.3. **Anti-KIR3DL1 antibody blocks binding of B57-NS1$_{26-34}$ TET.** (A) PBMC pre-incubated with anti-KIR3DL1 antibody for 30 min at 4^{o}C then with B57-NS1$_{26-34}$ TET for 50 min at 4^{o}C. (B) PBMC pre-incubated with B57-NS1$_{26-34}$ TET for 50 min at 4^{o}C then anti-KIR3DL1 antibody for 30 min at 4^{o}C. One representative experiment of five experiments is shown.

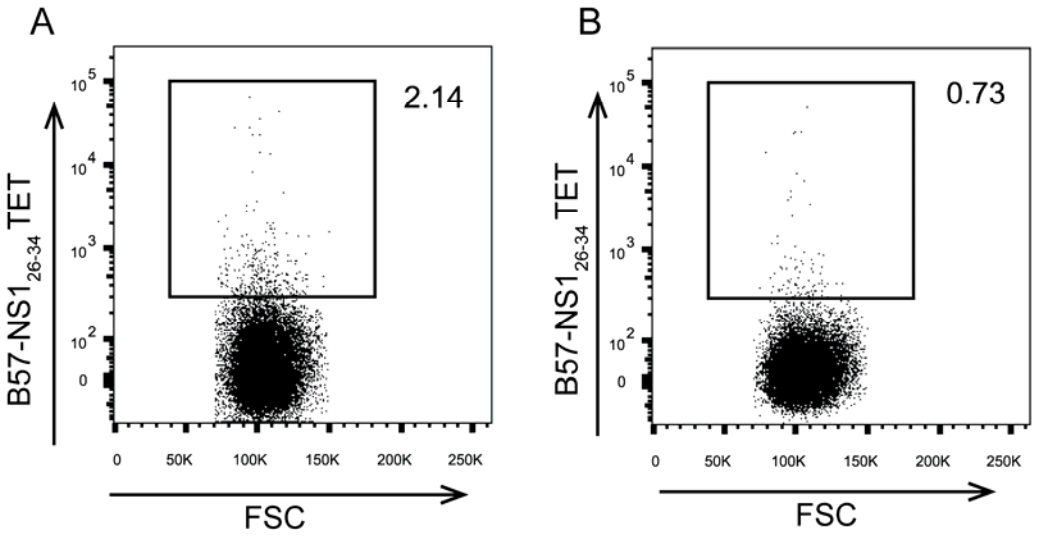


Figure 4.4. **Depletion of KIR3DL1+ cells decreases B57-NS1$_{26-34}$ TET binding.** (A) PBMC stained with B57-NS1$_{26-34}$ TET. (B) PBMC depleted of KIR3DL1^{+} cells by MACS and then stained with the B57-NS1$_{26-34}$ TET. One representative experiment of three experiments is shown.

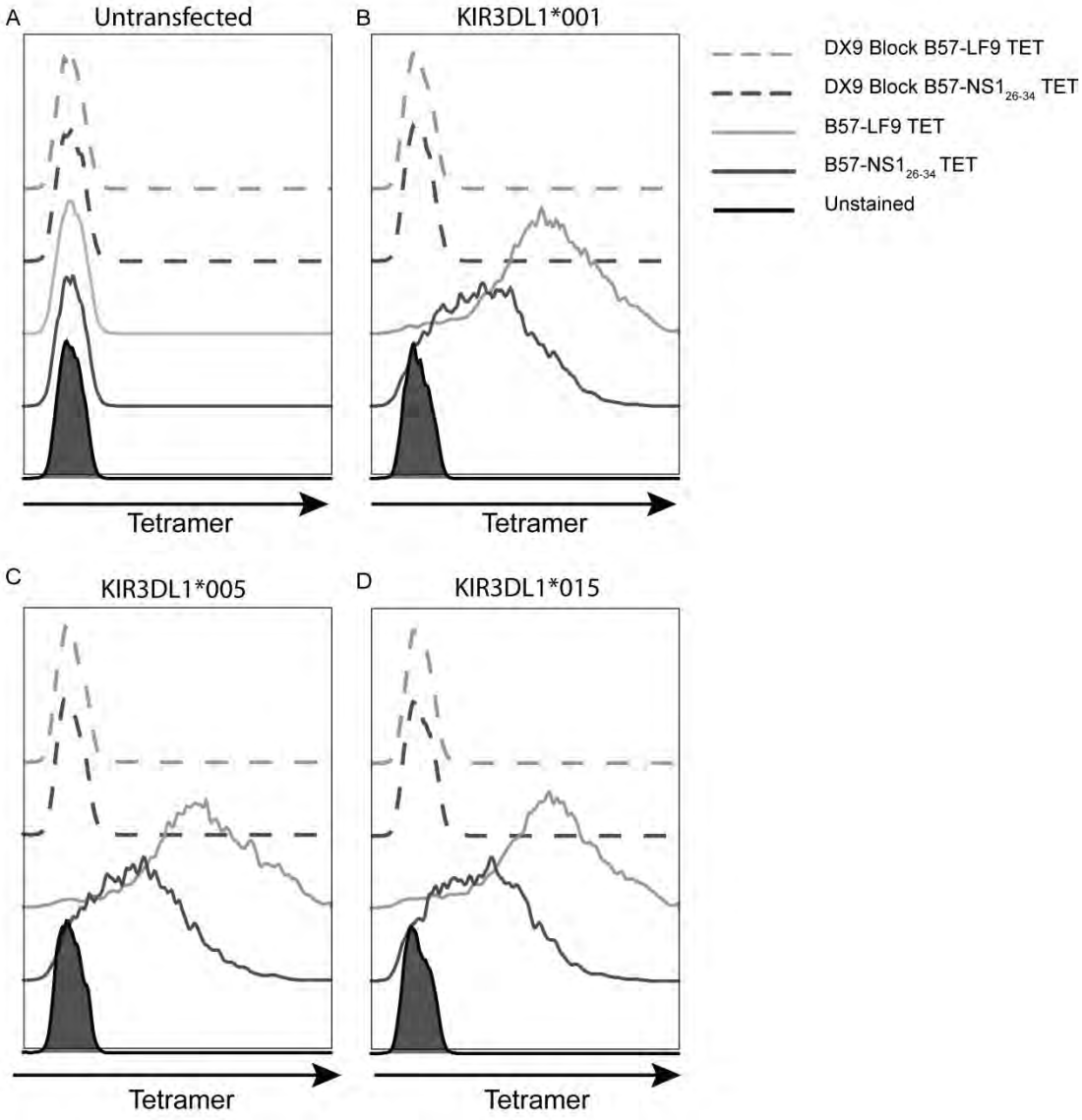


Figure 4.5. **B57-NS1$_{26-34}$ TET staining on KIR3DL1 transfectants.** Histograms showing B57-NS1$_{26-34}$ TET (black) as well as B57-LF9 TET (grey) binding (solid lines) to (A) an untransfected cell line, (B) KIR3DL1*001, (C) KIR3DL1*005, and (D) KIR3DL1*015 transfected cell lines. Binding of B57-NS1$_{26-34}$ 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 elsewhere[309] (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 KIR3DL1^{hi} and two donors were homozygous KIR3DL1^{low} (CHD02-073, KPP94-041).

In order to ascertain if B57-NS1$_{26\text{-}34}$ TET bound was bound to NK cells and assess if KIR3DL1 staining intensity or frequency affected B57-NS1$_{26\text{-}34}$ 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-NS1$_{26\text{-}34}$ 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-NS1$_{26\text{-}34}$ TET^{+} NK cells (Figure 4.6C).

Extended phenotyping of the B57-NS1$_{26\text{-}34}$ 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-NS1$_{26\text{-}34}$ TET^{+} NK cells (red dots) overlaid on the total NK cell population. Extended phenotyping of B57-NS1$_{26\text{-}34}$ 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-NS1$_{26\text{-}34}$ TET^{+} NK cells and the rest of the CD56^{dim} NK cells. B57-NS1$_{26\text{-}34}$ TET^{+} NK cells are phenotypically similar to total NK cells, but do not appear to occupy a subset identified by these markers.

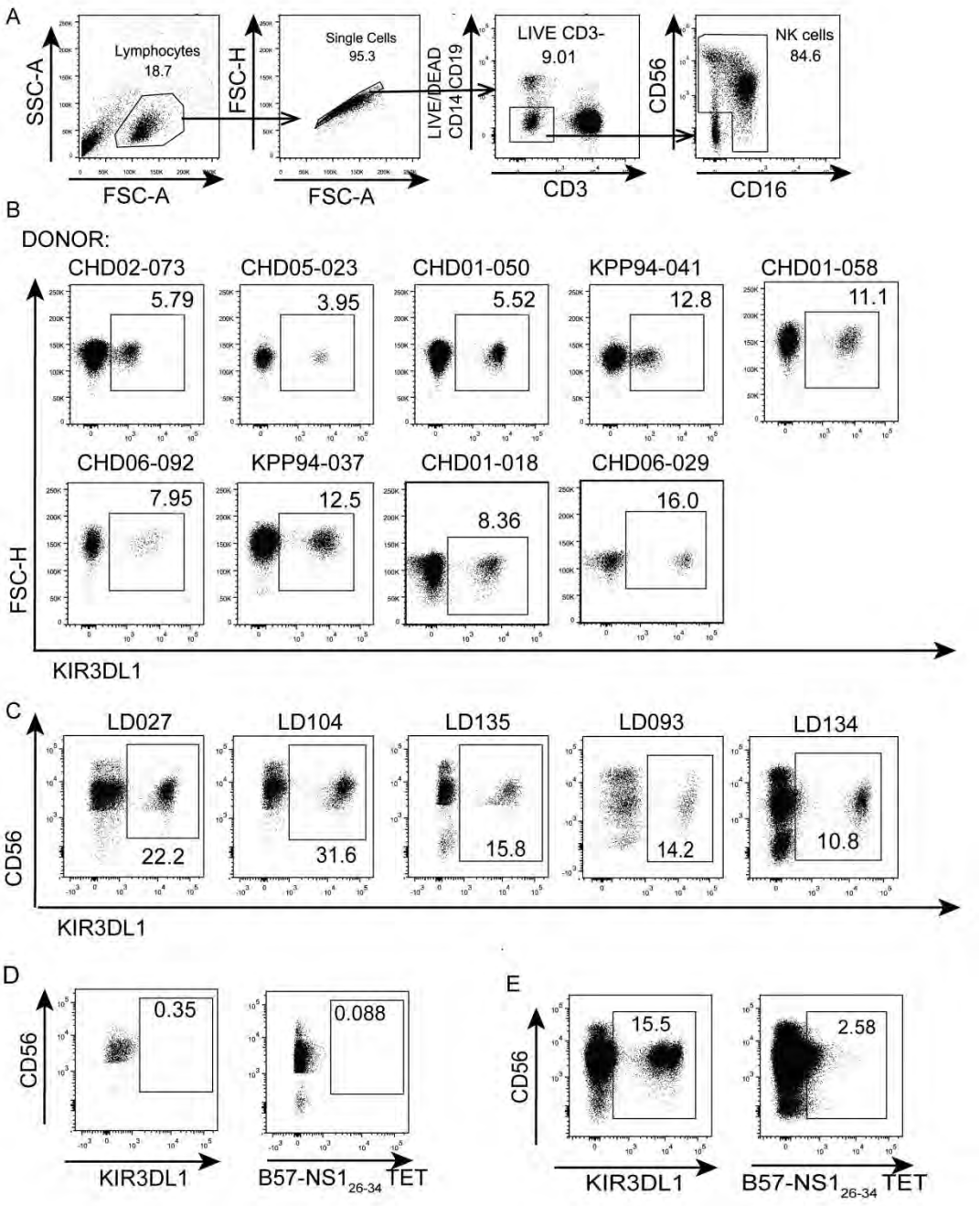


Figure 4.6. **KIR3DL1 staining on PBMC from healthy donors and Thai study cohort subjects**. (A) Gating strategy for the identification of CD56+ and/or CD16+ NK cells. (B) Frequency of KIR3DL1^{+} cells in the NK cell population of Thai Donors. (C) Frequency of KIR3DL1^{+} cells in the NK cell population of healthy donors. (D) Staining with anti-KIR3DL1 and B57-NS1$_{26-34}$ TET of PBMC from a KIR3DL1^{-} donor. (E) Binding of anti-KIR3DL1 or B57-NS1$_{26-34}$ TET to NK cells from a KIR3DL1^{+} donor

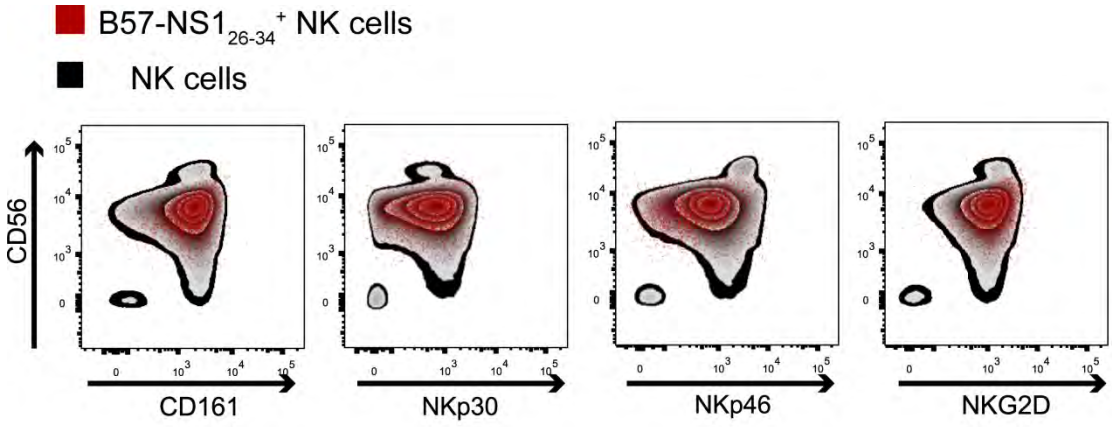


Figure 4.7. **Expression of NK cell markers on B57-NS1$_{26-34}$ TET^{+} cells.** The expression of CD161, NKp30, NKp46, NKG2D in the total NK cell population (zebra plot), with B57-NS1$_{26-34}$ TET^{+} NK cells overlaid (red dots). The expression pattern of CD161, NKp30, NKp46, NKG2D is consistent between the B57-NS1$_{26-34}$ TET^{+} 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^{dim} NK cells, but some CD56^{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$_{26\text{-}34}$. A CTL assay using a CD8^{+} T cell line, 3C11, specific for NS1$_{26\text{-}34}$ confirmed that, following peptide pulsing, NS1$_{26\text{-}34}$ 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$_{26\text{-}34}$, 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$_{26\text{-}34}$ when compared with their response to 221

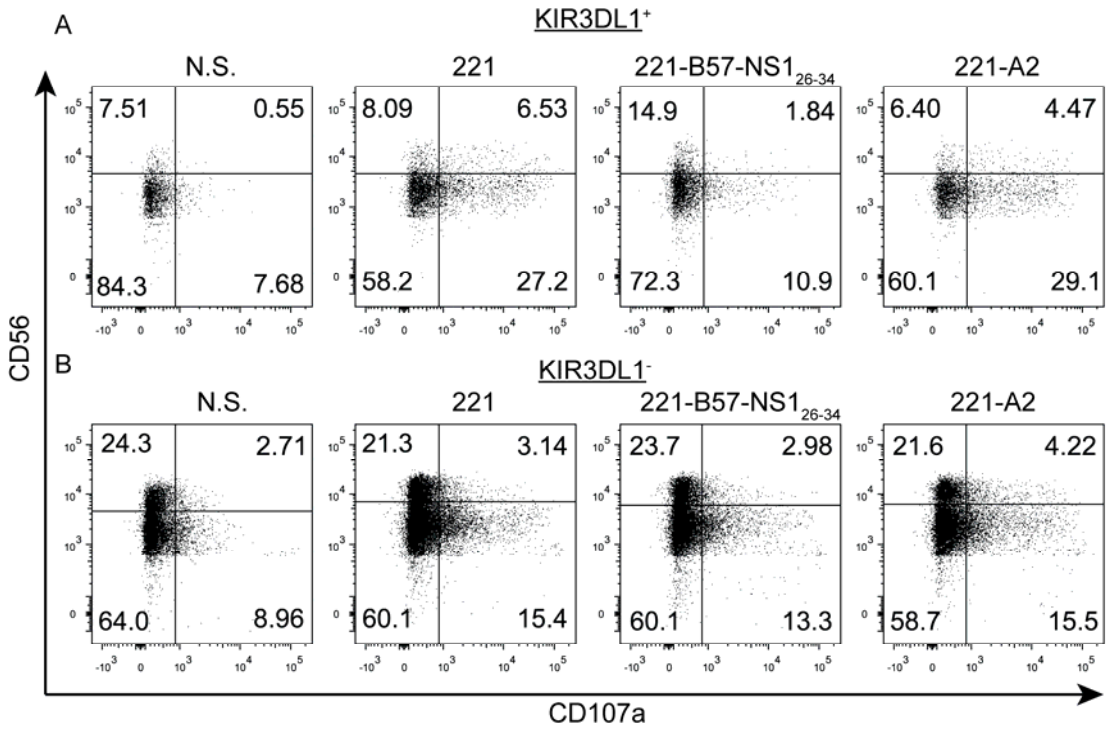


Figure 4.8. **Inhibition of KIR3DL1$^+$ NK cells by HLA-B57-NS1$_{26-34}$.** PBMC were incubated for six hours with anti-CD107a antibody either alone (N.S.) or in the presence of NK cell target lines 721.221 (221), 221-B57 pulsed with NS1$_{26-34}$ (221-B57-NS1$_{26-34}$), or 221-A2. Degranulation of (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$_{26-34}$. 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.

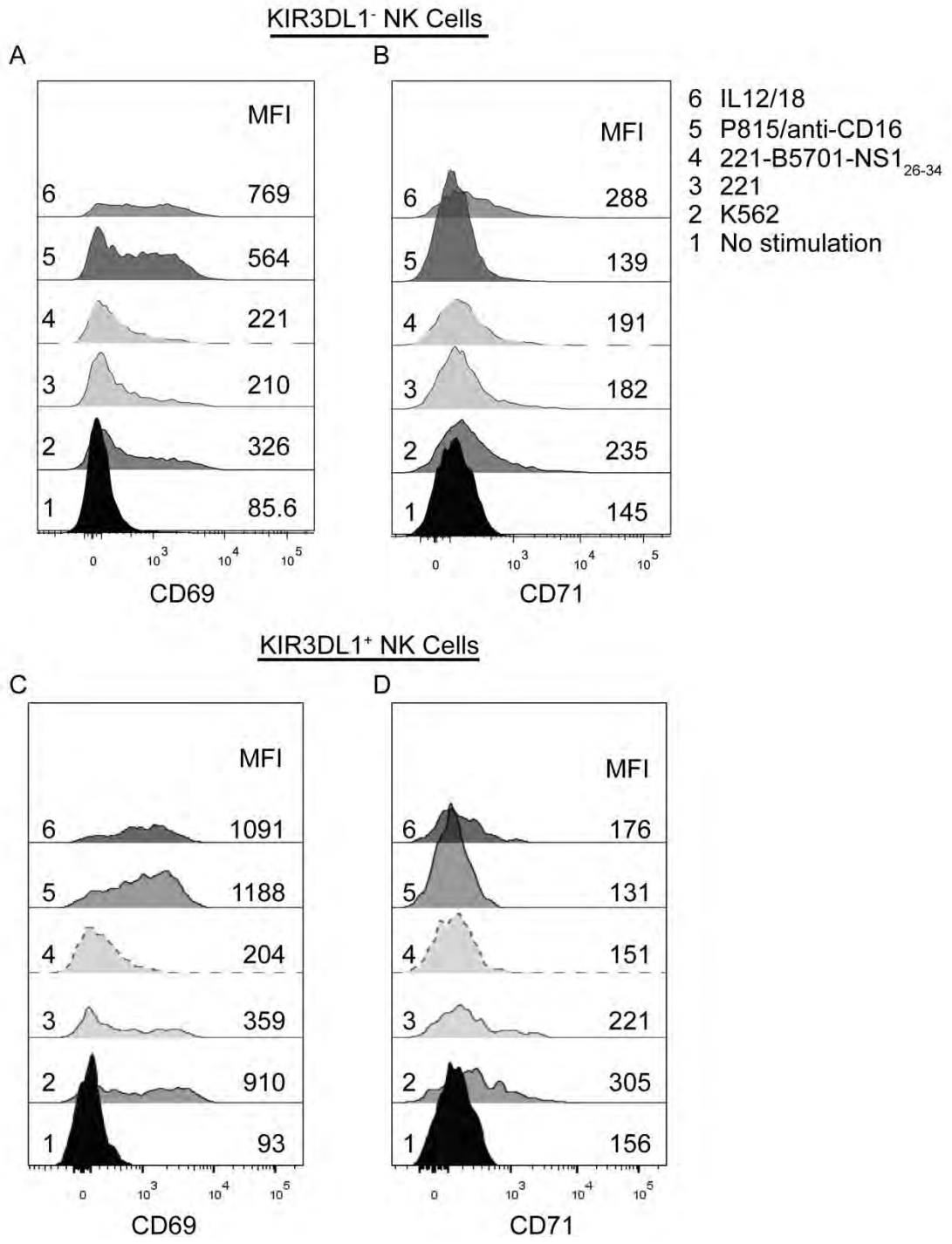


Figure 4.9. **Activation of NK cells through multiple pathways.** Expression of CD69 and CD71 on the KIR3DL1^{-} (A and B) and KIR3DL1^{+} (C and D) NK cells respectively. PBMC were = either (1) unstimulated or incubated with target cell lines K562 or 221 (2, 3), target cell line 221 transfected with 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 infection[327].

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 point[328]. 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.

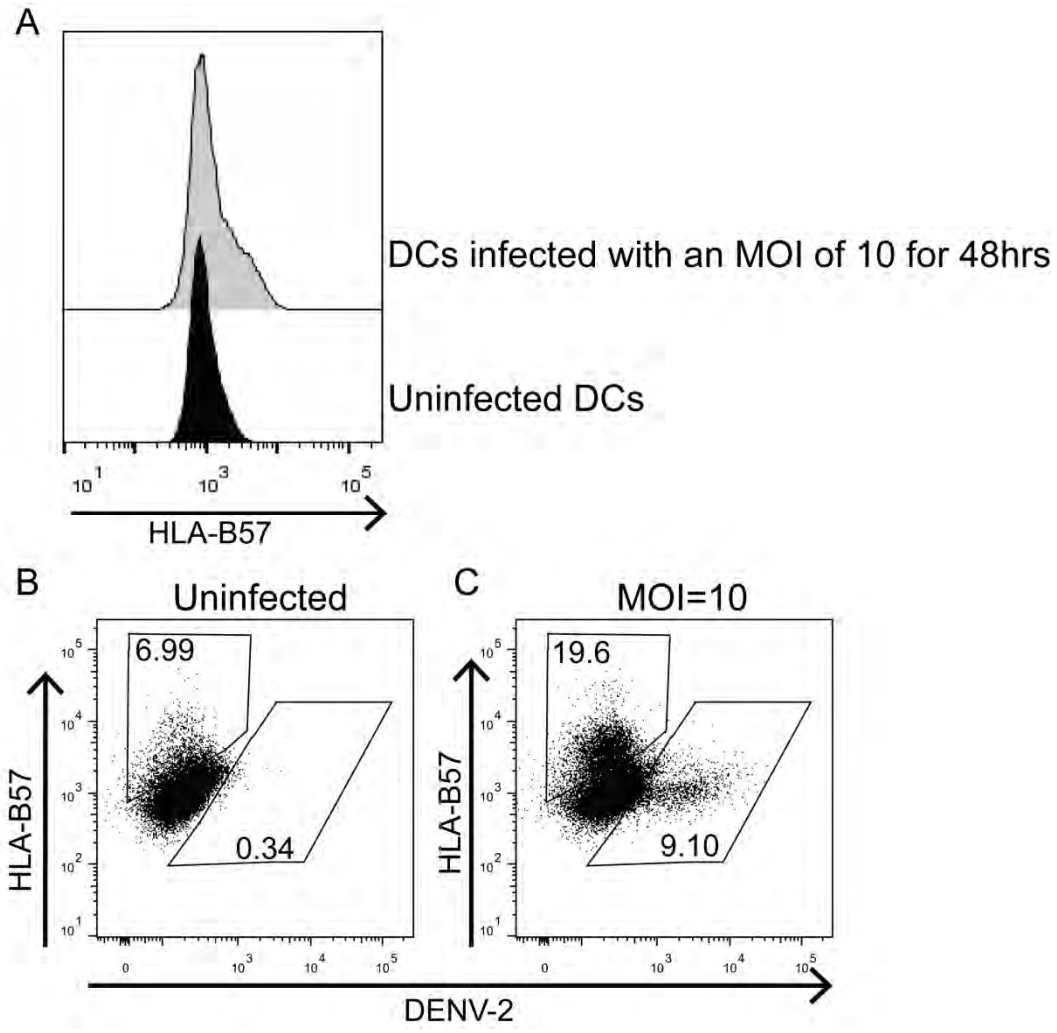
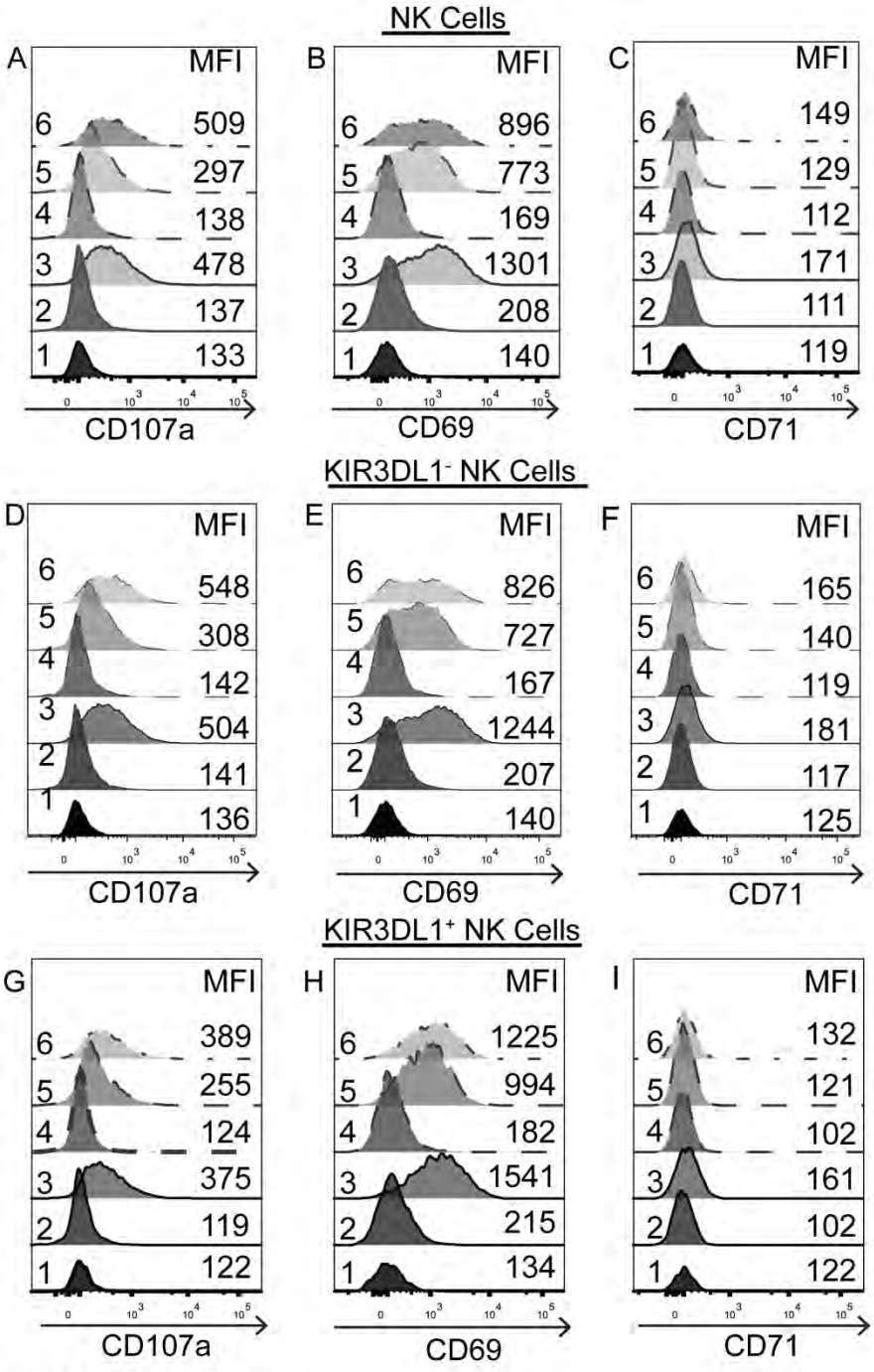


Figure 4.10. **Upregulation of MHC-I on DCs following DENV infection for 48 hours.** (A) Histogram showing expression of HLA-B57 on all DCs in DENV infected culture versus an uninfected culture. (B,C) HLA-B57 expression versus DENV-2 infection showing DCs in (B) uninfected culture or (C) infected culture.



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 *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$_{26\text{-}34}$ 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$_{26\text{-}34}$ 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$_{26\text{-}34}$ 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$_{26\text{-}34}$ 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 $CD38^{hi}$ and $CD38^{low}$ 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 $CD38^{hi}$ and $CD38^{low}$ expression. $CD38^{low}$ expression followed the same pattern as CD69 expression on NK cells. In contrast, $CD38^{hi}$ expression peaked at fever day +1 and returned to baseline at 1 year post-infection. The peak of $CD38^{hi}$ 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 $CD38^{hi}$ NK cells. Higher frequencies of $CD38^{hi}$-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 $CD38^{hi}$ 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

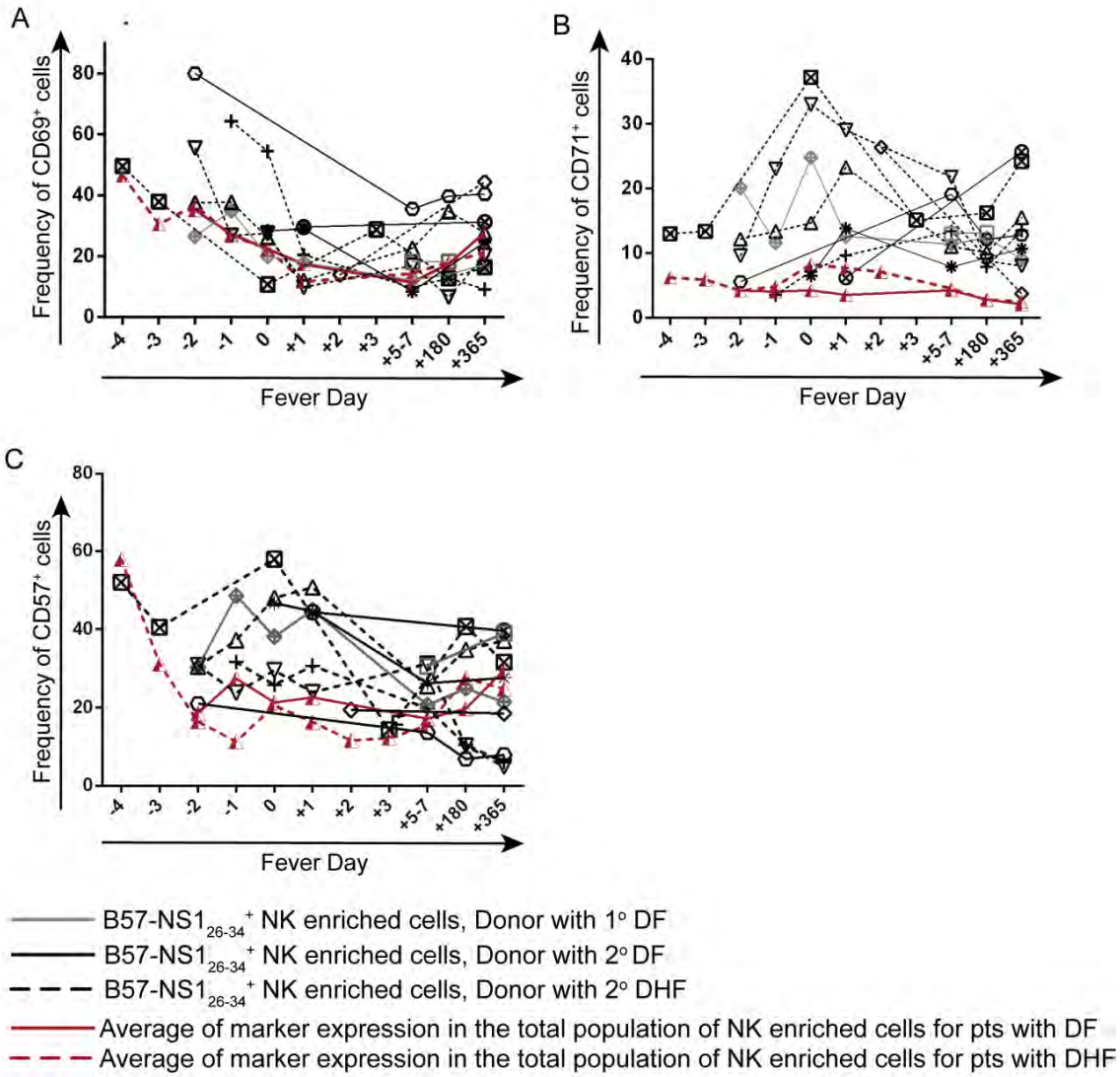


Figure 4.12. **Expression of surface activation markers over the course of acute DENV illness.** (A) The expression of CD69 (B) the expression of CD71 (C) the expression of CD57. The B57-NS1$_{26\text{-}34}$ TET^{+} NK-enriched cells from each donor at each time point are in grey for donors undergoing 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.

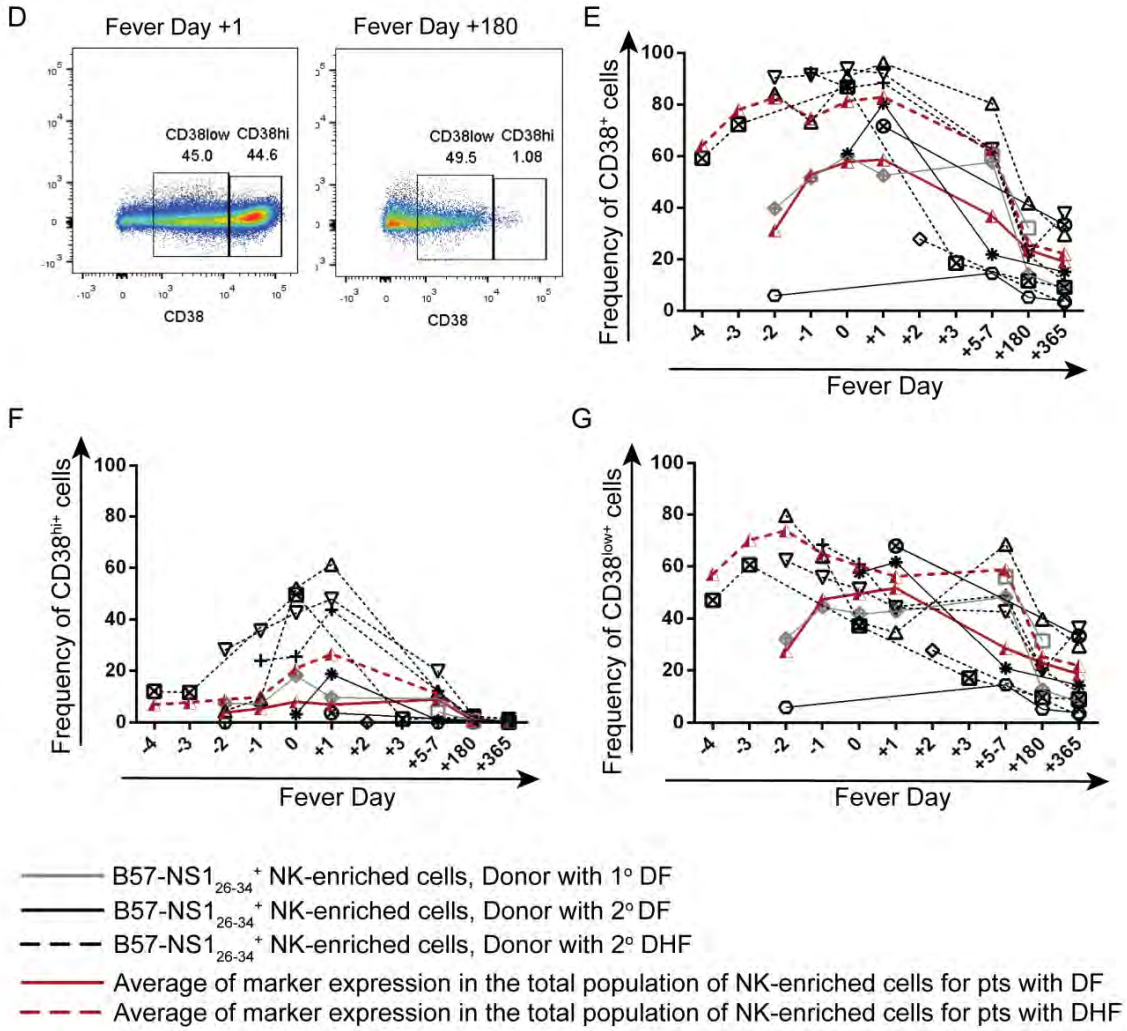


Figure 4.12 continued. **Expression of surface activation markers over the course of acute DENV illness.** (D) Representative CD38 staining showing the gating of $CD38^{hi}$ versus $CD38^{low}$ on the NK cell population. (E) The expression of CD38 (F) $CD38^{hi}$ and (G) $CD38^{low}$. The B57-$NS1_{26-34}$ 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[274, 286, 329]. 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[277]. 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[275, 276, 330]. 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-$NS1_{26\text{-}34}$ TET and KIR3DL1, an inhibitory receptor on NK cells. We found that B57-$NS1_{26\text{-}34}$ 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[244, 245, 331-335] and the development of autoimmune diseases[241, 243, 336, 337]. The interaction between HLA-B57 and KIR3DL1 has been extensively studied in the context of HIV[248, 250, 252, 253, 322, 338]. 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^{+}[321, 338].

We saw a striking activation phenotype of B57-NS1$_{26\text{-}34}$ 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-NS1$_{26\text{-}34}$ TET^{+} NK-enriched cells may reflect a role for the interaction between KIR3DL1 and B57-NS1$_{26\text{-}34}$ in modulating NK cell responses. Peak frequencies of CD38^{hi} 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, CD38^{hi} 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[339, 340]. Patients with DENV typically present to the clinic more than a week following the mosquito bite that initiated the infection[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 *ex vivo*. We therefore designed a series of *in vitro* experiments to complement our *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 *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-$NS1_{26\text{-}34}$ TET^{+} 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-$NS1_{26-34}$ 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[342]. IL-18 levels have also been shown to be increased in patients with DHF[127]. IL-12 levels, on the other hand, have been reported to be lower in patients with dengue compared to healthy controls[342], with very little IL-12 production in patients with DHF[127]. The findings of a $CD38^{hi}$ population, while very clear as a distinct population in *ex vivo* staining, was not observed after any *in vitro* stimulation. The expression of $CD38^{hi}$ 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 $NS1_{26-34}$ peptide did not disrupt this inhibition. In contrast, $KIR3DL1^{-}$ NK cells remained unaffected by the presence of B57-$NS1_{26-34}$. 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 $NS1_{26-34}$ 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 $NS1_{26-34}$ peptide on the function of $KIR3DL1^+$ NK cells, our data demonstrate that the presentation of $NS1_{26-34}$ 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[59]. 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-$NS1_{26-34}$ TET^+ NK cells were identified in $HLA\text{-}B57^+$ donors during acute DENV infection and at convalescent time points. We showed that HLA-B57 complexed with the DENV $NS1_{26-34}$ peptide (B57-$NS1_{26-34}$) 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 frame-work 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.

CHAPTER V

FINAL SUMMARY AND IMPLICATIONS

A. Thesis Summary

We identified a HLA-B57 DENV epitope ($NS1_{26-34}$) conserved across all four serotypes of DENV. We hypothesized that $CD8^+$ T cell responses to the $NS1_{26-34}$ 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 $NS1_{26-34}$ 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 ($NS1_{26-34}$) and when possible compare $NS1_{26-34}$-specific responses to other DENV epitope-specific $CD8^+$ T cell responses. We expected to find increased frequencies of B57-$NS1_{26-34}$ TET^+CD8^+ T cells compared to frequencies of A11-$NS3_{133-142}$ TET^+CD8^+ T cells or A2-$E_{213-221}$ TET^+CD8^+ T cells in all subjects undergoing secondary dengue. We also expected that activation of B57-$NS1_{26-34}$-specific T cells during secondary DENV-infection would be more consistent and occur more rapidly than activation of A11-$NS3_{133-142}$ or A2-$E_{213-221}$-specific T cells. However, we observed high frequencies of B57-$NS1_{26-34}$ 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-$NS1_{26-34}$-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-$NS1_{26\text{-}34}$ TET^+ and the A11-$NS3_{133\text{-}142}$ TET^+ /A2-$E_{213\text{-}221}$ 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-NS1$_{26-34}$ TET to CD8^{-} cells in all HLA-B57^{+} subjects from our Thai cohort and all healthy donors who were HLA-B57^{+}KIR3DL1^{+} 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^{hi} NK-enriched cells more frequent in subjects with DHF (p=0.057). Peak expression of CD38^{hi} 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^{hi} 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^{+} NK cells following stimulation with an NK-sensitive target cell 221, and inhibition of KIR3DL1^{+} NK cells in the presence of 221 target cells transfected with HLA-B57 and pulsed with the NS1$_{26-34}$ peptide. Our *in vitro* data suggest that the interaction between B57-NS1$_{26-34}$

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 $NS1_{26\text{-}34}$ 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 $NS1_{26\text{-}34}$ 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[274-277, 283, 286, 343]. Dampened 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-NS1$_{26\text{-}34}$ 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[92, 93] and a genome-wide association study identified MICB with susceptibility for DSS[94] (Table 1.2). These epidemiological relationships suggest that NK cells can contribute to dengue pathology.

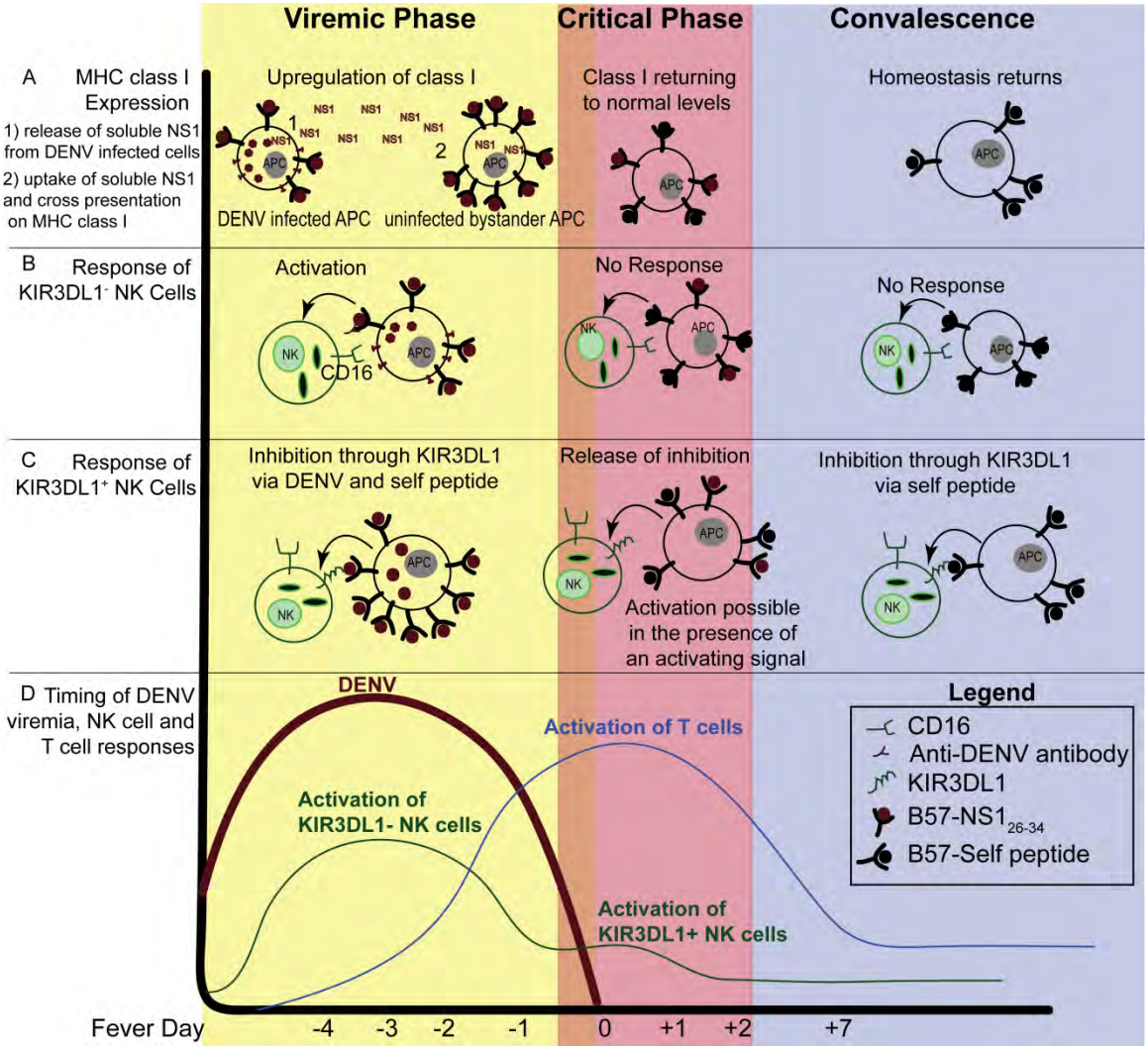


Figure 5.1. **Proposed model of the interaction between HLA-B57 and KIR3DL1 during DENV infection.** (A) DENV infection results in the upregulation of HLA-B57 expression on uninfected bystander APCs during the viremic phase (yellow). During the critical phase (red), when viremia has resolved, HLA-B57 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 $NS1_{26\text{-}34}$ 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[53, 54]. DENV is even beginning to re-emerge in the United States[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[58, 69-76]. NK cells have been shown to be important for the development of the adaptive responses to viral infections[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^{+} KIR3DL1^{+} subjects during DENV infection. This delay in NK cell activation could hamper the development of protective memory CD8^{+} 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[345]. Low levels of IL-12 have been reported in patients with DHF compared to healthy control or patients with DF[342]. Thus NK cell responses may explain why secondary $CD8^+$ T cell responses to this highly conserved epitope were no greater than primary responses in our Thai study cohort[346]. Additionally, NK cells are important for T cell development via the production of IFN-γ, IL-15, and IL-18[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[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 $KIR3DL1^+$ 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 $KIR3DL1^+$ NK cells, making these NK cells easier to activate near the resolution of DENV viremia. Not all $B57^+KIR3DL1^+$ 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[92, 93] and MICB which was associated with DSS in a genome-wide association study[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[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 [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[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[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[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[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.

CHAPTER VI

APPENDIX

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[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[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.

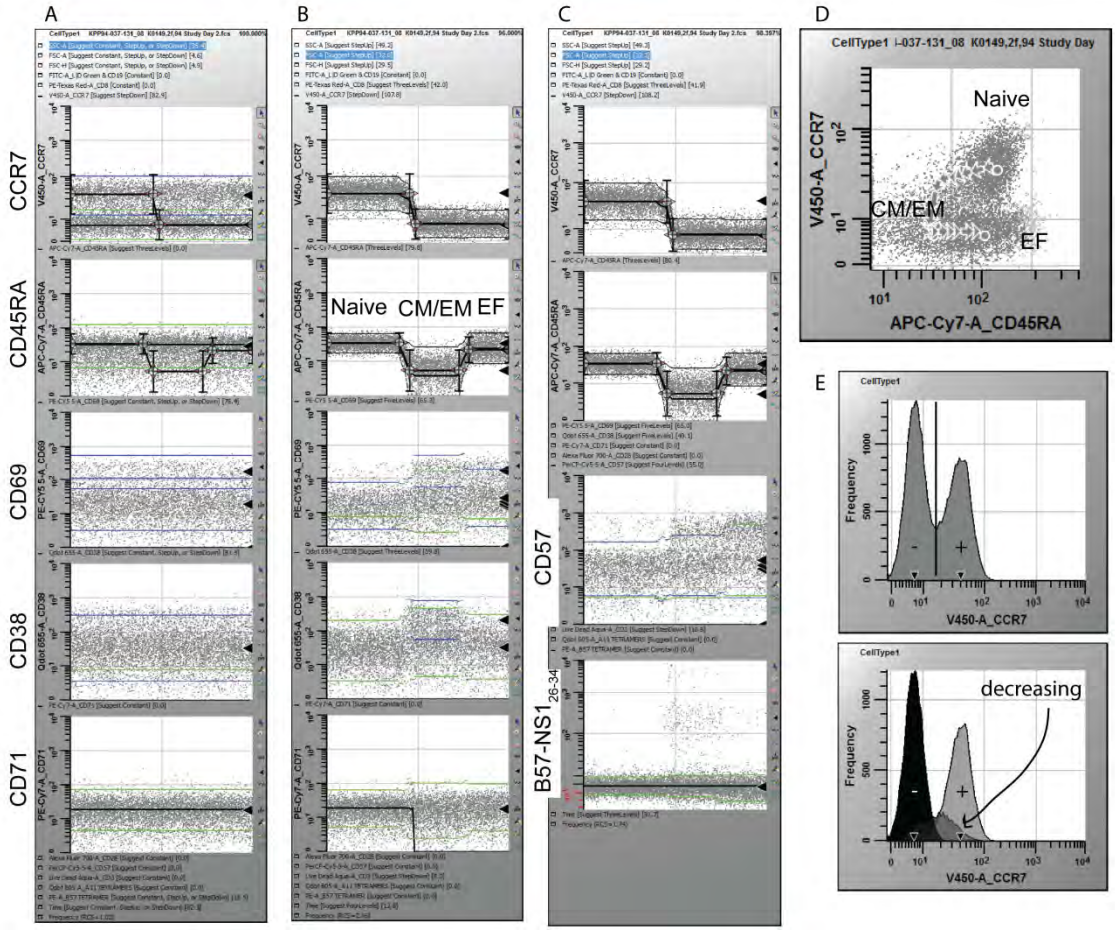


Figure 6.1. **Gemstone Analysis.** (A) Expression of CCR7, CD45RA, CD69, CD38, CD71 on $CD8^+$ T cells before any modeling. (B) Expression of CD69, CD38, and CD71 on $CD8^+$ T cells after modeling of CCR7 and CD45RA to identify naïve ($CCR7^+$, $CD45RA^+$ left) and memory ($CCR7^-$ right) populations 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-$NS1_{26-34}$ 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

REFERENCES

1. Lindenbach BD, Rice CM. Molecular biology of flaviviruses. Adv Virus Res 2003; 59:23-61.
2. Mukhopadhyay S, Kuhn RJ, Rossmann MG. A structural perspective of the flavivirus life cycle. Nat Rev Microbiol 2005; 3:13-22.
3. Diaz FJ, Black WCt, Farfan-Ale JA, Lorono-Pino MA, Olson KE, Beaty BJ. Dengue virus circulation and evolution in Mexico: a phylogenetic perspective. Arch Med Res 2006; 37:760-73.
4. Cahour A, Falgout B, Lai CJ. Cleavage of the dengue virus polyprotein at the NS3/NS4A and NS4B/NS5 junctions is mediated by viral protease NS2B-NS3, whereas NS4A/NS4B may be processed by a cellular protease. J Virol 1992; 66:1535-42.
5. Falgout B, Pethel M, Zhang YM, Lai CJ. Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. J Virol 1991; 65:2467-75.
6. Elshuber S, Allison SL, Heinz FX, Mandl CW. Cleavage of protein prM is necessary for infection of BHK-21 cells by tick-borne encephalitis virus. J Gen Virol 2003; 84:183-91.
7. Yu IM, Zhang W, Holdaway HA, *et al.* Structure of the immature dengue virus at low pH primes proteolytic maturation. Science 2008; 319:1834-7.
8. Li L, Lok SM, Yu IM, Zhang Y, Kuhn RJ, Chen J, Rossmann MG. The flavivirus precursor membrane-envelope protein complex: structure and maturation. Science 2008; 319:1830-4.
9. Yu IM, Holdaway HA, Chipman PR, Kuhn RJ, Rossmann MG, Chen J. Association of the pr peptides with dengue virus at acidic pH blocks membrane fusion. J Virol 2009; 83:12101-7.
10. Chu JJ, Ng ML. Infectious entry of West Nile virus occurs through a clathrin-mediated endocytic pathway. J Virol 2004; 78:10543-55.
11. Suksanpaisan L, Susantad T, Smith DR. Characterization of dengue virus entry into HepG2 cells. J Biomed Sci 2009; 16:17.
12. Se-Thoe SY, Ling AE, Ng MM. Alteration of virus entry mode: a neutralisation mechanism for Dengue-2 virus. J Med Virol 2000; 62:364-76.

13. Hase T, Summers PL, Cohen WH. A comparative study of entry modes into C6/36 cells by Semliki Forest and Japanese encephalitis viruses. Arch Virol 1989; 108:101-14.

14. Hase T, Summers PL, Eckels KH. Flavivirus entry into cultured mosquito cells and human peripheral blood monocytes. Arch Virol 1989; 104:129-43.

15. Heinz FX, Stiasny K, Allison SL. The entry machinery of flaviviruses. Arch Virol Suppl 2004:133-7.

16. Krishnan MN, Sukumaran B, Pal U, Agaisse H, Murray JL, Hodge TW, Fikrig E. Rab 5 is required for the cellular entry of dengue and West Nile viruses. J Virol 2007; 81:4881-5.

17. Tassaneetrithep B, Burgess TH, Granelli-Piperno A, *et al.* DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. J Exp Med 2003; 197:823-9.

18. Lozach PY, Burleigh L, Staropoli I, *et al.* Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN)-mediated enhancement of dengue virus infection is independent of DC-SIGN internalization signals. J Biol Chem 2005; 280:23698-708.

19. Germi R, Crance JM, Garin D, Guimet J, Lortat-Jacob H, Ruigrok RW, Zarski JP, Drouet E. Heparan sulfate-mediated binding of infectious dengue virus type 2 and yellow fever virus. Virology 2002; 292:162-8.

20. Alen MM, Dallmeier K, Balzarini J, Neyts J, Schols D. Crucial role of the N-glycans on the viral E-envelope glycoprotein in DC-SIGN-mediated dengue virus infection. Antiviral Res 2012; 96:280-7.

21. Mondotte JA, Lozach PY, Amara A, Gamarnik AV. Essential role of dengue virus envelope protein N glycosylation at asparagine-67 during viral propagation. J Virol 2007; 81:7136-48.

22. Dejnirattisai W, Webb AI, Chan V, Jumnainsong A, Davidson A, Mongkolsapaya J, Screaton G. Lectin switching during dengue virus infection. J Infect Dis 2011; 203:1775-83.

23. Durbin AP, Vargas MJ, Wanionek K, Hammond SN, Gordon A, Rocha C, Balmaseda A, Harris E. Phenotyping of peripheral blood mononuclear cells during acute dengue illness demonstrates infection and increased activation of monocytes in severe cases compared to classic dengue fever. Virology 2008; 376:429-35.

24. Jessie K, Fong MY, Devi S, Lam SK, Wong KT. Localization of dengue virus in naturally infected human tissues, by immunohistochemistry and in situ hybridization. J Infect Dis 2004; 189:1411-8.

25. de Araujo JM, Schatzmayr HG, de Filippis AM, Dos Santos FB, Cardoso MA, Britto C, Coelho JM, Nogueira RM. A retrospective survey of dengue virus

infection in fatal cases from an epidemic in Brazil. J Virol Methods 2009; 155:34-8.

26. Sun P, Kochel TJ. The battle between infection and host immune responses of dengue virus and its implication in dengue disease pathogenesis. ScientificWorldJournal 2013; 2013:843469.

27. Mackenzie JM, Khromykh AA, Jones MK, Westaway EG. Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A. Virology 1998; 245:203-15.

28. Luo D, Xu T, Hunke C, Gruber G, Vasudevan SG, Lescar J. Crystal structure of the NS3 protease-helicase from dengue virus. J Virol 2008; 82:173-83.

29. Luo D, Xu T, Watson RP, *et al.* Insights into RNA unwinding and ATP hydrolysis by the flavivirus NS3 protein. EMBO J 2008; 27:3209-19.

30. Yon C, Teramoto T, Mueller N, Phelan J, Ganesh VK, Murthy KH, Padmanabhan R. Modulation of the nucleoside triphosphatase/RNA helicase and 5'-RNA triphosphatase activities of Dengue virus type 2 nonstructural protein 3 (NS3) by interaction with NS5, the RNA-dependent RNA polymerase. J Biol Chem 2005; 280:27412-9.

31. Mazzon M, Jones M, Davidson A, Chain B, Jacobs M. Dengue virus NS5 inhibits interferon-alpha signaling by blocking signal transducer and activator of transcription 2 phosphorylation. J Infect Dis 2009; 200:1261-70.

32. Ashour J, Laurent-Rolle M, Shi PY, Garcia-Sastre A. NS5 of dengue virus mediates STAT2 binding and degradation. J Virol 2009; 83:5408-18.

33. Jones M, Davidson A, Hibbert L, Gruenwald P, Schlaak J, Ball S, Foster GR, Jacobs M. Dengue virus inhibits alpha interferon signaling by reducing STAT2 expression. J Virol 2005; 79:5414-20.

34. Munoz-Jordan JL, Sanchez-Burgos GG, Laurent-Rolle M, Garcia-Sastre A. Inhibition of interferon signaling by dengue virus. Proc Natl Acad Sci U S A 2003; 100:14333-8.

35. Mackenzie JM, Jones MK, Young PR. Immunolocalization of the dengue virus nonstructural glycoprotein NS1 suggests a role in viral RNA replication. Virology 1996; 220:232-40.

36. Flamand M, Megret F, Mathieu M, Lepault J, Rey FA, Deubel V. Dengue virus type 1 nonstructural glycoprotein NS1 is secreted from mammalian cells as a soluble hexamer in a glycosylation-dependent fashion. J Virol 1999; 73:6104-10.

37. Phuong HL, Thai KT, Nga TT, *et al.* Detection of dengue nonstructural 1 (NS1) protein in Vietnamese patients with fever. Diagn Microbiol Infect Dis 2009; 63:372-8.

38. Young PR, Hilditch PA, Bletchly C, Halloran W. An antigen capture enzyme-linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients. J Clin Microbiol 2000; 38:1053-7.

39. Alcon S, Talarmin A, Debruyne M, Falconar A, Deubel V, Flamand M. Enzyme-linked immunosorbent assay specific to Dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. J Clin Microbiol 2002; 40:376-81.

40. Gutsche I, Coulibaly F, Voss JE, *et al.* Secreted dengue virus nonstructural protein NS1 is an atypical barrel-shaped high-density lipoprotein. Proc Natl Acad Sci U S A 2011; 108:8003-8.

41. de la Cruz-Hernandez SI, Flores-Aguilar H, Gonzalez-Mateos S, Lopez-Martinez I, Alpuche-Aranda C, Ludert JE, del Angel RM. Determination of viremia and concentration of circulating nonstructural protein 1 in patients infected with dengue virus in Mexico. Am J Trop Med Hyg 2013; 88:446-54.

42. Erra EO, Korhonen EM, Voutilainen L, Huhtamo E, Vapalahti O, Kantele A. Dengue in travelers: kinetics of viremia and NS1 antigenemia and their associations with clinical parameters. PLoS One 2013; 8:e65900.

43. Libraty DH, Young PR, Pickering D, *et al.* High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. J Infect Dis 2002; 186:1165-8.

44. Avirutnan P, Punyadee N, Noisakran S, *et al.* Vascular leakage in severe dengue virus infections: a potential role for the nonstructural viral protein NS1 and complement. J Infect Dis 2006; 193:1078-88.

45. Falconar AK. The dengue virus nonstructural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin/adhesin proteins and binds to human endothelial cells: potential implications in haemorrhagic fever pathogenesis. Arch Virol 1997; 142:897-916.

46. Chuang YC, Wang SY, Lin YS, Chen HR, Yeh TM. Re-evaluation of the pathogenic roles of nonstructural protein 1 and its antibodies during dengue virus infection. J Biomed Sci 2013; 20:42.

47. Chuang YC, Lin YS, Liu HS, Wang JR, Yeh TM. Antibodies against thrombin in dengue patients contain both anti-thrombotic and pro-fibrinolytic activities. Thromb Haemost 2013; 110:358-65.

48. Chen CL, Lin CF, Wan SW, *et al.* Anti-dengue virus nonstructural protein 1 antibodies cause NO-mediated endothelial cell apoptosis via ceramide-regulated glycogen synthase kinase-3beta and NF-kappaB activation. J Immunol 2013; 191:1744-52.

49. In: Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control: New Edition. Geneva; 2009.

50. Normile D. Tropical medicine. Surprising new dengue virus throws a spanner in disease control efforts. Science 2013; 342:415.

51. Brathwaite Dick O, San Martin JL, Montoya RH, del Diego J, Zambrano B, Dayan GH. The history of dengue outbreaks in the Americas. Am J Trop Med Hyg 2012; 87:584-93.

52. Rush A. An account of the bilious remitting fever, as it appeared in Philadelphia in the summer and autumn of the year 1780. Medical Inquiries and Observations 1789:104-17.

53. WHO. Dengue and severe dengue fact sheet. 2013.

54. Bhatt S, Gething PW, Brady OJ, *et al.* The global distribution and burden of dengue. Nature 2013; 496:504-7.

55. Rico-Hesse R. Dengue virus virulence and transmission determinants. Curr Top Microbiol Immunol 2010; 338:45-55.

56. Srikiatkhachorn A, Rothman AL, Gibbons RV, Sittisombut N, Malasit P, Ennis FA, Nimmannitya S, Kalayanarooj S. Dengue--how best to classify it. Clin Infect Dis 2011; 53:563-7.

57. World Health Organization. Dengue heaemorrhagi fever: diganosis, treatment, prevention, and control. 1997:1-58p.

58. Vaughn DW, Green S, Kalayanarooj S, *et al.* Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J Infect Dis 2000; 181:2-9.

59. Vaughn DW, Green S, Kalayanarooj S, *et al.* Dengue in the early febrile phase: viremia and antibody responses. J Infect Dis 1997; 176:322-30.

60. Srikiatkhachorn A, Krautrachue A, Ratanaprakarn W, *et al.* Natural history of plasma leakage in dengue hemorrhagic fever: a serial ultrasonographic study. Pediatr Infect Dis J 2007; 26:283-90; discussion 91-2.

61. Durbin AP, Whitehead SS. Dengue vaccine candidates in development. Curr Top Microbiol Immunol 2010; 338:129-43.

62. Martins RM, Maia Mde L, Farias RH, *et al.* 17DD yellow fever vaccine: a double blind, randomized clinical trial of immunogenicity and safety on a dose-response study. Hum Vaccin Immunother 2013; 9:879-88.

63. Appaiahgari MB, Vrati S. IMOJEV((R)): a Yellow fever virus-based novel Japanese encephalitis vaccine. Expert Rev Vaccines 2010; 9:1371-84.

64. Sabchareon A, Wallace D, Sirivichayakul C, *et al.* Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai

schoolchildren: a randomised, controlled phase 2b trial. Lancet 2012; 380:1559-67.

65. McArthur MA, Sztein MB, Edelman R. Dengue vaccines: recent developments, ongoing challenges and current candidates. Expert Rev Vaccines 2013; 12:933-53.

66. Thomas SJ, Endy TP. Critical issues in dengue vaccine development. Curr Opin Infect Dis 2011; 24:442-50.

67. Murphy BR, Whitehead SS. Immune response to dengue virus and prospects for a vaccine. Annu Rev Immunol 2011; 29:587-619.

68. Lindow JC, Borochoff-Porte N, Durbin AP, Whitehead SS, Fimlaid KA, Bunn JY, Kirkpatrick BD. Primary vaccination with low dose live dengue 1 virus generates a proinflammatory, multifunctional T cell response in humans. PLoS Negl Trop Dis 2012; 6:e1742.

69. Sangkawibha N, Rojanasuphot S, Ahandrik S, Viriyapongse S, Jatanasen S, Salitul V, Phanthumachinda B, Halstead SB. Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. Am J Epidemiol 1984; 120:653-69.

70. Tee HP, How SH, Jamalludin AR, Safhan MN, Sapian MM, Kuan YC, Sapari S. Risk factors associated with development of dengue haemorrhagic fever or dengue shock syndrome in adults in Hospital Tengku Ampuan Afzan Kuantan. Med J Malaysia 2009; 64:316-20.

71. Nguyen TH, Nguyen TL, Lei HY, *et al.* Association between sex, nutritional status, severity of dengue hemorrhagic fever, and immune status in infants with dengue hemorrhagic fever. Am J Trop Med Hyg 2005; 72:370-4.

72. Guzman MG, Kouri G, Valdes L, Bravo J, Alvarez M, Vazques S, Delgado I, Halstead SB. Epidemiologic studies on Dengue in Santiago de Cuba, 1997. Am J Epidemiol 2000; 152:793-9; discussion 804.

73. Halstead SB, Nimmannitya S, Cohen SN. Observations related to pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. Yale J Biol Med 1970; 42:311-28.

74. Fried JR, Gibbons RV, Kalayanarooj S, *et al.* Serotype-specific differences in the risk of dengue hemorrhagic fever: an analysis of data collected in Bangkok, Thailand from 1994 to 2006. PLoS Negl Trop Dis 2010; 4:e617.

75. Burke DS, Nisalak A, Johnson DE, Scott RM. A prospective study of dengue infections in Bangkok. Am J Trop Med Hyg 1988; 38:172-80.

76. Halstead SB, Yamarat C. Recent Epidemics of Hemorrhagic Fever in Thailand. Observations Related to Pathogenesis of a "New" Dengue Disease. Am J Public Health Nations Health 1965; 55:1386-95.

77. Nisalak A, Endy TP, Nimmannitya S, *et al.* Serotype-specific dengue virus circulation and dengue disease in Bangkok, Thailand from 1973 to 1999. Am J Trop Med Hyg 2003; 68:191-202.

78. Suwandono A, Kosasih H, Nurhayati, *et al.* Four dengue virus serotypes found circulating during an outbreak of dengue fever and dengue haemorrhagic fever in Jakarta, Indonesia, during 2004. Trans R Soc Trop Med Hyg 2006; 100:855-62.

79. Anantapreecha S, Chanama S, A An, Naemkhunthot S, Sa-Ngasang A, Sawanpanyalert P, Kurane I. Serological and virological features of dengue fever and dengue haemorrhagic fever in Thailand from 1999 to 2002. Epidemiol Infect 2005; 133:503-7.

80. Gibbons RV, Kalanarooj S, Jarman RG, Nisalak A, Vaughn DW, Endy TP, Mammen MP, Jr., Srikiatkhachorn A. Analysis of repeat hospital admissions for dengue to estimate the frequency of third or fourth dengue infections resulting in admissions and dengue hemorrhagic fever, and serotype sequences. Am J Trop Med Hyg 2007; 77:910-3.

81. Watts DM, Porter KR, Putvatana P, Vasquez B, Calampa C, Hayes CG, Halstead SB. Failure of secondary infection with American genotype dengue 2 to cause dengue haemorrhagic fever. Lancet 1999; 354:1431-4.

82. Gubler DJ, Clark GG. Dengue/dengue hemorrhagic fever: the emergence of a global health problem. Emerg Infect Dis 1995; 1:55-7.

83. Rothman AL. T lymphocyte responses to heterologous secondary dengue virus infections. Ann N Y Acad Sci 2009; 1171 Suppl 1:E36-41.

84. Patkar C, Giaya K, Libraty DH. Dengue virus type 2 modulates endothelial barrier function through CD73. Am J Trop Med Hyg 2013; 88:89-94.

85. Liu P, Woda M, Ennis FA, Libraty DH. Dengue virus infection differentially regulates endothelial barrier function over time through type I interferon effects. J Infect Dis 2009; 200:191-201.

86. Mehta D, Malik AB. Signaling mechanisms regulating endothelial permeability. Physiol Rev 2006; 86:279-367.

87. Srikiatkhachorn A, Green S. Markers of dengue disease severity. Curr Top Microbiol Immunol 2010; 338:67-82.

88. Stephens HA. HLA and other gene associations with dengue disease severity. Curr Top Microbiol Immunol 2010; 338:99-114.

89. Stephens HA, Klaythong R, Sirikong M, *et al.* HLA-A and -B allele associations with secondary dengue virus infections correlate with disease severity and the infecting viral serotype in ethnic Thais. Tissue Antigens 2002; 60:309-18.

90. Vejbaesya S, Luangtrakool P, Luangtrakool K, *et al.* TNF and LTA gene, allele, and extended HLA haplotype associations with severe dengue virus infection in ethnic Thais. J Infect Dis 2009; 199:1442-8.

91. Nguyen TP, Kikuchi M, Vu TQ, *et al.* Protective and enhancing HLA alleles, HLA-DRB1*0901 and HLA-A*24, for severe forms of dengue virus infection, dengue hemorrhagic fever and dengue shock syndrome. PLoS Negl Trop Dis 2008; 2:e304.

92. Garcia G, del Puerto F, Perez AB, *et al.* Association of MICA and MICB alleles with symptomatic dengue infection. Hum Immunol 2011; 72:904-7.

93. Whitehorn J, Chau TN, Nguyet NM, *et al.* Genetic variants of MICB and PLCE1 and associations with non-severe dengue. PLoS One 2013; 8:e59067.

94. Khor CC, Chau TN, Pang J, *et al.* Genome-wide association study identifies susceptibility loci for dengue shock syndrome at MICB and PLCE1. Nat Genet 2011; 43:1139-41.

95. Zwirner NW, Fuertes MB, Girart MV, Domaica CI, Rossi LE. Cytokine-driven regulation of NK cell functions in tumor immunity: role of the MICA-NKG2D system. Cytokine Growth Factor Rev 2007; 18:159-70.

96. Halstead SB. Pathogenesis of dengue: challenges to molecular biology. Science 1988; 239:476-81.

97. Guzman MG, Alvarez M, Halstead SB. Secondary infection as a risk factor for dengue hemorrhagic fever/dengue shock syndrome: an historical perspective and role of antibody-dependent enhancement of infection. Arch Virol 2013; 158:1445-59.

98. Rothman AL. Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. Nat Rev Immunol 2011; 11:532-43.

99. Burton DR, Poignard P, Stanfield RL, Wilson IA. Broadly neutralizing antibodies present new prospects to counter highly antigenically diverse viruses. Science 2012; 337:183-6.

100. Dowd KA, Pierson TC. Antibody-mediated neutralization of flaviviruses: a reductionist view. Virology 2011; 411:306-15.

101. Flipse J, Wilschut J, Smit JM. Molecular mechanisms involved in antibody-dependent enhancement of dengue virus infection in humans. Traffic 2013; 14:25-35.

102. Garcia G, Arango M, Perez AB, *et al.* Antibodies from patients with dengue viral infection mediate cellular cytotoxicity. J Clin Virol 2006; 37:53-7.

103. Laoprasopwattana K, Libraty DH, Endy TP, Nisalak A, Chunsuttiwat S, Ennis FA, Rothman AL, Green S. Antibody-dependent cellular cytotoxicity mediated by

plasma obtained before secondary dengue virus infections: potential involvement in early control of viral replication. J Infect Dis 2007; 195:1108-16.

104. Dejnirattisai W, Jumnainsong A, Onsirisakul N, *et al.* Cross-reacting antibodies enhance dengue virus infection in humans. Science 2010; 328:745-8.

105. Halstead SB, O'Rourke EJ. Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. J Exp Med 1977; 146:201-17.

106. Halstead SB, O'Rourke EJ. Antibody-enhanced dengue virus infection in primate leukocytes. Nature 1977; 265:739-41.

107. Kurane I, Rothman AL, Livingston PG, *et al.* Immunopathologic mechanisms of dengue hemorrhagic fever and dengue shock syndrome. Arch Virol Suppl 1994; 9:59-64.

108. Libraty DH, Acosta LP, Tallo V, *et al.* A prospective nested case-control study of Dengue in infants: rethinking and refining the antibody-dependent enhancement dengue hemorrhagic fever model. PLoS Med 2009; 6:e1000171.

109. Halstead SB, Mahalingam S, Marovich MA, Ubol S, Mosser DM. Intrinsic antibody-dependent enhancement of microbial infection in macrophages: disease regulation by immune complexes. Lancet Infect Dis 2010; 10:712-22.

110. Ubol S, Phuklia W, Kalayanarooj S, Modhiran N. Mechanisms of immune evasion induced by a complex of dengue virus and preexisting enhancing antibodies. J Infect Dis 2010; 201:923-35.

111. Ubol S, Halstead SB. How innate immune mechanisms contribute to antibody-enhanced viral infections. Clin Vaccine Immunol 2010; 17:1829-35.

112. Balsitis SJ, Williams KL, Lachica R, *et al.* Lethal antibody enhancement of dengue disease in mice is prevented by Fc modification. PLoS Pathog 2010; 6:e1000790.

113. Huang KJ, Li SY, Chen SC, Liu HS, Lin YS, Yeh TM, Liu CC, Lei HY. Manifestation of thrombocytopenia in dengue-2-virus-infected mice. J Gen Virol 2000; 81:2177-82.

114. Mongkolsapaya J, Dejnirattisai W, Xu XN, *et al.* Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. Nat Med 2003; 9:921-7.

115. Green S, Pichyangkul S, Vaughn DW, *et al.* Early CD69 expression on peripheral blood lymphocytes from children with dengue hemorrhagic fever. J Infect Dis 1999; 180:1429-35.

116. Chau TN, Quyen NT, Thuy TT, *et al.* Dengue in Vietnamese infants--results of infection-enhancement assays correlate with age-related disease epidemiology,

and cellular immune responses correlate with disease severity. J Infect Dis 2008; 198:516-24.

117. Dung NT, Duyen HT, Thuy NT, *et al.* Timing of CD8+ T cell responses in relation to commencement of capillary leakage in children with dengue. J Immunol 2010; 184:7281-7.

118. Imrie A, Meeks J, Gurary A, Sukhbataar M, Kitsutani P, Effler P, Zhao Z. Differential functional avidity of dengue virus-specific T-cell clones for variant peptides representing heterologous and previously encountered serotypes. J Virol 2007; 81:10081-91.

119. Mangada MM, Rothman AL. Altered cytokine responses of dengue-specific CD4+ T cells to heterologous serotypes. J Immunol 2005; 175:2676-83.

120. Friberg H, Burns L, Woda M, *et al.* Memory CD8+ T cells from naturally acquired primary dengue virus infection are highly cross-reactive. Immunol Cell Biol 2011; 89:122-9.

121. Gagnon SJ, Mori M, Kurane I, *et al.* Cytokine gene expression and protein production in peripheral blood mononuclear cells of children with acute dengue virus infections. Journal of Medical Virology 2002; 67:41-6.

122. Green S, Vaughn DW, Kalayanarooj S, *et al.* Early immune activation in acute dengue is related to development of plasma leakage and disease severity. Journal of Infectious Diseases 1999; 179:755-62.

123. Green S, Vaughn DW, Kalayanarooj S, Nimmannitya S, Suntayakorn S, Nisalak A, Rothman AL, Ennis FA. Elevated plasma interleukin-10 levels in acute dengue correlate with disease severity. Journal of Medical Virology 1999; 59:329-34.

124. Hober D, Delannoy AS, Benyoucef S, De Groote D, Wattre P. High levels of sTNFR p75 and TNF alpha in dengue-infected patients. Microbiol Immunol 1996; 40:569-73.

125. Hober D, Poli L, Roblin B, *et al.* Serum levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) in dengue-infected patients. American Journal of Tropical Medicine and Hygiene 1993; 48:324-31.

126. Mathew A, Rothman AL. Understanding the contribution of cellular immunity to dengue disease pathogenesis. Immunol Rev 2008; 225:300-13.

127. Mustafa AS, Elbishbishi EA, Agarwal R, Chaturvedi UC. Elevated levels of interleukin-13 and IL-18 in patients with dengue hemorrhagic fever. FEMS Immunol Med Microbiol 2001; 30:229-33.

128. Srikiatkhachorn A, Ajariyakhajorn C, Endy TP, Kalayanarooj S, Libraty DH, Green S, Ennis FA, Rothman AL. Virus-induced decline in soluble vascular endothelial growth receptor 2 is associated with plasma leakage in dengue hemorrhagic Fever. J Virol 2007; 81:1592-600.

129. Zivna I, Green S, Vaughn DW, *et al.* T cell responses to an HLA B*07-restricted epitope on the dengue NS3 protein correlate with disease severity. Journal of Immunology 2002; 168:5959-65.

130. Simmons CP, Dong T, Chau NV, *et al.* Early T-cell responses to dengue virus epitopes in Vietnamese adults with secondary dengue virus infections. J Virol 2005; 79:5665-75.

131. Mongkolsapaya J, Duangchinda T, Dejnirattisai W, *et al.* T cell responses in dengue hemorrhagic fever: are cross-reactive T cells suboptimal? J Immunol 2006; 176:3821-9.

132. Duangchinda T, Dejnirattisai W, Vasanawathana S, Limpitikul W, Tangthawornchaikul N, Malasit P, Mongkolsapaya J, Screaton G. Immunodominant T-cell responses to dengue virus NS3 are associated with DHF. Proc Natl Acad Sci U S A 2010; 107:16922-7.

133. Hatch S, Endy TP, Thomas S, *et al.* Intracellular cytokine production by dengue virus-specific T cells correlates with subclinical secondary infection. J Infect Dis 2010; 203:1282-91.

134. Friberg H, Bashyam H, Toyosaki-Maeda T, *et al.* Cross-reactivity and expansion of dengue-specific T cells during acute primary and secondary infections in humans. Sci Rep 2011; 1:51.

135. Malavige GN, McGowan S, Atukorale V, Salimi M, Peelawatta M, Fernando N, Jayaratne SD, Ogg G. Identification of serotype-specific T cell responses to highly conserved regions of the dengue viruses. Clin Exp Immunol 2012; 168:215-23.

136. Soundravally R, Hoti SL. Immunopathogenesis of dengue hemorrhagic fever and shock syndrome: role of TAP and HPA gene polymorphism. Hum Immunol 2007; 68:973-9.

137. Brown MG, Salas RA, Vickers IE, Heslop OD, Smikle MF. Dengue HLA associations in Jamaicans. West Indian Med J 2011; 60:126-31.

138. Monteiro SP, Brasil PE, Cabello GM, Souza RV, Brasil P, Georg I, Cabello PH, De Castro L. HLA-A*01 allele: a risk factor for dengue haemorrhagic fever in Brazil's population. Mem Inst Oswaldo Cruz 2012; 107:224-30.

139. Beltrame LM, Sell AM, Moliterno RA, Clementino SL, Cardozo DM, Dalalio MM, Fonzar UJ, Visentainer JE. Influence of KIR genes and their HLA ligands in susceptibility to dengue in a population from southern Brazil. Tissue Antigens 2013; 82:397-404.

140. Xavier Eurico de Alencar L, de Mendonca Braga-Neto U, Jose Moura do Nascimento E, *et al.* HLA-B *44 Is Associated with Dengue Severity Caused by DENV-3 in a Brazilian Population. J Trop Med 2013; 2013:648475.

141. Hertz T, Nolan D, James I, *et al.* Mapping the landscape of host-pathogen coevolution: HLA class I binding and its relationship with evolutionary conservation in human and viral proteins. J Virol 2011; 85:1310-21.

142. Appanna R, Huat TL, See LL, Tan PL, Vadivelu J, Devi S. Cross-reactive T-cell responses to the nonstructural regions of dengue viruses among dengue fever and dengue hemorrhagic fever patients in Malaysia. Clin Vaccine Immunol 2007; 14:969-77.

143. Weiskopf D, Angelo MA, de Azeredo EL, *et al.* Comprehensive analysis of dengue virus-specific responses supports an HLA-linked protective role for CD8+ T cells. Proc Natl Acad Sci U S A 2013; 110:E2046-53.

144. Bashyam HS, Green S, Rothman AL. Dengue virus-reactive CD8+ T cells display quantitative and qualitative differences in their response to variant epitopes of heterologous viral serotypes. J Immunol 2006; 176:2817-24.

145. Zivny J, DeFronzo M, Jarry W, Jameson J, Cruz J, Ennis FA, Rothman AL. Partial agonist effect influences the CTL response to a heterologous dengue virus serotype. Journal of Immunology 1999; 163:2754-60.

146. Mathew A, Kurane I, Green S, *et al.* Predominance of HLA-restricted cytotoxic T-lymphocyte responses to serotype-cross-reactive epitopes on nonstructural proteins following natural secondary dengue virus infection. J Virol 1998; 72:3999-4004.

147. Livingston PG, Kurane I, Dai LC, *et al.* Dengue virus-specific, HLA-B35-restricted, human CD8+ cytotoxic T lymphocyte (CTL) clones. Recognition of NS3 amino acids 500 to 508 by CTL clones of two different serotype specificities. Journal of Immunology 1995; 154:1287-95.

148. Wen J, Duan Z, Jiang L. Identification of a dengue virus-specific HLA-A*0201-restricted CD8+ T cell epitope. J Med Virol 2010; 82:642-8.

149. Mangada MM, Endy TP, Nisalak A, *et al.* Dengue-specific T cell responses in peripheral blood mononuclear cells obtained prior to secondary dengue virus infections in Thai schoolchildren. JID 2002; 185:1697-703.

150. Gunther VJ, Putnak R, Eckels KH, Mammen MP, Scherer JM, Lyons A, Sztein MB, Sun W. A human challenge model for dengue infection reveals a possible protective role for sustained interferon gamma levels during the acute phase of illness. Vaccine 2011; 29:3895-904.

151. Yauch LE, Zellweger RM, Kotturi MF, *et al.* A protective role for dengue virus-specific CD8+ T cells. J Immunol 2009; 182:4865-73.

152. Yauch LE, Prestwood TR, May MM, Morar MM, Zellweger RM, Peters B, Sette A, Shresta S. CD4+ T cells are not required for the induction of dengue virus-specific CD8+ T cell or antibody responses but contribute to protection after vaccination. J Immunol 2010; 185:5405-16.

153. Gil L, Lopez C, Blanco A, *et al.* The cellular immune response plays an important role in protecting against dengue virus in the mouse encephalitis model. Viral Immunol 2009; 22:23-30.

154. Gil L, Lopez C, Lazo L, *et al.* Recombinant nucleocapsid-like particles from dengue-2 virus induce protective CD4+ and CD8+ cells against viral encephalitis in mice. Int Immunol 2009; 21:1175-83.

155. Seder RA, Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. Nat Rev Immunol 2008; 8:247-58.

156. Ho LP, Yit PS, Ng LH, *et al.* The road to memory: an early rest for the long journey. J Immunol 2013; 191:5603-14.

157. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol 2000; 182:311-22.

158. Speiser DE, Migliaccio M, Pittet MJ, *et al.* Human CD8(+) T cells expressing HLA-DR and CD28 show telomerase activity and are distinct from cytolytic effector T cells. Eur J Immunol 2001; 31:459-66.

159. Cesano A, Visonneau S, Deaglio S, Malavasi F, Santoli D. Role of CD38 and its ligand in the regulation of MHC-nonrestricted cytotoxic T cells. J Immunol 1998; 160:1106-15.

160. Rasmussen AM, Blomhoff HK, Stokke T, Horejsi V, Smeland EB. Cross-linking of CD53 promotes activation of resting human B lymphocytes. J Immunol 1994; 153:4997-5007.

161. Testi R, Phillips JH, Lanier LL. T cell activation via Leu-23 (CD69). J Immunol 1989; 143:1123-8.

162. Santis AG, Campanero MR, Alonso JL, Tugores A, Alonso MA, Yague E, Pivel JP, Sanchez-Madrid F. Tumor necrosis factor-alpha production induced in T lymphocytes through the AIM/CD69 activation pathway. Eur J Immunol 1992; 22:1253-9.

163. Nakamura S, Sung SS, Bjorndahl JM, Fu SM. Human T cell activation. IV. T cell activation and proliferation via the early activation antigen EA 1. J Exp Med 1989; 169:677-89.

164. Lauzurica P, Sancho D, Torres M, *et al.* Phenotypic and functional characteristics of hematopoietic cell lineages in CD69-deficient mice. Blood 2000; 95:2312-20.

165. Deaglio S, Mallone R, Baj G, *et al.* Human CD38 and its ligand CD31 define a unique lamina propria T lymphocyte signaling pathway. FASEB J 2001; 15:580-2.

166. Morra M, Zubiaur M, Terhorst C, Sancho J, Malavasi F. CD38 is functionally dependent on the TCR/CD3 complex in human T cells. FASEB J 1998; 12:581-92.

167. Mehta K, Shahid U, Malavasi F. Human CD38, a cell-surface protein with multiple functions. FASEB J 1996; 10:1408-17.

168. Focosi D, Bestagno M, Burrone O, Petrini M. CD57+ T lymphocytes and functional immune deficiency. J Leukoc Biol 2010; 87:107-16.

169. Piazza P, McMurtrey CP, Lelic A, *et al.* Surface phenotype and functionality of WNV specific T cells differ with age and disease severity. PLoS One 2010; 5:e15343.

170. Krutzik PO, Clutter MR, Nolan GP. Coordinate analysis of murine immune cell surface markers and intracellular phosphoproteins by flow cytometry. J Immunol 2005; 175:2357-65.

171. Neckers LM, Cossman J. Transferrin receptor induction in mitogen-stimulated human T lymphocytes is required for DNA synthesis and cell division and is regulated by interleukin 2. Proc Natl Acad Sci U S A 1983; 80:3494-8.

172. Batista A, Millan J, Mittelbrunn M, Sanchez-Madrid F, Alonso MA. Recruitment of transferrin receptor to immunological synapse in response to TCR engagement. J Immunol 2004; 172:6709-14.

173. Starska K, Glowacka E, Kulig A, Lewy-Trenda I, Brys M, Lewkowicz P. Prognostic value of the immunological phenomena and relationship with clinicopathological characteristics of the tumor--the expression of the early CD69+, CD71+ and the late CD25+, CD26+, HLA/DR+ activation markers on T CD4+ and CD8+ lymphocytes in squamous cell laryngeal carcinoma. Part II. Folia Histochem Cytobiol 2011; 49:593-603.

174. Starska K, Glowacka E, Kulig A, Lewy-Trenda I, Brys M, Lewkowicz P. The role of tumor cells in the modification of T lymphocytes activity--the expression of the early CD69+, CD71+ and the late CD25+, CD26+, HLA/DR+ activation markers on T CD4+ and CD8+ cells in squamous cell laryngeal carcinoma. Part I. Folia Histochem Cytobiol 2011; 49:579-92.

175. Weitz M, Kiessling C, Friedrich M, *et al.* Persistent CMV infection correlates with disease activity and dominates the phenotype of peripheral CD8+ T cells in psoriasis. Exp Dermatol 2011; 20:561-7.

176. Meintjes G, Wilkinson KA, Rangaka MX, *et al.* Type 1 helper T cells and FoxP3-positive T cells in HIV-tuberculosis-associated immune reconstitution inflammatory syndrome. Am J Respir Crit Care Med 2008; 178:1083-9.

177. Vesela R, Dolezalova L, Pytlik R, Rychtrmocova H, Mareckova H, Trneny M. The evaluation of survival and proliferation of lymphocytes in autologous mixed leukocyte reaction with dendritic cells. The comparison of incorporation of (3)H-thymidine and differential gating method. Cell Immunol 2011; 271:78-84.

178. Cassetti MC, Durbin A, Harris E, *et al.* Report of an NIAID workshop on dengue animal models. Vaccine 2010; 28:4229-34.

179. Yauch LE, Shresta S. Mouse models of dengue virus infection and disease. Antiviral Res 2008; 80:87-93.

180. Zompi S, Harris E. Animal models of dengue virus infection. Viruses 2012; 4:62-82.

181. Johnson AJ, Roehrig JT. New mouse model for dengue virus vaccine testing. J Virol 1999; 73:783-6.

182. Mota J, Rico-Hesse R. Dengue virus tropism in humanized mice recapitulates human dengue fever. PLoS One 2011; 6:e20762.

183. Jaiswal S, Pearson T, Friberg H, Shultz LD, Greiner DL, Rothman AL, Mathew A. Dengue virus infection and virus-specific HLA-A2 restricted immune responses in humanized NOD-scid IL2rgammanull mice. PLoS One 2009; 4:e7251.

184. Jaiswal S, Pazoles P, Woda M, Shultz LD, Greiner DL, Brehm MA, Mathew A. Enhanced humoral and HLA-A2-restricted dengue virus-specific T-cell responses in humanized BLT NSG mice. Immunology 2012; 136:334-43.

185. Goncalvez AP, Engle RE, St Claire M, Purcell RH, Lai CJ. Monoclonal antibody-mediated enhancement of dengue virus infection in vitro and in vivo and strategies for prevention. Proc Natl Acad Sci U S A 2007; 104:9422-7.

186. Halstead SB. In vivo enhancement of dengue virus infection in rhesus monkeys by passively transferred antibody. J Infect Dis 1979; 140:527-33.

187. Marchette NJ, Halstead SB, Falkler WA, Jr., Stenhouse A, Nash D. Studies on the pathogenesis of dengue infection in monkeys. 3. Sequential distribution of virus in primary and heterologous infections. J Infect Dis 1973; 128:23-30.

188. Scherer WF, Russell PK, Rosen L, Casals J, Dickerman RW. Experimental infection of chimpanzees with dengue viruses. Am J Trop Med Hyg 1978; 27:590-9.

189. Halstead SB, Shotwell H, Casals J. Studies on the pathogenesis of dengue infection in monkeys. II. Clinical laboratory responses to heterologous infection. J Infect Dis 1973; 128:15-22.

190. Halstead SB, Shotwell H, Casals J. Studies on the pathogenesis of dengue infection in monkeys. I. Clinical laboratory responses to primary infection. J Infect Dis 1973; 128:7-14.

191. Koraka P, Benton S, van Amerongen G, Stittelaar KJ, Osterhaus AD. Characterization of humoral and cellular immune responses in cynomolgus macaques upon primary and subsequent heterologous infections with dengue viruses. Microbes Infect 2007; 9:940-6.

192. Mladinich KM, Piaskowski SM, Rudersdorf R, *et al.* Dengue virus-specific CD4+ and CD8+ T lymphocytes target NS1, NS3 and NS5 in infected Indian rhesus macaques. Immunogenetics 2012; 64:111-21.

193. Raviprakash K, Kochel TJ, Ewing D, Simmons M, Phillips I, Hayes CG, Porter KR. Immunogenicity of dengue virus type 1 DNA vaccines expressing truncated and full length envelope protein. Vaccine 2000; 18:2426-34.

194. Guy B, Barban V, Mantel N, *et al.* Evaluation of interferences between dengue vaccine serotypes in a monkey model. Am J Trop Med Hyg 2009; 80:302-11.

195. Koraka P, Benton S, van Amerongen G, Stittelaar KJ, Osterhaus AD. Efficacy of a live attenuated tetravalent candidate dengue vaccine in naive and previously infected cynomolgus macaques. Vaccine 2007; 25:5409-16.

196. Osorio JE, Brewoo JN, Silengo SJ, *et al.* Efficacy of a tetravalent chimeric dengue vaccine (DENVax) in Cynomolgus macaques. Am J Trop Med Hyg 2011; 84:978-87.

197. Xagorari A, Chlichlia K. Toll-like receptors and viruses: induction of innate antiviral immune responses. Open Microbiol J 2008; 2:49-59.

198. Hober D, Shen L, Benyoucef S, De Groote D, Deubel V, Wattre P. Enhanced TNF alpha production by monocytic-like cells exposed to dengue virus antigens. Immunol Lett 1996; 53:115-20.

199. Nightingale ZD, Patkar C, Rothman AL. Viral replication and paracrine effects result in distinct, functional responses of dendritic cells following infection with dengue 2 virus. J Leukoc Biol 2008; 84:1028-38.

200. Sun P, Fernandez S, Marovich MA, *et al.* Functional characterization of ex vivo blood myeloid and plasmacytoid dendritic cells after infection with dengue virus. Virology 2009; 383:207-15.

201. Sun P, Bauza K, Pal S, Liang Z, Wu SJ, Beckett C, Burgess T, Porter K. Infection and activation of human peripheral blood monocytes by dengue viruses through the mechanism of antibody-dependent enhancement. Virology 2011; 421:245-52.

202. Sun P, Celluzzi CM, Marovich M, *et al.* CD40 ligand enhances dengue viral infection of dendritic cells: a possible mechanism for T cell-mediated immunopathology. J Immunol 2006; 177:6497-503.

203. Kurane I, Hebblewaite D, Ennis FA. Characterization with monoclonal antibodies of human lymphocytes active in natural killing and antibody-dependent cell-mediated cytotoxicity of dengue virus-infected cells. Immunology 1986; 58:429-36.

204. Romee R, Foley B, Lenvik T, *et al.* NK cell CD16 surface expression and function is regulated by a disintegrin and metalloprotease-17 (ADAM17). Blood 2013; 121:3599-608.

205. Yoshida T, Omatsu T, Saito A, *et al.* CD16(+) natural killer cells play a limited role against primary dengue virus infection in tamarins. Arch Virol 2012; 157:363-8.

206. Azeredo EL, De Oliveira-Pinto LM, Zagne SM, Cerqueira DI, Nogueira RM, Kubelka CF. NK cells, displaying early activation, cytotoxicity and adhesion molecules, are associated with mild dengue disease. Clin Exp Immunol 2006; 143:345-56.

207. Sung JM, Lee CK, Wu-Hsieh BA. Intrahepatic infiltrating NK and CD8 T cells cause liver cell death in different phases of dengue virus infection. PLoS One 2012; 7:e46292.

208. Hershkovitz O, Zilka A, Bar-Ilan A, *et al.* Dengue virus replicon expressing the nonstructural proteins suffices to enhance membrane expression of HLA class I and inhibit lysis by human NK cells. J Virol 2008; 82:7666-76.

209. Momburg F, Mullbacher A, Lobigs M. Modulation of transporter associated with antigen processing (TAP)-mediated peptide import into the endoplasmic reticulum by flavivirus infection. J Virol 2001; 75:5663-71.

210. Yossef R, Rosental B, Appel MY, Hershkovitz O, Porgador A. Upregulation of MHC class I expression following dengue virus infection: the mechanism at the promoter level. Expert Rev Anti Infect Ther 2012; 10:285-7.

211. Petersen JL, Morris CR, Solheim JC. Virus evasion of MHC class I molecule presentation. J Immunol 2003; 171:4473-8.

212. Libraty DH, Pichyangkul S, Ajariyakhajorn C, Endy TP, Ennis FA. Human dendritic cells are activated by dengue virus infection: enhancement by gamma interferon and implications for disease pathogenesis. J Virol 2001; 75:3501-8.

213. Warke RV, Becerra A, Zawadzka A, *et al.* Efficient dengue virus (DENV) infection of human muscle satellite cells upregulates type I interferon response genes and differentially modulates MHC I expression on bystander and DENV-infected cells. J Gen Virol 2008; 89:1605-15.

214. Mullbacher A, Lobigs M. Up-regulation of MHC class I by flavivirus-induced peptide translocation into the endoplasmic reticulum. Immunity 1995; 3:207-14.

215. King NJ, Kesson AM. Interaction of flaviviruses with cells of the vertebrate host and decoy of the immune response. Immunol Cell Biol 2003; 81:207-16.

216. Hershkovitz O, Rosental B, Rosenberg LA, *et al.* NKp44 receptor mediates interaction of the envelope glycoproteins from the West Nile and dengue viruses with NK cells. J Immunol 2009; 183:2610-21.

217. Abdul-Careem MF, Lee AJ, Pek EA, Gill N, Gillgrass AE, Chew MV, Reid S, Ashkar AA. Genital HSV-2 infection induces short-term NK cell memory. PLoS One 2012; 7:e32821.

218. Paust S, Gill HS, Wang BZ, *et al.* Critical role for the chemokine receptor CXCR6 in NK cell-mediated antigen-specific memory of haptens and viruses. Nat Immunol 2010; 11:1127-35.

219. Gillard GO, Bivas-Benita M, Hovav AH, Grandpre LE, Panas MW, Seaman MS, Haynes BF, Letvin NL. Thy1+ NK [corrected] cells from vaccinia virus-primed mice confer protection against vaccinia virus challenge in the absence of adaptive lymphocytes. PLoS Pathog 2011; 7:e1002141.

220. O'Leary JG, Goodarzi M, Drayton DL, von Andrian UH. T cell- and B cell-independent adaptive immunity mediated by natural killer cells. Nat Immunol 2006; 7:507-16.

221. Peng H, Jiang X, Chen Y, *et al.* Liver-resident NK cells confer adaptive immunity in skin-contact inflammation. J Clin Invest 2013; 123:1444-56.

222. Greenberg AH, Playfair JH. Spontaneously arising cytotoxicity to the P-815-Y mastocytoma in NZB mice. Clin Exp Immunol 1974; 16:99-109.

223. Rosenberg EB, Herberman RB, Levine PH, Halterman RH, McCoy JL, Wunderlich JR. Lymphocyte cytotoxicity reactions to leukemia-associated antigens in identical twins. Int J Cancer 1972; 9:648-58.

224. Herberman RB, Nunn ME, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. Int J Cancer 1975; 16:216-29.

225. Bjorkstrom NK, Ljunggren HG, Sandberg JK. CD56 negative NK cells: origin, function, and role in chronic viral disease. Trends Immunol 2010; 31:401-6.

226. Nielsen CM, White MJ, Goodier MR, Riley EM. Functional Significance of CD57 Expression on Human NK Cells and Relevance to Disease. Front Immunol 2013; 4:422.

227. Borrego F, Pena J, Solana R. Regulation of CD69 expression on human natural killer cells: differential involvement of protein kinase C and protein tyrosine kinases. Eur J Immunol 1993; 23:1039-43.

228. Borrego F, Robertson MJ, Ritz J, Pena J, Solana R. CD69 is a stimulatory receptor for natural killer cell and its cytotoxic effect is blocked by CD94 inhibitory receptor. Immunology 1999; 97:159-65.

229. Deaglio S, Zubiaur M, Gregorini A, Bottarel F, Ausiello CM, Dianzani U, Sancho J, Malavasi F. Human CD38 and CD16 are functionally dependent and physically associated in natural killer cells. Blood 2002; 99:2490-8.

230. Mallone R, Funaro A, Zubiaur M, *et al.* Signaling through CD38 induces NK cell activation. Int Immunol 2001; 13:397-409.

231. Deaglio S, Mehta K, Malavasi F. Human CD38: a (r)evolutionary story of enzymes and receptors. Leuk Res 2001; 25:1-12.

232. Sconocchia G, Titus JA, Mazzoni A, Visintin A, Pericle F, Hicks SW, Malavasi F, Segal DM. CD38 triggers cytotoxic responses in activated human natural killer cells. Blood 1999; 94:3864-71.

233. Bryceson YT, Ljunggren HG, Long EO. Minimal requirement for induction of natural cytotoxicity and intersection of activation signals by inhibitory receptors. Blood 2009; 114:2657-66.

234. Bryceson YT, March ME, Barber DF, Ljunggren HG, Long EO. Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells. J Exp Med 2005; 202:1001-12.

235. Anfossi N, Andre P, Guia S, *et al.* Human NK cell education by inhibitory receptors for MHC class I. Immunity 2006; 25:331-42.

236. Brodin P, Lakshmikanth T, Johansson S, Karre K, Hoglund P. The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells. Blood 2009; 113:2434-41.

237. Jiang Y, Chen O, Cui C, *et al.* KIR3DS1/L1 and HLA-Bw4-80I are associated with HIV disease progression among HIV typical progressors and long-term nonprogressors. BMC Infect Dis 2013; 13:405.

238. Ozturk OG, Sahin G, Karacor ED, Kucukgoz U. Evaluation of KIR genes in recurrent miscarriage. J Assist Reprod Genet 2012; 29:933-8.

239. Bettencourt A, Silva AM, Carvalho C, Leal B, Santos E, Costa PP, Silva BM. The role of KIR2DS1 in multiple sclerosis: KIR in Portuguese MS patients. J Neuroimmunol 2014.

240. Seymour LA, Nourse JP, Crooks P, Wockner L, Bird R, Tran H, Gandhi MK. The presence of KIR2DS5 confers protection against adult immune thrombocytopenia. Tissue Antigens 2014; 83:154-60.

241. Ramirez-De los Santos S, Sanchez-Hernandez PE, Munoz-Valle JF, Palafox-Sanchez CA, Rosales-Rivera LY, Garcia-Iglesias T, Daneri-Navarro A, Ramirez-Duenas MG. Associations of killer cell immunoglobulin- like receptor genes with rheumatoid arthritis. Dis Markers 2012; 33:201-6.

242. Ito M, Okuno T, Fujii T, Mutoh K, Oguro K, Shiraishi H, Shirasaka Y, Mikawa H. ACTH therapy in infantile spasms: relationship between dose of ACTH and initial effect or long-term prognosis. Pediatr Neurol 1990; 6:240-4.

243. Kusnierczyk P. Killer cell immunoglobulin-like receptor gene associations with autoimmune and allergic diseases, recurrent spontaneous abortion, and neoplasms. Front Immunol 2013; 4:8.

244. de Vasconcelos JM, de Jesus Maues Pereira Moia L, Amaral Ido S, *et al.* Association of killer cell immunoglobulin-like receptor polymorphisms with chronic hepatitis C and responses to therapy in Brazil. Genet Mol Biol 2013; 36:22-7.

245. Marangon AV, Silva GF, de Moraes CF, *et al.* KIR genes and their human leukocyte antigen ligands in the progression to cirrhosis in patients with chronic hepatitis C. Hum Immunol 2011; 72:1074-8.

246. Zuniga J, Romero V, Azocar J, *et al.* Protective KIR-HLA interactions for HCV infection in intravenous drug users. Mol Immunol 2009; 46:2723-7.

247. Vivian JP, Duncan RC, Berry R, *et al.* Killer cell immunoglobulin-like receptor 3DL1-mediated recognition of human leukocyte antigen B. Nature 2011; 479:401-5.

248. Thomas R, Yamada E, Alter G, *et al.* Novel KIR3DL1 alleles and their expression levels on NK cells: convergent evolution of KIR3DL1 phenotype variation? J Immunol 2008; 180:6743-50.

249. Gagne K, Willem C, Legrand N, *et al.* Both the nature of KIR3DL1 alleles and the KIR3DL1/S1 allele combination affect the KIR3DL1 NK-cell repertoire in the French population. Eur J Immunol 2013; 43:1085-98.

250. Fadda L, O'Connor GM, Kumar S, Piechocka-Trocha A, Gardiner CM, Carrington M, McVicar DW, Altfeld M. Common HIV-1 peptide variants mediate differential binding of KIR3DL1 to HLA-Bw4 molecules. J Virol 2011; 85:5970-4.

251. Boulet S, Song R, Kamya P, Bruneau J, Shoukry NH, Tsoukas CM, Bernard NF. HIV protective KIR3DL1 and HLA-B genotypes influence NK cell function following stimulation with HLA-devoid cells. J Immunol 2010; 184:2057-64.

252. Boulet S, Kleyman M, Kim JY, *et al.* A combined genotype of KIR3DL1 high expressing alleles and HLA-B*57 is associated with a reduced risk of HIV infection. AIDS 2008; 22:1487-91.

253. Alter G, Rihn S, Walter K, *et al.* HLA class I subtype-dependent expansion of KIR3DS1+ and KIR3DL1+ NK cells during acute human immunodeficiency virus type 1 infection. J Virol 2009; 83:6798-805.

254. Colonna M, Borsellino G, Falco M, Ferrara GB, Strominger JL. HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. Proc Natl Acad Sci U S A 1993; 90:12000-4.

255. Maenaka K, Juji T, Stuart DI, Jones EY. Crystal structure of the human p58 killer cell inhibitory receptor (KIR2DL3) specific for HLA-Cw3-related MHC class I. Structure 1999; 7:391-8.

256. Faure M, Long EO. KIR2DL4 (CD158d), an NK cell-activating receptor with inhibitory potential. J Immunol 2002; 168:6208-14.

257. Rajagopalan S, Long EO. KIR2DL4 (CD158d): An activation receptor for HLA-G. Front Immunol 2012; 3:258.

258. Moretta A, Sivori S, Vitale M, Pende D, Morelli L, Augugliaro R, Bottino C, Moretta L. Existence of both inhibitory (p58) and activatory (p50) receptors for HLA-C molecules in human natural killer cells. J Exp Med 1995; 182:875-84.

259. Graef T, Moesta AK, Norman PJ, *et al.* KIR2DS4 is a product of gene conversion with KIR3DL2 that introduced specificity for HLA-A*11 while diminishing avidity for HLA-C. J Exp Med 2009; 206:2557-72.

260. Hansasuta P, Dong T, Thananchai H, Weekes M, Willberg C, Aldemir H, Rowland-Jones S, Braud VM. Recognition of HLA-A3 and HLA-A11 by KIR3DL2 is peptide-specific. Eur J Immunol 2004; 34:1673-9.

261. Wong-Baeza I, Ridley A, Shaw J, *et al.* KIR3DL2 binds to HLA-B27 dimers and free H chains more strongly than other HLA class I and promotes the expansion of T cells in ankylosing spondylitis. J Immunol 2013; 190:3216-24.

262. Braud VM, Allan DS, O'Callaghan CA, *et al.* HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature 1998; 391:795-9.

263. Latchman Y, McKay PF, Reiser H. Identification of the 2B4 molecule as a counter-receptor for CD48. J Immunol 1998; 161:5809-12.

264. Brown MH, Boles K, van der Merwe PA, Kumar V, Mathew PA, Barclay AN. 2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48. J Exp Med 1998; 188:2083-90.

265. Brown D, Trowsdale J, Allen R. The LILR family: modulators of innate and adaptive immune pathways in health and disease. Tissue Antigens 2004; 64:215-25.

266. Lanier LL, Ruitenberg JJ, Phillips JH. Functional and biochemical analysis of CD16 antigen on natural killer cells and granulocytes. J Immunol 1988; 141:3478-85.

267. Kaiser BK, Barahmand-Pour F, Paulsene W, Medley S, Geraghty DE, Strong RK. Interactions between NKG2x immunoreceptors and HLA-E ligands display overlapping affinities and thermodynamics. J Immunol 2005; 174:2878-84.

268. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, Spies T. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 1999; 285:727-9.

269. Pende D, Parolini S, Pessino A, *et al.* Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. J Exp Med 1999; 190:1505-16.

270. Cantoni C, Bottino C, Vitale M, *et al.* NKp44, a triggering receptor involved in tumor cell lysis by activated human natural killer cells, is a novel member of the immunoglobulin superfamily. J Exp Med 1999; 189:787-96.

271. Pessino A, Sivori S, Bottino C, Malaspina A, Morelli L, Moretta L, Biassoni R, Moretta A. Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. J Exp Med 1998; 188:953-60.

272. Ljutic B, Carlyle JR, Filipp D, Nakagawa R, Julius M, Zuniga-Pflucker JC. Functional requirements for signaling through the stimulatory and inhibitory mouse NKR-P1 (CD161) NK cell receptors. J Immunol 2005; 174:4789-96.

273. Pozo D, Vales-Gomez M, Mavaddat N, Williamson SC, Chisholm SE, Reyburn H. CD161 (human NKR-P1A) signaling in NK cells involves the activation of acid sphingomyelinase. J Immunol 2006; 176:2397-406.

274. Raulet DH. Interplay of natural killer cells and their receptors with the adaptive immune response. Nat Immunol 2004; 5:996-1002.

275. Waggoner SN, Cornberg M, Selin LK, Welsh RM. Natural killer cells act as rheostats modulating antiviral T cells. Nature 2012; 481:394-8.

276. Waggoner SN, Daniels KA, Welsh RM. Therapeutic depletion of natural killer cells controls persistent infection. J Virol 2013.

277. Pembroke TP, Gallimore AM, Godkin A. Rapid innate control of antigen abrogates adaptive immunity. Immunology 2013; 138:293-7.

278. De Rose V, Cappello P, Sorbello V, Ceccarini B, Gani F, Bosticardo M, Fassio S, Novelli F. IFN-gamma inhibits the proliferation of allergen-activated T lymphocytes from atopic, asthmatic patients by inducing Fas/FasL-mediated apoptosis. J Leukoc Biol 2004; 76:423-32.

279. Moretta A, Marcenaro E, Sivori S, Della Chiesa M, Vitale M, Moretta L. Early liaisons between cells of the innate immune system in inflamed peripheral tissues. Trends Immunol 2005; 26:668-75.

280. Ferlazzo G. Natural killer and dendritic cell liaison: recent insights and open questions. Immunol Lett 2005; 101:12-7.

281. Walzer T, Dalod M, Vivier E, Zitvogel L. Natural killer cell-dendritic cell crosstalk in the initiation of immune responses. Expert Opin Biol Ther 2005; 5 Suppl 1:S49-59.

282. Wilson JL, Heffler LC, Charo J, Scheynius A, Bejarano MT, Ljunggren HG. Targeting of human dendritic cells by autologous NK cells. J Immunol 1999; 163:6365-70.

283. Ferlazzo G, Morandi B, D'Agostino A, Meazza R, Melioli G, Moretta A, Moretta L. The interaction between NK cells and dendritic cells in bacterial infections results in rapid induction of NK cell activation and in the lysis of uninfected dendritic cells. Eur J Immunol 2003; 33:306-13.

284. Ferlazzo G, Tsang ML, Moretta L, Melioli G, Steinman RM, Munz C. Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. J Exp Med 2002; 195:343-51.

285. Alter G, Altfeld M. Mutiny or scrutiny: NK cell modulation of DC function in HIV-1 infection. Trends Immunol 2011; 32:219-24.

286. Alter G, Kavanagh D, Rihn S, Luteijn R, Brooks D, Oldstone M, van Lunzen J, Altfeld M. IL-10 induces aberrant deletion of dendritic cells by natural killer cells in the context of HIV infection. J Clin Invest 2010; 120:1905-13.

287. Mavilio D, Lombardo G, Kinter A, *et al.* Characterization of the defective interaction between a subset of natural killer cells and dendritic cells in HIV-1 infection. J Exp Med 2006; 203:2339-50.

288. Tasca S, Tambussi G, Nozza S, *et al.* Escape of monocyte-derived dendritic cells of HIV-1 infected individuals from natural killer cell-mediated lysis. AIDS 2003; 17:2291-8.

289. Kalayanarooj S, Vaughn DW, Nimmannitya S, *et al.* Early clinical and laboratory indicators of acute dengue illness. J Infect Dis 1997; 176:313-21.

290. Kang W, Shin EC. Colorimetric focus-forming assay with automated focus counting by image analysis for quantification of infectious hepatitis C virions. PLoS One 2012; 7:e43960.

291. O'Connor GM, Vivian JP, Widjaja JM, *et al.* Mutational and Structural Analysis of KIR3DL1 Reveals a Lineage-Defining Allotypic Dimorphism That Impacts Both HLA and Peptide Sensitivity. J Immunol 2014; 192:2875-84.

292. Borghans JA, Molgaard A, de Boer RJ, Kesmir C. HLA alleles associated with slow progression to AIDS truly prefer to present HIV-1 p24. PLoS One 2007; 2:e920.

293. Kim AY, Kuntzen T, Timm J, *et al.* Spontaneous control of HCV is associated with expression of HLA-B 57 and preservation of targeted epitopes. Gastroenterology 2011; 140:686-96 e1.

294. Stephens HA. HIV-1 diversity versus HLA class I polymorphism. Trends Immunol 2005; 26:41-7.

295. Illing PT, Vivian JP, Dudek NL, *et al.* Immune self-reactivity triggered by drug-modified HLA-peptide repertoire. Nature 2012; 486:554-8.

296. Reinherz EL. Pharmacology: A false sense of non-self. Nature 2012; 486:479-81.

297. Kosmrlj A, Read EL, Qi Y, *et al.* Effects of thymic selection of the T-cell repertoire on HLA class I-associated control of HIV infection. Nature 2010; 465:350-4.

298. Huebers HA, Finch CA. The physiology of transferrin and transferrin receptors. Physiol Rev 1987; 67:520-82.

299. Inokuma MS, Maino VC, Bagwell CB. Probability state modeling of memory CD8(+) T-cell differentiation. J Immunol Methods 2013; 397:8-17.

300. Beaumier CM, Mathew A, Bashyam HS, Rothman AL. Cross-reactive memory CD8(+) T cells alter the immune response to heterologous secondary dengue virus infections in mice in a sequence-specific manner. J Infect Dis 2008; 197:608-17.

301. Lazaro E, Godfrey SB, Stamegna P, Ogbechie T, Kerrigan C, Zhang M, Walker BD, Le Gall S. Differential HIV epitope processing in monocytes and CD4 T cells affects cytotoxic T lymphocyte recognition. J Infect Dis 2009; 200:236-43.

302. Akondy RS, Monson ND, Miller JD, *et al.* The yellow fever virus vaccine induces a broad and polyfunctional human memory CD8+ T cell response. J Immunol 2009; 183:7919-30.

303. May WS, Jr., Cuatrecasas P. Transferrin receptor: its biological significance. J Membr Biol 1985; 88:205-15.

304. Salmeron A, Borroto A, Fresno M, Crumpton MJ, Ley SC, Alarcon B. Transferrin receptor induces tyrosine phosphorylation in T cells and is physically associated with the TCR zeta-chain. J Immunol 1995; 154:1675-83.

305. Bayer AL, Baliga P, Woodward JE. Transferrin receptor in T cell activation and transplantation. J Leukoc Biol 1998; 64:19-24.

306. Pattanapanyasat K, Hoy TG. Expression of cell surface transferrin receptor and intracellular ferritin after in vitro stimulation of peripheral blood T lymphocytes. Eur J Haematol 1991; 47:140-5.

307. Pelosi E, Testa U, Louache F, Thomopoulos P, Salvo G, Samoggia P, Peschle C. Expression of transferrin receptors in phytohemagglutinin-stimulated human T-lymphocytes. Evidence for a three-step model. J Biol Chem 1986; 261:3036-42.

308. Gonzalez-Galarza FF, Christmas S, Middleton D, Jones AR. Allele frequency net: a database and online repository for immune gene frequencies in worldwide populations. Nucleic Acids Res 2011; 39:D913-9.

309. Gardiner CM, Guethlein LA, Shilling HG, Pando M, Carr WH, Rajalingam R, Vilches C, Parham P. Different NK cell surface phenotypes defined by the DX9 antibody are due to KIR3DL1 gene polymorphism. J Immunol 2001; 166:2992-3001.

310. Jacobs R, Hintzen G, Kemper A, Beul K, Kempf S, Behrens G, Sykora KW, Schmidt RE. CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells. Eur J Immunol 2001; 31:3121-7.

311. KIR3DL1 Alleles. EMBL-EBI Immune Polymorphism Database 2014.

312. Middleton D, Gonzelez F. The extensive polymorphism of KIR genes. Immunology 2010; 129:8-19.

313. Robinson J, Halliwell JA, McWilliam H, Lopez R, Marsh SG. IPD--the Immuno Polymorphism Database. Nucleic Acids Res 2013; 41:D1234-40.

314. Nomenclature: HLA Antigens Bw4 and Bw6 associated specificities. Anthony Nolan Research Institute 2013.

315. Martin MP, Qi Y, Gao X, *et al.* Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. Nat Genet 2007; 39:733-40.

316. Thananchai H, Gillespie G, Martin MP, *et al.* Cutting Edge: Allele-specific and peptide-dependent interactions between KIR3DL1 and HLA-A and HLA-B. J Immunol 2007; 178:33-7.

317. Peruzzi M, Parker KC, Long EO, Malnati MS. Peptide sequence requirements for the recognition of HLA-B*2705 by specific natural killer cells. J Immunol 1996; 157:3350-6.

318. Rajagopalan S, Long EO. The direct binding of a p58 killer cell inhibitory receptor to human histocompatibility leukocyte antigen (HLA)-Cw4 exhibits peptide selectivity. J Exp Med 1997; 185:1523-8.

319. Fadda L, Korner C, Kumar S, van Teijlingen NH, Piechocka-Trocha A, Carrington M, Altfeld M. HLA-Cw*0102-restricted HIV-1 p24 epitope variants can modulate the binding of the inhibitory KIR2DL2 receptor and primary NK cell function. PLoS Pathog 2012; 8:e1002805.

320. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. Immunol Today 1990; 11:237-44.

321. Carrington M, Alter G. Innate immune control of HIV. Cold Spring Harb Perspect Med 2012; 2:a007070.

322. Alter G, Martin MP, Teigen N, *et al.* Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. J Exp Med 2007; 204:3027-36.

323. Colantonio AD, Bimber BN, Neidermyer WJ, Jr., *et al.* KIR polymorphisms modulate peptide-dependent binding to an MHC class I ligand with a Bw6 motif. PLoS Pathog 2011; 7:e1001316.

324. Malnati MS, Peruzzi M, Parker KC, Biddison WE, Ciccone E, Moretta A, Long EO. Peptide specificity in the recognition of MHC class I by natural killer cell clones. Science 1995; 267:1016-8.

325. Zappacosta F, Borrego F, Brooks AG, Parker KC, Coligan JE. Peptides isolated from HLA-Cw*0304 confer different degrees of protection from natural killer cell-mediated lysis. Proc Natl Acad Sci U S A 1997; 94:6313-8.

326. Mandelboim O, Wilson SB, Vales-Gomez M, Reyburn HT, Strominger JL. Self and viral peptides can initiate lysis by autologous natural killer cells. Proc Natl Acad Sci U S A 1997; 94:4604-9.

327. Marovich M, Grouard-Vogel G, Louder M, *et al.* Human dendritic cells as targets of dengue virus infection. J Investig Dermatol Symp Proc 2001; 6:219-24.

328. Yu J, Heller G, Chewning J, Kim S, Yokoyama WM, Hsu KC. Hierarchy of the human natural killer cell response is determined by class and quantity of inhibitory receptors for self-HLA-B and HLA-C ligands. J Immunol 2007; 179:5977-89.

329. Degli-Esposti MA, Smyth MJ. Close encounters of different kinds: dendritic cells and NK cells take centre stage. Nat Rev Immunol 2005; 5:112-24.

330. Welsh RM, Waggoner SN. NK cells controlling virus-specific T cells: Rheostats for acute vs. persistent infections. Virology 2013; 435:37-45.

331. Martin MP, Carrington M. Immunogenetics of viral infections. Curr Opin Immunol 2005; 17:510-6.

332. Talledo M, Lopez G, Huyghe JR, *et al.* Role of killer cell immunoglobulin-like receptor gene content and human leukocyte antigen-C group in susceptibility to human T-lymphotropic virus 1-associated myelopathy/tropical spastic paraparesis in Peru. Hum Immunol 2010; 71:804-8.

333. Rivero-Juarez A, Gonzalez R, Camacho A, *et al.* Natural killer KIR3DS1 is closely associated with HCV viral clearance and sustained virological response in HIV/HCV patients. PLoS One 2013; 8:e61992.

334. Rauch A, Laird R, McKinnon E, *et al.* Influence of inhibitory killer immunoglobulin-like receptors and their HLA-C ligands on resolving hepatitis C virus infection. Tissue Antigens 2007; 69 Suppl 1:237-40.

335. Cariani E, Pilli M, Zerbini A, *et al.* HLA and killer immunoglobulin-like receptor genes as outcome predictors of hepatitis C virus-related hepatocellular carcinoma. Clin Cancer Res 2013; 19:5465-73.

336. Jelcic I, Hsu KC, Kakalacheva K, *et al.* Killer immunoglobulin-like receptor locus polymorphisms in multiple sclerosis. Mult Scler 2012; 18:951-8.

337. Wang S, Li G, Ge R, *et al.* Association of KIR genotype with susceptibility to HLA-B27-positive ankylosing spondylitis. Mod Rheumatol 2013; 23:538-41.

338. Martin MP, Gao X, Lee JH, *et al.* Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. Nat Genet 2002; 31:429-34.

339. Littwitz E, Francois S, Dittmer U, Gibbert K. Distinct roles of NK cells in viral immunity during different phases of acute Friend retrovirus infection. Retrovirology 2013; 10:127.

340. Shresta S, Kyle JL, Robert Beatty P, Harris E. Early activation of natural killer and B cells in response to primary dengue virus infection in A/J mice. Virology 2004; 319:262-73.

341. Chan M, Johansson MA. The incubation periods of Dengue viruses. PLoS One 2012; 7:e50972.

342. Rathakrishnan A, Wang SM, Hu Y, Khan AM, Ponnampalavanar S, Lum LC, Manikam R, Sekaran SD. Cytokine expression profile of dengue patients at different phases of illness. PLoS One 2012; 7:e52215.

343. Crome SQ, Lang PA, Lang KS, Ohashi PS. Natural killer cells regulate diverse T cell responses. Trends Immunol 2013; 34:342-9.

344. Anez G, Rios M. Dengue in the United States of America: a worsening scenario? Biomed Res Int 2013; 2013:678645.

345. Gerosa F, Baldani-Guerra B, Nisii C, Marchesini V, Carra G, Trinchieri G. Reciprocal activating interaction between natural killer cells and dendritic cells. J Exp Med 2002; 195:327-33.

346. Townsley E, Woda M, Thomas SJ, *et al.* Distinct Activation Phenotype of a Highly Conserved Novel HLA-B57-Restricted Epitope during Dengue Virus Infection. Immunology 2013.

347. Vankayalapati R, Klucar P, Wizel B, Weis SE, Samten B, Safi H, Shams H, Barnes PF. NK cells regulate CD8+ T cell effector function in response to an intracellular pathogen. J Immunol 2004; 172:130-7.

348. Charles A Janeway J, Paul Travers, Mark Walport, and Mark J Shlomchik. Immunobiology: The Immune System in Health and Disease. 5th edition. Antigen recognition by T cells. . New York: Garland Science 2001.

349. Rodriguez-Rodero S, Gonzalez S, Rodrigo L, Fernandez-Morera JL, Martinez-Borra J, Lopez-Vazquez A, Lopez-Larrea C. Transcriptional regulation of MICA and MICB: a novel polymorphism in MICB promoter alters transcriptional regulation by Sp1. Eur J Immunol 2007; 37:1938-53.

350. Marcus A, Raulet DH. Evidence for natural killer cell memory. Curr Biol 2013; 23:R817-20.

351. Sun JC, Madera S, Bezman NA, Beilke JN, Kaplan MH, Lanier LL. Proinflammatory cytokine signaling required for the generation of natural killer cell memory. J Exp Med 2012; 209:947-54.

352. Sun LX, Lin ZB, Duan XS, *et al.* polysaccharides counteract inhibition on CD71 and FasL expression by culture supernatant of B16F10 cells upon lymphocyte activation. Exp Ther Med 2013; 5:1117-22.

353. Kneissl S, Zhou Q, Schwenkert M, Cosset FL, Verhoeyen E, Buchholz CJ. CD19 and CD20 targeted vectors induce minimal activation of resting B lymphocytes. PLoS One 2013; 8:e79047.

354. Millrud CR, Mansson Kvarnhammar A, Uddman R, Bjornsson S, Riesbeck K, Cardell LO. The activation pattern of blood leukocytes in head and neck squamous cell carcinoma is correlated to survival. PLoS One 2012; 7:e51120.

355. Bendall SC, Simonds EF, Qiu P, *et al.* Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. Science 2011; 332:687-96.

356. Maecker HT, Rinfret A, D'Souza P, *et al.* Standardization of cytokine flow cytometry assays. BMC Immunol 2005; 6:13.

357. Aghaeepour N, Finak G, Flow CAPC, *et al.* Critical assessment of automated flow cytometry data analysis techniques. Nat Methods 2013; 10:228-38.

358. Herbert DJ, Miller DT, Bruce Bagwell C. Automated analysis of flow cytometric data for CD34+ stem cell enumeration using a probability state model. Cytometry B Clin Cytom 2012; 82:313-8.