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CD8^{+} T CELL SEROTYPE-CROSS-REACTIVITY IS A PREDOMINANT FEATURE OF DENGUE VIRUS INFECTIONS IN HUMANS

A Dissertation Presented

By

HEATHER L. FRIBERG-ROBERTSON

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

NOVEMBER 30, 2010

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CD8$^+$ T CELL SEROTYPE-CROSS-REACTIVITY IS A PREDOMINANT FEATURE OF DENGUE VIRUS INFECTIONS IN HUMANS

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ABSTRACT

The four serotypes of dengue virus (DENV 1-4) have a significant and growing impact on global health. Dengue disease encompasses a wide range of clinical symptoms, usually presenting as an uncomplicated febrile illness lasting 5-7 days; however, a small percentage of infections are associated with plasma leakage and bleeding tendency (called dengue hemorrhagic fever, DHF), which can result in shock. Epidemiological studies indicate that severe dengue disease most often occurs during secondary heterotypic DENV infection. Additionally, plasma leakage (the hallmark of DHF) coincides with defervescence and viral clearance, suggesting that severe disease arises from the immune response to infection rather than a direct effect of the virus.

A number of studies have found increased levels of markers of immune cell activation in patients with DHF compared to patients with the less severe form of disease (DF). These markers include IFNγ, TNFα, soluble CD8, soluble IL-2 receptor, soluble TNF receptor, and CD69, which support a role for T cells in mediating immunopathology. Because of the high homology of DENV 1-4, some degree of serotype-cross-reactivity is seen for most T cell epitopes. A high percentage of DENV-specific T cells recognize multiple DENV serotypes, as demonstrated by peptide-MHC (pMHC) tetramer binding and *in vitro* functional assays performed on PBMC from subjects vaccinated with an experimental DENV vaccine or naturally-infected subjects with secondary (>1) DENV infection.

This thesis sought to address several gaps in the literature, specifically whether T cell responses differ in primary versus secondary (natural) infection. We studied the frequency, phenotype, and function of DENV-specific T cells. We demonstrated substantial serotype-cross-reactivity of antigen-specific T cells generated in response to naturally-acquired primary as well as secondary DENV infection. The frequency of A11-$NS3_{133}$ epitope-specific T cells during acute infection did not correlate with disease severity. However, the peak frequency occurred earlier in primary infection while the frequency of $CD45RA^{+}$ T cells declined quicker in secondary infection, suggesting the expansion of DENV-specific memory T cells. DENV-immune T cells exhibited different functional capabilities that were dependent on the particular serotype of infection. Specifically, DENV-1 or -3 stimulation of A11-$NS3_{133}$ epitope-specific T cell lines resulted in robust function that included IFNγ production, whereas DENV-2 stimulation resulted in limited function that often included MIP-1β but not IFNγ production. These data support a role for T cells in DENV infection and offer new insights into their potential contribution to dengue pathology.

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ABBREVIATIONS

1º	primary
2º	secondary
^{51}Cr	radio-labeled chromium
Ab	antibody
ADE	antibody dependent enhancement
ann	artificial neural network algorithm
AP-1	activating protein 1
APC	allophycocyanin
APC	antigen presenting cell
APL	altered peptide ligand
arb	average relative binding algorithm
AST	aspartate aminotransferase
Bcl-2	B-cell lymphoma 2
BLCL	B-lymphoblastoid cell line
C	capsid protein
CDR3	complementarity-determining region 3
CSA	cyclosporin A
CTL	cytolysis
DAG	diacylglycerol
DC	dendritic cell

DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing non-integrin
DENV	dengue virus
DF	dengue fever
DHF	dengue hemorrhagic fever
DSS	dengue shock syndrome
E	envelope protein
EBV	Epstein Barr virus
ER	endoplasmic reticulum
ERK1/2	extracellular signal-regulated kinase ½
FBS	fetal bovine serum
FITC	fluorescein
Flu	influenza virus
FSC	forward scatter
gMFI	geometric mean fluorescence intensity
GADS	Grb2-related adapter protein
Grb2	growth-factor-receptor-bound protein 2
GSK	GlaxoSmithKline
HAI	hemagglutination-inhibition
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
ICS	intracellular cytokine staining

IFN	interferon
Ig	immunoglobulin
IκB	inhibitor of NFκB
IL	interleukin
IP3	inositol 3,4,5 trisphosphate
ITAM	immunoreceptor tyrosine-based activation motif
LAT	linker for the activation of T cells
LCMV	lymphocytic choriomeningitis virus
LT	lymphotoxin
LTA	lymphotoxin gene
M	membrane protein
mAbs	monoclonal antibodies
MHC	major histocompatibility complex
MIP-1β	macrophage inflammatory protein 1β
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor-κB
NIH	National Institutes of Health
NS	no stimulation
NS#	non-structural protein #
P#	amino acid position #
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline

pCD3ζ	phosphorylated CD3 ζ chain
PE	phycoerythrin
PEI	pleural effusion index
PerCP	Peridinin-chlorophyll protein
pERK1/2	phosphorylated ERK1/2
PIP2	phosphatidylinositol 4,5 bisphosphate
PKC	protein kinase C
PLCγ1	phospholipase Cγ1
pMHC	peptide-MHC complex
preM	precursor form of M protein
PV	Pichinde virus
Qdot	quantum dot
RNA	ribonucleic acid
RPMI	Rosewell Park Memorial Institute cell culture medium
SCID	severe combined immunodeficiency
SD	standard deviation
SH2	Src homology 2
SLP76	SH2-domain-containing leukocyte protein-76
smm	stabilized matrix method algorithm
SNP	single nucleotide polymorphism
SP	Acambis/Sanofi Pasteur
SSC	side scatter

TCR	T cell receptor
TGFβ	transforming growth factor β
TNF	tumor necrosis factor
UTR	untranslated region
V_β	variable gene segment β
VV	vaccinia virus
WHO	World Health Organization
WRAIR	Walter Reed Army Institute of Research
Zap70	ζ chain-associated protein of 70kDa

PREFACE

Parts of this thesis have appeared in separate publications:

Chapter III:
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Chapter IV:
Friberg H, Burns L, Woda M, Kalayanarooj S, Endy TP, Stephens HAF, Green S, Rothman AL, Mathew A (2010). Memory $CD8^+$ T cells from naturally-acquired primary dengue virus infection are highly cross-reactive. *Immunology and Cell Biology*. PMID: 20421879 [Epub ahead of print].

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> *I contributed to this work by developing the immunohistochemistry assay performed in the paper as well as generating the* in vitro *infection data.*

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> *I contributed to this study by independently confirming the viral epitopes identified in this paper while performing* in vivo *infections on HLA-A2 transgenic B6 mice.*

CHAPTER I

INTRODUCTION

A. Global impact of dengue

The tropical and subtropical regions of the world are home to an array of diseases transmitted by arthropod vectors. Among them are the dengue viruses (DENV), a collection of four related but genetically-distinct viruses (termed serotypes 1-4) which are transmitted by mosquitoes and cause disease in nearly half of their infected hosts. The global reach of dengue has expanded dramatically over the past forty years and currently extends into >100 countries (World Health Organization, Dengue and Dengue Hemorrhagic Fever Fact Sheet, <http://www.who.int/mediacentre/factsheets/fs117/en /index.html>). Approximately 3.6 billion people currently live in areas at risk of DENV transmission, resulting in an estimated 70-500 million infections, 2.1 million severe cases of disease, and over 20,000 deaths each year (Mark Beatty, Pediatric Dengue Vaccine Initiative, Global Burden of Dengue, <http://www.pdvi.org/about_dengue/GBD.asp>). The spread of dengue is largely due to increasing globalization and urbanization, which allow the principle mosquito vector for DENV, *Aedes aegypti*, a greater area to establish its home (Wilder-Smith and Gubler, 2008). Increasing global trade and travel provide an easy route to physically transport an infected mosquito or the virus itself via infected blood (on which mosquitoes feed) to new regions, and densely-populated urban centers offer plenty of opportunities for standing water, which serves as a breeding ground for the potential DENV vectors.

The transmission cycle of DENV is largely maintained through mosquito-human contact (reviewed in Gubler and Kuno, 1997). The anthropophilic nature of *Aedes aegypti* prompted the species to adapt to human behavior, resulting in its domestication and, thus, its role as primary DENV vector. Other *Aedes* species also contribute to DENV transmission (e.g. *A. albopictus*), some even sustaining limited sylvatic cycles with non-human primates in Africa and Asia (Rodhain, 1991). Once mosquitoes are infected with DENV they are able to transmit the virus throughout the remainder of their lifetime, which, according to laboratory tests, may last over 100 days (Gubler, 1970). Not only can the vectors pass DENV on to humans, but evidence for vertical and sexual transmission amongst the mosquitoes themselves has also been found (Rosen, 1987; Freier and Rosen, 1987, 1988). Transmission efficiency varies according to a number of factors including ambient temperature, which affects the duration of gonotrophic cycles, frequency of blood meals, and the external incubation period (the time between DENV-infection of the mosquito and its ability to subsequently pass infectious DENV to humans) (Rodhain and Rosen, 1997). While *A. aegypti* is largely limited to warm tropical and subtropical climates, the less efficient DENV vector *A. albopictus* can withstand lower temperatures. *A. albopictus*, which is now established in many parts of the continental U.S. and Europe, is also more susceptible to oral DENV infection and can become infected on persons with lower viremia levels (Kuno, 1997). There is no evidence of chronic DENV infection in humans; the extent of anthropophilia among *Aedes* species, and the ever-increasing human population, allow for maintenance of DENV transmission.

Recent outbreaks of DENV infection have renewed domestic interest in this viral threat. In 2006-2007, significant dengue outbreaks in the Americas affected Mexico, Latin America, the Caribbean, and South America with over 1 million reported cases (PAHO, 2007). Outbreaks have occurred in Hawaii (Effler, *et al.*, 2005) and Puerto Rico (Rigau-Perez and Laufer, 2006) during the past decade. DENV has been virtually eradicated from the continental U.S. since the 1940s. However, occasional DENV infections have been reported in the southern-most parts of Texas, tracking with outbreaks that occurred in nearby Mexican cities (CDC, 1996, 2001, 2007). Most recently, DENV infections have occurred in Key West, Florida, with a seroprevalence of about 5% recorded in the local population (CDC, 2010). If DENV makes its way up the Florida coast, its presence would mark its true reestablishment in the continental U.S.

With the relatively rapid spread of DENV around the world, there is an increased awareness of the economic, not to mention human, cost to society. A recent report assessing the economic burden of DENV on eight countries spanning Asia and the Americas estimated that hundreds of millions of U.S. dollars were spent each year to treat DENV-infected patients (Suaya, *et al.*, 2009). Considering that many of the countries harboring DENV-infected mosquitoes are developing nations, the cost to an average family can be overwhelming. In addition, mosquitoes tend to migrate within a relatively confined area (Harrington, *et al.*, 2005; Mammen, *et al.*, 2008), leading to the possibility that multiple family members will become infected, further increasing the expense. Such issues serve to highlight the need for more research on DENV infection and ways to treat or prevent disease.

B. Dengue disease and treatment

Dengue disease ranges in severity and can manifest as a variety of clinical symptoms. The most common form of disease, dengue fever (DF), is typically characterized by a high fever, retro-orbital headache and severe myalgias that last for five to seven days and are occasionally accompanied by a rash (George, 1997). Some patients suffer from dengue hemorrhagic fever (DHF), a more serious condition in which the patient experiences DF symptoms as well as bleeding tendencies and plasma leakage, and is at higher risk for developing shock. According to established World Health Organization (WHO) guidelines, DHF is subdivided into four grades of severity based on the presence and extent of spontaneous bleeding and shock (WHO, 1997). The most severe cases (grades III and IV) are classified as dengue shock syndrome or DSS. More recent recommendations by the WHO acknowledge the breadth and complexity of disease symptoms and simply classify cases of DENV infection as uncomplicated or severe (WHO, 2009).

Several clinical variables are tracked throughout hospitalization of DENV-infected patients and serve as indicators of disease severity. Tourniquet tests are performed to determine the presence and extent of vascular fragility. Where resources are available, platelet counts are measured daily to check for thrombocytopenia ($<100,000$ platelets/mm^3 is a criterion for classification as DHF); elevated aspartate aminotransferase (AST) values indicate liver damage. A $\geq 20\%$ rise in hematocrit compared to baseline and evidence of pleural effusion or ascites are markers of plasma leakage, the hallmark of DHF (Nimmannitya, 1987). The new WHO criteria for severe

dengue include some of these measures and are intended to help coordinate case reporting world-wide.

Distinguishing cases of DENV infection from other acute febrile illnesses is initially challenging, as many viral infections result in fever as well as head and body aches. As dengue disease progresses, however, indicators such as hemorrhage (including a positive tourniquet test), thrombocytopenia, leukopenia and pleural effusion help to suggest DENV as the pathological agent (Nimmannitya, 1987; Kalayanarooj, 1999b). Further difficulty arises when trying to predict whether a patient will recover without complications or suffer DHF upon defervescence. Severe clinical symptoms indicative of DHF (e.g. plasma leakage) occur suddenly and concurrent with viral clearance (Vaughn, *et al.*, 1997; Vaughn, *et al.*, 2000), so vigilant monitoring of patients around the time of defervescence is critical (Nimmannitya, 1987; Kalayanarooj, 1999a).

Unfortunately, no vaccine for DENV is yet available nor is there a specific treatment for dengue disease. However, experienced physicians will closely observe hospitalized DENV-infected patients and administer fluid replacement therapy at the first sign of plasma leakage (Nimmannitya, 1987; Kalayanarooj, 1999a). Such aggressive intervention has been shown to meaningfully reduce the mortality rate, which can otherwise reach up to 20% of patients with DHF (Kalayanarooj, 1999a). Recent efforts to design vaccines effective against all four DENV serotypes are discussed below.

C. Characteristics of dengue virus

The four serotypes of DENV (DENV 1-4) are members of the virus family *Flaviviridae* (named after the prototype virus, yellow fever) and the genus flavivirus (reviewed in Lindenbach, *et al.,* 2007). Flaviviruses have small, enveloped virions with a single-stranded, positive-sense RNA genome that is approximately 11,000 nucleotides long. Members of this genus all share a similar virion structure, which is made up of three structural proteins (capsid [C], membrane [M], and envelope [E]). The genome also encodes seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). DENV 1-4 share approximately 70% homology at the amino acid level, depending on which proteins are being compared; E is quite variable across serotypes, whereas the non-structural proteins retain more conserved sequences (Irie*, et al.*, 1989).

The DENV virion is composed of a lipid bilayer containing E and M glycoproteins which encapsulate the nucleocapsid made up of C protein together with the RNA genome. The envelope protein E facilitates binding to cells and virus fusion with the host cell membrane, mediating viral entry into the cell. While a definitive receptor remains elusive, it is known that the cell-surface protein dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) can mediate attachment and productive infection (Navarro-Sanchez*, et al.*, 2003; Tassaneetrithep*, et al.*, 2003), although DC-SIGN internalization is unnecessary for DENV entry (Lozach*, et al.*, 2005). Once inside a host cell, the low pH of the endocytic compartment allows for fusion of the viral and host membranes, releasing the nucleocapsid into the cytoplasm. The single-stranded, positive-sense RNA genome is immediately ready for translation

and is directed toward the endoplasmic reticulum (ER). The ten viral proteins are translated as a single open reading frame along the ER membrane. Host and viral proteases then cleave the polyprotein to release the individual proteins for further processing.

The seven non-structural proteins of DENV play a variety of roles involving RNA replication (NS1, NS3, NS4a, NS5), virus assembly (likely NS2a and NS3) and serine protease activity (NS2b, NS3). They are also known to interfere with anti-viral immune responses of the host, such as type 1 interferon (IFN) signaling (NS2a, NS4a, NS4b). NS1 is secreted from the cell during infection, its accumulation in patient sera correlating with disease severity (Libraty*, et al.*, 2002b). Once outside the cell, soluble NS1 can bind the plasma membrane of both infected and uninfected cells (Avirutnan*, et al.*, 2007), in particular endothelial cells, and thus target them for complement-mediated lysis (Lin*, et al.*, 2003). NS3 has been shown to induce apoptosis *in vitro*, using NS2b as a cofactor (Shafee and AbuBakar, 2003). NS5 induces the production of interleukin (IL)-8 by infected cells, which may increase the spread of the virus by recruiting inflammatory target cells to the site of infection (Medin*, et al.*, 2005). The 5' and 3' untranslated regions (UTRs) of the viral genome are also involved in efficient DENV infection. These regions form highly conserved stem-loop secondary structures which are important for viral replication (Alvarez*, et al.*, 2005a; Alvarez*, et al.*, 2005b; Pankhong*, et al.*, 2009); UTR mutations can affect the virulence of progeny virions (Cahour*, et al.*, 1995; Men*, et al.*, 1996; Zeng*, et al.*, 1998).

DENV can infect a variety of cell types. *In vitro* experiments using primary human cells have demonstrated the susceptibility of monocytes, macrophages, dendritic cells (DC), endothelial cells, and lung epithelium to DENV infection (Avirutnan*, et al.*, 1998; Huang*, et al.*, 2000; Wu*, et al.*, 2000; Chen and Wang, 2002; Lee*, et al.*, 2007). Liver-resident macrophages can also become infected, but no viral progeny are subsequently produced (Marianneau*, et al.*, 1999). Staining of patient tissues from autopsy and/or biopsy samples has confirmed the presence of DENV antigen in blood-resident monocytes, macrophages (from spleen, lung, and liver), skin-resident DC, lymphocytes found in the spleen and blood and endothelial cells found in the liver and lung (Wu*, et al.*, 2000; Jessie*, et al.*, 2004; Limonta*, et al.*, 2007). Unfortunately, no DENV-infected, immunocompetent animal model accurately exhibits the disease observed in humans so tracking virus *in vivo* during acute infection is a challenge. Nevertheless, the above cell types were also shown to be susceptible in DENV-infected mice (Chen*, et al.*, 2007; Kyle*, et al.*, 2007; Balsitis*, et al.*, 2009; Zellweger*, et al.*, 2010), affording some confidence in these models, which typically result in inconsistent and low level infection with few, if any, signs of disease. The continued development and recent successful DENV infection of “humanized” mice appears promising for generating a useful animal model for DENV infection (Bente*, et al.*, 2005; Kuruvilla*, et al.*, 2007; Jaiswal*, et al.*, 2009; Mota and Rico-Hesse, 2009).

D. Risk factors for severe dengue disease

One of the earliest descriptions of DHF noted that patients with a secondary-type antibody response were at greater risk for developing the severe form of disease than those exposed to the virus for the first time (Halstead, *et al.*, 1970). This observation has since been confirmed in many studies of DENV patients from all over the world infected with any of the four serotypes (Burke, *et al.*, 1988; Guzman, *et al.*, 1990; Thein, *et al.*, 1997). While primary DENV infection results in long-term protective immunity to the infecting serotype, it only provides partial and transient protection against the other, heterologous, serotypes (Sabin, 1952). This means that an individual can, in theory, experience up to four DENV infections in their lifetime with an increased risk for severe disease upon subsequent (secondary) exposure to a heterologous DENV serotype. Prior immunity, therefore, seems to increase the risk for severe dengue disease, implicating a role for adaptive immune responses (i.e. T and B cells) in contributing to disease pathogenesis. Details regarding the involvement of these factors are discussed later in this chapter.

In addition to prior immunity, many other factors also help shape the outcome of disease in DENV-infected individuals. Such influential features include demographic, host genetic, and virus-specific factors that demonstrate associations with patients experiencing mild versus severe disease. Each of these elements likely contributes to the final outcome of DENV-infected patients, but their extent of involvement in dengue pathology remains unclear.

i. Demographic factors

A number of studies have noted that certain demographic populations, unrelated to genetic background, are at higher or lower risk for severe dengue disease. Studies on the nutritional status of infants (Nguyen, *et al.*, 2005) and children (Thisyakorn and Nimmannitya, 1993) report an underrepresentation of DHF/DSS cases in those with malnutrition, and this was attributed to dampened cellular immunity. This is in contrast to another study which demonstrated that, while malnourished children were at lower risk of contracting DENV infection, those who did were at higher risk of developing shock (Kalayanarooj and Nimmannitya, 2005). On the other hand, obese children are at higher risk of both contracting DENV infection (Kalayanarooj and Nimmannitya, 2005) and developing severe disease (Kalayanarooj and Nimmannitya, 2005; Pichainarong, *et al.*, 2006). Age appears to influence the symptoms of disease, as one study showed that children had a higher incidence of cough and hepatomegaly compared to adults, who more often experienced headache, myalgias/arthralgias and petechiae (Wichmann, *et al.*, 2004). In other reports, socio-economic status also correlated with severe disease, in particular patients from high income or highly educated homes (Blanton, *et al.*, 2008; Figueiredo, *et al.*, 2010). In general, those with chronic disease or other complicating factors such as diabetes, allergies, or hypertension are at increased risk for DHF (Bravo, *et al.*, 1987; Figueiredo, *et al.*, 2010).

ii. Host genetic factors

A relatively small fraction (~6% of clinical cases) of DENV-infected individuals experiences severe dengue disease (Mark Beatty, Pediatric Dengue Vaccine Initiative, Global Burden of Dengue, <http://www.pdvi.org/about_dengue/GBD.asp>) implicating host-specific genetic factors in contributing to disease susceptibility. For example, patients with blood type AB were at higher risk for developing DHF compared to those with other blood types in studies of Thai children (Kalayanarooj, *et al.*, 2007; Fried, *et al.*, 2010). Race and/or ethnic associations with dengue disease have regularly been noted, specifically identifying “white” individuals at higher risk for severe disease than “black” individuals (Bravo, *et al.*, 1987; Sierra, *et al.*, 2007b; Figueiredo, *et al.*, 2010). These demographic differences may be caused by genetic variations affecting human leukocyte antigen (HLA) haplotypes or other genes which affect the immune response to DENV infection (Sierra, *et al.*, 2006; Restrepo, *et al.*, 2008).

A number of studies have linked specific HLA class I, II, and III alleles with different dengue disease manifestations. HLA-A2 is a common class I allele found throughout many populations worldwide (The Allele Frequency Net Database <http://www.allelefrequencies.net/>) and was found to be associated with clinically evident secondary DENV infection in pediatric Thai subjects (Chiewsilp, *et al.*, 1981; Stephens, *et al.*, 2002). More detailed analysis found that HLA-A*0203 in particular correlated with mild disease (DF) as compared to HLA-A*0207, which correlated with severe disease (DHF) (Stephens, *et al.*, 2002). This report and others identified additional HLA class I and II correlations with protective (HLA-A29, A33, B13, B14,

B44, B52, B62, B76, B77, DRB1*04, DRB1*07, DRB1*09) or pathologic (HLA-A1, A24, A31, B15, B46, B51, DQ1) outcomes (Chiewsilp*, et al.*, 1981; Paradoa Perez*, et al.*, 1987; Loke*, et al.*, 2001; LaFleur*, et al.*, 2002; Stephens*, et al.*, 2002; Polizel*, et al.*, 2004; Sierra*, et al.*, 2007a; Nguyen*, et al.*, 2008).

Studies analyzing HLA class III or other genes involved in immune responses, including cytokine production, also detected correlations with particular disease outcomes. Separate studies found that the A allele of the single nucleotide polymorphism (SNP) at position 308 in the gene for tumor necrosis factor (TNF)α is associated with DHF (Fernandez-Mestre*, et al.*, 2004; Perez*, et al.*, 2010). TNF-308A has been linked to higher levels of TNFα protein than other alleles (Wilson*, et al.*, 1997), supporting a role for TNFα in dengue pathogenesis. These same studies also reported that particular SNP alleles of the gene for IL-10, which are associated with low levels of IL-10 protein production, correlate with DHF (Fernandez-Mestre*, et al.*, 2004; Perez*, et al.*, 2010). These groups suggested that high TNFα/low IL-10 production helped mediate severe dengue disease. Another study reported a strong genetic linkage between the SNP allele –238A in the TNFα gene and the lymphotoxin-α (LTA)-3 haplotype, which is associated with high TNFα and LTα production during acute viremia in DENV-infected patients (Gagnon*, et al.*, 2002; Vejbaesya*, et al.*, 2009). Patients with TNF –238A and LTA-3 were at greater risk for developing DHF compared to DF, and DHF patients with these alleles were nearly all shown to have HLA-B48 and/or HLA-B57 (Vejbaesya*, et al.*, 2009), otherwise rare HLA haplotypes in this (Thai) population (Stephens*, et al.*, 2002). Such analyses underscore the need to address extended haplotypes in future genetic

studies of dengue patients in order to better understand potential mechanisms for the immunopathology observed in DENV infection. Additional genes with allelic variants associated with different dengue disease outcomes include those for mannose-binding lectin (Acioli-Santos, *et al.*, 2008), the vitamin D receptor, Fcγ receptor II (Loke, *et al.*, 2002), transporter associated with antigen processing, human platelet antigens (Soundravally and Hoti, 2007), and DC-SIGN (Sakuntabhai, *et al.*, 2005).

iii. Viral factors

As an accurate animal model of dengue disease remains to be established, differences in virulence among various virus strains are explored by isolating viruses from viremic patients, comparing the viral sequences, and associating specific sequence mutations with the patients' final outcome (Rico-Hesse, 2003). Most viral isolates come from those who are ill enough to come to medical attention, which is a limitation of this approach; however, significant correlations have been made. For example, large outbreaks of severe disease are often associated with DENV serotypes 2 and 3, whereas DENV-4 is less commonly detected in clinically evident cases of dengue and then mostly in those with secondary DENV infections (Nisalak, *et al.*, 2003; Fried, *et al.*, 2010). DENV-2 is also more often seen in patients experiencing a secondary DENV infection than in those with primary infections. On the other hand, DENV-1 and DENV-3 cause clinically evident dengue in those with either primary or secondary DENV infections.

Certain serotypes of DENV have also been correlated with particular disease symptoms. One study comparing DENV-2 versus DENV-3 infection in adults from

Taiwan found that DENV-3 tended to cause myalgias, ascites and skin rash whereas DENV-2 infection was more often associated with bone pain (Tsai, *et al.*, 2009). A different study in Thai children found more pleural effusions and a higher incidence of ascites in subjects infected with DENV-2 compared to other serotypes (Fried, *et al.*, 2010).

Furthermore, phylogenetic analysis of different virus isolates has identified several viral genotypes within each DENV serotype. For example, DENV-2 (the most well studied serotype due to its wide transmission pattern) comprises four genotypes, including those that originated in West Africa, the Americas, Southeast Asia and Malaysia/Indian subcontinent (Rico-Hesse, *et al.*, 1998; Rico-Hesse, 2003). West African viruses are thought to have the lowest epidemiological impact due to their sequence proximity to sylvatic viruses and relatively rare encounter by humans. American and Malaysian/Indian viruses are typically associated with cases of DF, whereas DHF cases are associated with Southeast Asian viruses (Rico-Hesse, *et al.*, 1997). The Southeast Asian viral strains seem to be replacing other DENV-2 genotypes worldwide, possibly due to a greater ability to replicate in both mosquito vectors and primary human DC, higher infection efficiency (of mosquitoes themselves), and thus higher transmission rates (Armstrong and Rico-Hesse, 2001, 2003; Cologna, *et al.*, 2005; Anderson and Rico-Hesse, 2006). Differences in virulence among the different genotypes of DENV-2 have been attributed to particular nucleotide changes and RNA folding patterns in the 5' and 3' UTRs of the virus as well as amino acid changes in a variety of viral proteins (Leitmeyer, *et al.*, 1999). Replacement of the 5' and 3' UTRs

together with amino acid 390 of E protein in a Southeast Asian virus with those found in an American strain lowered the level of replication of the virus in primary human DC cultures to that seen for the wild-type American virus (Cologna and Rico-Hesse, 2003). This resulted in a 10-fold decrease in viral yield compared to the wild-type Southeast Asian virus. Higher viral loads are often associated with more severe disease (Murgue*, et al.*, 2000; Vaughn*, et al.*, 2000; Libraty*, et al.*, 2002a; Libraty*, et al.*, 2002b), though this pattern is not always seen (Sudiro*, et al.*, 2001). Genotype classification of serotypes 1, 3, and 4 is less well established, although Southeast Asian strains of DENV-3 have been associated with large DHF outbreaks (Chungue*, et al.*, 1993; Rico-Hesse, 2003). DENV-3 strains from the Indian subcontinent have been shown to evolve to become more virulent (Lanciotti*, et al.*, 1994), and American strains are associated with outbreaks of milder disease (Rico-Hesse, 2003). Studies like that coordinated by the Broad Institute (of Massachusetts Institute of Technology and Harvard University) intend to vastly increase the database of DENV sequences in order to assess viral diversity across continents as well as within infected individuals (Broad Institute, Dengue Virus Portal, <http://www.broadinstitute.org/annotation/viral/Dengue/>). Such analyses should help identify specific elements of the virus that contribute to mild versus severe disease.

E. DENV vaccine initiatives

Considering the potential for all four DENV serotypes to cause severe disease, and the increased risk for severe disease in secondary heterologous DENV infection, strategies for developing a DENV vaccine have been directed at achieving tetravalent

immunity (Webster, *et al.*, 2009). Numerous strategies are currently being enlisted to develop live-attenuated, inactivated, chimeric, infectious clone, and/or subunit vaccines. Several vaccine candidates are now in various stages of clinical trials due to the efforts of investigators at Walter Reed Army Institute of Research (WRAIR), GlaxoSmithKline (GSK), Acambis/Sanofi Pasteur (SP), and the National Institutes of Health (NIH), among others.

Live-attenuated vaccines have several theoretical advantages over other strategies including efficacy, cost, and long-term immunity (Webster, *et al.*, 2009). The yellow fever virus vaccine YF-17D is a live-attenuated virus that has proven to be extremely effective at inducing robust T and B cell responses (Poland, *et al.*, 1981; Akondy, *et al.*, 2009) and serves as a model for successful vaccines. SP researchers used the YF-17D genome as a backbone into which the coding sequences for the precursor form of M (preM) and E proteins from each of the four DENV serotypes were placed (Guirakhoo, *et al.*, 2000; Guirakhoo, *et al.*, 2001). These live, chimeric viruses were genetically stable, immunogenic, and provided protection from subsequent DENV challenge in non-human primates. Clinical trials in humans have demonstrated high seroconversion rates among various populations, including adults from the U.S.A., children from Mexico, and flavivirus-immune individuals from the Philippines (Lang, 2009). Vaccination induced DENV-specific neutralizing antibodies (Guirakhoo, *et al.*, 2006) as well as $IFN\gamma^{+}CD8^{+}$ T cell responses (thought to be a measure of protection) toward all four serotypes (Guy, *et al.*, 2008). This promising vaccine is currently in Phase IIb efficacy trials in a region of Thailand with high DENV incidence. Live-attenuated versions of DENV have also been

developed by serial passage in cell culture. One such vaccine, developed by WRAIR and subsequently licensed to GSK, induced tetravalent neutralizing antibody responses in a small pediatric cohort (Simasathien*, et al.*, 2008), but further development is currently on hold. The NIH attenuated DENV by deleting 30 nucleotides from the 3' UTR of DENV-1 and -4 and by replacing the preM and E coding sequences of the attenuated DENV-4 with those from DENV-2 or -3 (Men*, et al.*, 1996; Whitehead*, et al.*, 2003a; Whitehead*, et al.*, 2003b; Blaney*, et al.*, 2004). The monovalent vaccines were immunogenic in small cohorts of healthy American adults (Durbin*, et al.*, 2001; Durbin*, et al.*, 2005; Durbin*, et al.*, 2006a; Durbin*, et al.*, 2006b), and a tetravalent formulation containing all four viruses induced neutralizing antibodies to all four DENV serotypes in rhesus monkeys (Blaney*, et al.*, 2005). A Phase I clinical trial of this vaccine is currently underway.

F. T cell cross-reactivity

T cells 'see' antigen via their T cell receptors (TCR), which bind to class I or class II major histocompatibility complexes (MHC) presenting an immunogenic peptide (pMHC) usually 8-12 amino acids in length (reviewed in Janeway, 2005). TCR-pMHC binding is considered highly specific, and T cell coreceptors (i.e. CD4 and CD8) act to enhance this interaction through direct contact with the MHC itself. The concept of T cell cross-reactivity, or 'polyspecificity', refers to the ability of a T cell, expressing a single TCR that is defined as specific for a particular pMHC, to engage and respond to a different pMHC (Wucherpfennig*, et al.*, 2007). An increasing body of evidence indicates

that while TCR-pMHC interactions are specific (i.e. only recognize a fraction of available ligands), an extraordinary amount of cross-reactivity exists in nature. Mathematical calculations predict that a single TCR can bind at least 10^6 different pMHC complexes (Mason, 1998). The presence of such degeneracy is thought to be necessary to accommodate the myriad epitopes (~$5x10^{11}$ just for 9mer peptides) confronted throughout a human lifetime by the relatively limited number of T cells (~10^{12} total) that circulate in a given individual (Wucherpfennig, *et al.*, 2007).

T cell cross-reactivity can occur via a number of mechanisms related to the interaction between TCRs and pMHC molecules (Yin and Mariuzza, 2009). TCRs are heterodimers consisting of α and β chains, which are generated by rearrangement of multiple gene segments (including V, J, D, and C segments) and tested for self-reactivity by positive and negative selection in the thymus (Janeway, 2005). During gene rearrangement, TCRs are also subject to random insertions of nucleotides between the gene segments of each chain that serve to ultimately increase the diversity of the TCR repertoire. The most variable region of the TCR comprises the complementarity-determining region (CDR)3 loop, which sits in the middle of the pMHC binding site. The flexibility of the CDR3 loop allows for the TCR to adjust to fit the particular pMHC complex that is bound (Armstrong, *et al.*, 2008); this is the 'induced fit' mechanism of cross-reactivity (Yin and Mariuzza, 2009). Other mechanisms include differential TCR docking, structural degeneracy, molecular mimicry, and antigen-dependent tuning of pMHC flexibility. The variety of peptide ligands and the role of T cell cross-reactivity in immunity are discussed further below.

G. T cell signaling – an overview

The engagement of a TCR by a pMHC complex initiates a series of protein phosphorylation events that culminates into the T cell effector response (reviewed in Cisznak, *et al.,* 2007). The TCR is associated with the CD3 complex on the surface of T cells, which includes the γ, δ, ε, and ζ chains that contain immunoreceptor tyrosine-based activation motifs (ITAMs). Although the events that immediately follow TCR-pMHC engagement are still unclear, the ITAMs of the CD3 complex become phosphorylated (Figure 1.1). The extent of CD3ζ phosphorylation (CD3ζ is a homodimer, each with three ITAMs) determines the number of binding sites for Zap70, which harbors an SH2-domain. Upon binding, Zap70 becomes itself phosphorylated and thus activated. Activated Zap70 uses its kinase activity to subsequently phosphorylate and activate other proteins, including LAT and SLP-76, which continue the phosphorylation cascade. Two major pathways are involved, which ultimately activate transcription factors that will initiate the transcription of immune response genes. Phosphorylated SLP-76 activates phospholipase Cγ1 (PLCγ1), which cleaves phosphatidylinositol bisphosphate (PIP2) into diacylglycerol (DAG) and inositol trisphosphate (IP3). IP3 up-regulates the concentration of intracellular calcium, helping to activate calcineurin, which leads to NFAT activation. DAG together with calcium activates protein kinase C (PKC), leading to NFkB-mediated transcription. Phosphorylated SLP-76 also activates the MAP kinase pathway via Ras, leading to AP-1 activation. The interaction of these transcription factors with their target genes, as well as with each other, leads to an effector response

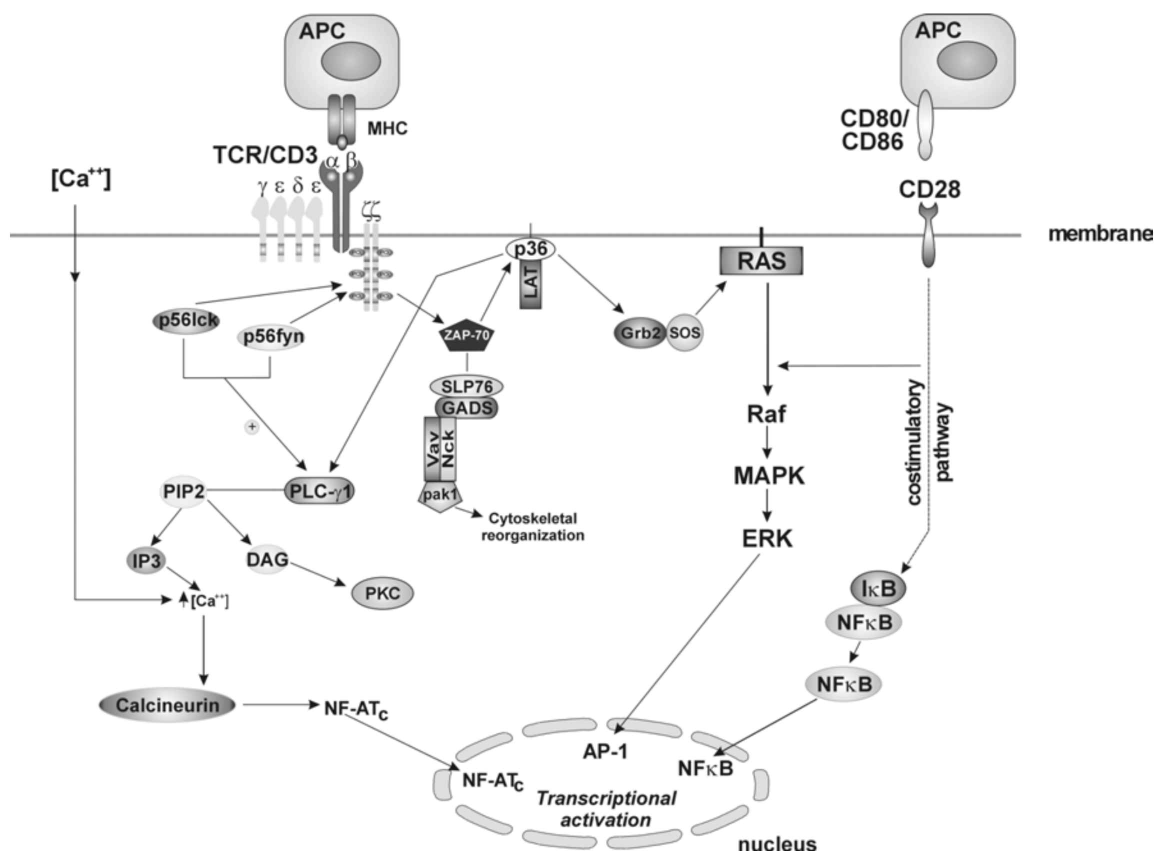

FIGURE 1.1. TCR signal transduction pathways.

Following TCR engagement, members of Src family kinases, Lck and Fyn, are activated, leading to the phosphorylation of tandemly arranged tyrosine residues within the ITAMs of the CD3 γ, δ, ε, and ζ chains. Phosphorylated ITAMs in the CD3 γ, δ, ε, and ζ chains function as docking sites for the recruitment of ZAP-70, which subsequently phosphorylates an adapter protein, such as LAT and SLP-76. The phosphorylation of adapter proteins initiates the Ras/Raf/MAPK (ERK) and PLCγ1/calcineurin/calcium pathways and promotes the formation of the SLP-76-Vav-Nck-Pak1 complex, which may be important in the regulation of cytoskeletal rearrangements. Sustained activation of these pathways is required for transactivation of the transcription factor AP-1 complex and NF-AT, leading to IL-2 production, T cell proliferation, and effector responses. LAT: linker for the activation of T cells; SLP76: Src homology 2 (SH2)-domain-containing leukocyte protein-76; PLC-γ1: phospholipase Cγ1; PIP2: phosphatidylinositol 4,5 bisphosphate; IP3: inositol 3,4,5 trisphosphate; PKC: protein kinase C; DAG: diacylglycerol; Grb2: growth-factor-receptor-bound protein 2; SOS: Ras GTPase guanine nucleotide exchange factor; GADS: Grb2-related adapter protein; MAPK: mitogen-activated protein kinase; ERK: extracellular signal-regulated kinases; NFATc: nuclear factor of activated T cells; AP-1: activating protein 1; NFκB: nuclear factor-κB; IκB: inhibitor of nuclear factor-κB.

This figure was reprinted with permission from Cisznak, et al., 2007.

specific for a given stimulus. Changes in the strength and/or type of signal, which involves not only TCR engagement but coreceptor engagement and cytokine signaling, can affect the T cell response.

H. Altered peptide ligands

The epitopes to which a particular T cell responds are numerous and can differ in their primary structure by one or many amino acids. Early biochemical studies used amino acid substitutions of immunogenic peptides to assess the contribution of particular amino acid positions to recognition by T cells. These substituted peptide variants were termed altered peptide ligands (APL) and shown to induce T cell responses that often differed from T cell recognition of the originally-identified, cognate peptide (Evavold, *et al.*, 1993a). For instance, APL stimulation of a $CD4^+$ T cell clone induced cytolysis and up-regulation of surface IL-2 receptors but not proliferation or cytokine production, as seen for the cognate ligand (Evavold, *et al.*, 1993b). In addition, viral isolates from chronically-infected patients demonstrated the dose-dependent capacity of naturally-derived APL to inhibit the cytotoxic T cell response elicited by the original epitope ligand (Bertoletti, *et al.*, 1994; Klenerman, *et al.*, 1994). Classes of APL were subsequently characterized according to the T cell response they elicited relative to the cognate peptide: agonists (those that elicited a similar response as the cognate ligand), super-agonists (those that elicited a stronger response), partial agonists (those that elicited a weaker or altered response), and antagonists (those that disrupted T cell responses).

The mechanism of partial agonism was found to encompass a "continuum of T cell signaling" (Evavold, *et al.*, 1993a), covering a spectrum between agonism and antagonism, which affected both the quality and quantitative threshold of TCR-mediated signaling. Partial agonist ligands impact early events in T cell signaling that propagate through to altered effector responses. For example, agonistic stimulation of a murine $CD4^+$ T cell clone resulted in full phosphorylation of CD3ζ that showed a band of about 21kDa on a Western Blot, whereas stimulation with a partial agonist resulted in a large band at 18kDa (indicating partial phosphorylation of the CD3ζ homodimer) and less of the 21kDa band (Sloan-Lancaster, *et al.*, 1994). This was confirmed by another group which also demonstrated that partial agonist stimulation of a murine $CD4^+$ T cell clone failed to phosphorylate CD3ε (Madrenas, *et al.*, 1995). Both groups found that these altered phosphorylation states affected the recruitment and activation of Zap70. A separate study showed that the intracellular calcium flux induced by partial agonist stimulation reached lower levels and was sustained for a shorter period of time compared to that measured after stimulation with an agonist ligand (Sloan-Lancaster, *et al.*, 1996). These results highlight the fact that partial agonists affect both the strength and type of signal they send through the TCR in order to fine tune their ultimate effect on T cell responses.

I. Sequential heterologous infections

Relatively recent data have demonstrated a significant role for cross-reactive T cells that recognize naturally-occurring APL in both murine models and human instances

of sequential infection with unrelated pathogens (Welsh*, et al.*, 2010). Mice immune to lymphocytic choriomeningitis virus (LCMV) or Pichinde virus (PV) clear virus more quickly upon subsequent infection with doses of vaccinia virus (VV) that are lethal in naïve mice (Selin*, et al.*, 1998). This protection was shown to be mediated by LCMV- or PV-specific memory T cells that responded to VV epitopes. Interestingly, while LCMV-immune mice quickly cleared VV upon challenge, they also developed acute fat necrosis (Yang*, et al.*, 1985; Selin*, et al.*, 1998), an immunopathological condition mediated by enhanced IFNγ levels circulating in these mice. This condition was not detected after VV challenge of PV-immune mice, however (Selin*, et al.*, 1998), indicating that not all cross-reactive T cell responses are equal.

In humans, human immunodeficiency virus (HIV) and hepatitis C virus (HCV)-specific T cell responses have been found in HIV and HCV seronegative individuals (Kaul*, et al.*, 2001; Wedemeyer*, et al.*, 2001). These analyses may be identifying cross-reactive memory T cells generated in response to previous, unrelated infections, which may mediate protection from HIV or HCV infection in high risk individuals (Welsh and Selin, 2002). In contrast, HCV patients with severe manifestations of disease were found to have a high frequency of epitope-specific $CD8^+$ T cells which were functionally cross-reactive with an influenza virus epitope (Urbani*, et al.*, 2005). These cross-reactive T cells were not detected in patients with milder disease, suggesting that reactivation of memory influenza virus-specific T cells during acute HCV infection contributes to immunopathology. Similarly, particular Epstein Barr virus (EBV)-specific T cell responses (compared to other EBV-, human cytomegalovirus- or influenza virus-specific

T cell responses) were found to be higher in patients with multiple sclerosis compared to healthy EBV-infected individuals (Lunemann*, et al.*, 2010).

In all of these cases, heterologous immunity mediated by T cells cross-reactive to unrelated viruses heavily influenced the outcome of infection. DENV has multiple closely-related serotypes which co-circulate, and infection results in a spectrum of clinical manifestations; therefore, it is easy to speculate that serotype-cross-reactive memory T cells play a role in shaping the course of disease.

J. Cytokines and disease

As T cells respond to an invading pathogen, they exhibit a range of effector functions, including cytotoxicity and cytokine production. These responses serve to eliminate the pathogen and prevent or mitigate symptoms of disease experienced by the host. However, as noted above, T cell effector responses themselves can sometimes cause or exacerbate disease. Such instances often occur due to unchecked cytokine production.

T cells produce multiple cytokines upon activation, including IFNγ, MIP-1β, and TNFα. IFNγ is one of the most common cytokines utilized to identify antigen-specific T cell responses. The expression of IFNγ often correlates with protection from viral or bacterial infections. For example, preexisting IFNγ responses to mycobacterial antigens correlated with protection from subsequent HIV-related tuberculosis in adults from Tanzania (Lahey, *et al.,* 2010). In contrast, during chronic HCV infection, $IFN\gamma^{+}CD8^{+}$ T cells contribute to the development of hepatitis (Thimme, *et al.,* 2001), similar to the

immunopathology observed after VV challenge of LCMV-immune mice (Yang, *et al.,* 1985; Selin, *et al.,* 1998), as mentioned above.

Macrophage inflammatory protein (MIP)-1β is a chemoattractant that recruits pro-inflammatory cells (e.g. T cells, macrophages, DCs) that express its receptor, CCR5, to the site of infection, thus orchestrating localized immune responses (Maurer and von Stebut, 2004). By way of binding CCR5, MIP-1β has also been shown to interfere with HIV infection, which uses CCR5 as a co-receptor for entry into target $CD4^+$ T cells (Lehner, 2002). The production of MIP-1β by T cells seems to require a low threshold for activation, leading some to suggest that it is a better marker of antigen-specific T cells than IFNγ (De Rosa, *et al.,* 2004; Betts, *et al.,* 2006).

TNFα can also contribute to both protective and immunopathologic responses. In general, TNFα helps contain localized infections (Janeway, 2005). However, systemic release of TNFα, or its overproduction, can lead to septic shock (Tracey and Cerami, 1993; Janeway, 2005). TNFα has also been implicated as a critical mediator of inflammatory responses in autoimmune diseases, such as rheumatoid arthritis (Maini, *et al.,* 1995). IFNγ, MIP-1β, and TNFα, together with IL-2 and other cytokines, contribute to the T cell response toward countless immunological insults. Whether individual cytokines will contribute to protection or immunopathology (or both) is determined by the timing, magnitude, location, and interaction of cytokine responses.

K. Identification of virus-specific memory T cells

In a typical acute infection, innate immunity first responds to non-specific features of the invading pathogen in an effort to slow its spread. Soon thereafter, the pathogen-specific adaptive immune response initiates and takes over to clear the pathogen from the host. One of the unique features of adaptive immunity is its ability to generate memory to particular pathogens. Memory T cells have lower thresholds for activation and are quicker to respond than naïve T cells (Pihlgren, *et al.*, 1996; Curtsinger, *et al.*, 1998; Cho, *et al.*, 1999; Veiga-Fernandes, *et al.*, 2000); thus, they are important for limiting the impact of recurrent pathogens over the lifetime of the host.

Identifying antigen-specific memory T cells, however, has been a challenge. Early studies indicated that different isoforms of CD45 could differentiate between naïve and memory T cell subsets. Specifically, CD45RA marked naïve T cells, whereas CD45RO was expressed by memory T cells (Young, *et al.*, 1997). Another study soon identified a lymph node homing marker, CCR7, which further stratified memory T cells into central memory ($CCR7^{+}CD45RA^{-}$) and effector memory ($CCR7^{-}CD45RA^{-}$) phenotypes (Sallusto, *et al.*, 1999). This same study also noticed that $CD8^{+}$ T cells had a third memory subset ($CCR7^{-}CD45RA^{+}$), which they and others suggested was terminally differentiated due to loss of the T cells' proliferative potential (Sallusto, *et al.*, 1999; Champagne, *et al.*, 2001). However, recent data indicated that these cells are, in fact, fully functional (Miller, *et al.*, 2008; Akondy, *et al.*, 2009). CD127, the IL-7 receptor α chain, has been used as a marker of quality memory after murine studies revealed its utility in identifying long-lived memory T cells (Kaech, *et al.*, 2003). Studies assessing

antigen-specific $CD8^+$ T cells over the course of acute infection and convalescence in humans indicated that CD45RA, CCR7, and CD127 were down-regulated to various extents during acute infection and were re-expressed in memory T cells following distinct kinetics (Miller, *et al.*, 2008; Akondy, *et al.*, 2009). Independent re-expression of these markers was suggested to reflect a gradual and continuous process of T cell differentiation into memory. While these markers are commonly utilized in studies characterizing memory T cells, their ability to identify specific T cell subsets appears to be dependent on the model employed for experimentation as well as the particular combination of these and other markers that are used. Thus, the strategy for identifying memory T cells continues to be refined.

L. Protective immune responses to DENV infection

Infection with one DENV serotype is thought to confer life-long protection from re-challenge with that same serotype. Mechanisms of immune protection are difficult to ascertain in DENV-infected patients due to the complexity of delineating protective versus pathologic immune responses, among other challenges. Nonetheless, analyses of patient sera have shown that the neutralizing antibody response comprises antibodies that block DENV attachment (He, *et al.*, 1995). This is consistent with findings in mice in which anti-E antibodies inhibit viral binding (Roehrig, *et al.*, 1998) and, when passively transferred to mice, provide protection from subsequent DENV challenge (Kaufman, *et al.*, 1987). Antibodies specific for preM and NS1 proteins have also been shown to

provide protection from DENV challenge in mouse models (Henchal, *et al.*, 1988; Kaufman, *et al.*, 1989).

The role of T cells in mediating protection from DENV infection is somewhat more complicated. DENV infection of IFNα/β receptor knockout mice demonstrated higher viral loads upon depletion of $CD8^+$ T cells (Yauch, *et al.*, 2009) but not after depletion of $CD4^+$ T cells (Yauch, *et al.*, 2010). Additionally, immunization of the mice with $CD8^+$ or $CD4^+$ epitopes enhanced viral clearance upon subsequent DENV challenge, supporting a protective role for T cells. A similar result was seen after lethal DENV challenge of DENV-immunized BALB/c mice, shown to provide 100% protection, which demonstrated increased mortality after depletion of $CD8^+$ T cells (Gil, *et al.*, 2009). However, a different mouse model (HepG2-grafted SCID) suggested that DENV-specific $CD8^+$ T cells have both protective and pathogenic roles (An, *et al.*, 2004). Specifically, mice inoculated with DENV-specific $CD8^+$ T cells and subsequently challenged with a lethal dose of DENV showed reduced mortality compared to uninoculated mice (80% versus 100%), but the mice that died did so much more quickly (day 12.8 versus day 17.4).

The studies above examined mechanisms of protective immunity in mouse models during primary DENV infection or upon homologous virus re-challenge. A different study considered the immune response to heterologous DENV infection and used IFNα/β and -γ receptor knockout mice to demonstrate with adoptive transfer experiments that serotype-cross-reactive antibody was more widely protective compared to cross-reactive cell-mediated immune responses (Kyle, *et al.*, 2008). In general, while

T cells likely contribute to protective immune responses by viral clearance and enhancement of B cell responses, the antibody response seems to play a greater role in mediating protection from DENV infection.

M. Immunopathological mechanisms in dengue pathogenesis

Severe dengue disease (DHF/DSS) is characterized by plasma leakage, a phenomenon that involves increasing the permeability of capillaries such that blood and plasma can leak into body cavities. However, pathological studies indicate that vascular endothelial cells are relatively structurally intact in DHF/DSS patients (Sahaphong, *et al.*, 1980; Kurane and Ennis, 1992). Since dengue patients typically experience a rapid recovery, it is thought that plasma leakage occurs due to endothelial cell malfunction rather than lysis of infected endothelial cells (Kurane and Ennis, 1992). Thus, DHF-associated plasma leakage is hypothesized to occur as a result of excessive cytokine levels circulating in DHF/DSS patients, which are produced by highly activated immune cells triggered by high viral loads (Mathew and Rothman, 2008). The precise etiology of this vascular leak syndrome is under intense investigation, which has led to two prevailing, but not mutually exclusive, hypotheses: antibody-dependent enhancement and altered cytokine production by serotype-cross-reactive T cells. Considering the markedly increased risk for severe disease in secondary heterologous DENV infection, these models assume a role for preexisting adaptive immune responses.

i. Antibody-dependent enhancement

Epidemiological studies on dengue in Thailand in the 1960s first noted two populations of patients that experienced DHF: infants with primary DENV infections and children with secondary DENV infections (Halstead*, et al.*, 1969; Nimmannitya*, et al.*, 1969; Halstead*, et al.*, 1970). Both groups would be expected to have preexisting DENV-specific antibodies, as infants born to DENV-immune mothers acquire maternally-derived antibodies *in utero* and children with secondary infections have antibodies from their first DENV infection. This led researchers to consider a pathogenic role for serotype-cross-reactive anti-DENV antibodies in facilitating severe dengue disease via antibody-dependent enhancement (ADE) (Halstead, 1970; Halstead and O'Rourke, 1977). ADE involves antigen-specific, though sub-neutralizing, antibodies which attach to the virus and increase uptake into Fc receptor-bearing cells, thereby increasing the viral load.

In vitro experiments that pre-incubated monocytes with immune sera containing anti-flavivirus antibodies, then infected the cells with DENV-2, demonstrated higher viral titers compared to infection without immune sera (Halstead*, et al.*, 1980). This was supported by later experiments showing *in vivo* antibody-mediated enhancement of DENV infection in rhesus monkeys (Goncalvez*, et al.*, 2007). In keeping with the decline in levels of passively-transferred maternal antibodies over time, clinical studies have demonstrated a correlation between the lowered neutralization activity of maternal antibody and the infant age at the time of DENV infection (Kliks*, et al.*, 1988; Libraty*, et al.*, 2009). The ability of antibody-containing sera to enhance *in vitro* infection of susceptible cells was also correlated with the age of onset of infant DHF (Chau*, et al.*,

2008; Libraty, *et al.*, 2009), supporting a role for ADE in DHF pathogenesis. In addition, some prospective studies noted a correlation between low preexisting neutralizing anti-DENV antibody titers (Endy, *et al.*, 2004), or high preexisting enhancing antibody titers (Kliks, *et al.*, 1989), and the severity of disease experienced during subsequent DENV infection of Thai children. However, a study that compared infants with DF to those with DHF detected no differences between these groups in either the neutralizing or enhancing activity of maternal antibody (Libraty, *et al.*, 2009). In support of this, a study assessing the enhancing activity of patient sera obtained prior to secondary infection detected no correlation with the severity of disease experienced by these individuals upon subsequent DENV infection (Laoprasopwattana, *et al.*, 2005). These data suggest that factors other than, or in addition to, ADE contribute to DHF pathogenesis.

ii. Altered cytokine production by serotype-cross-reactive T cells

Analysis of sera from DENV-infected patients revealed the presence of a number of immune cell activation markers. IFNγ and IL-2 were highly produced during DENV infection, and their levels in serum peaked just before the onset of plasma leakage in subjects with DHF (Kurane, *et al.*, 1991). Soluble IL-2 receptors were also found in the sera of dengue patients, with higher levels detected in those with DHF versus those with DF (Kurane, *et al.*, 1991; Green, *et al.*, 1999b; Libraty, *et al.*, 2002a). TNFα is a cytokine known to cause capillary leakage syndrome (Tracey and Cerami, 1993), and its levels were consistently higher in patients experiencing more severe dengue disease (Hober, *et al.*, 1993; Kuno and Bailey, 1994; Bethell, *et al.*, 1998; Green, *et al.*, 1999b).

Soluble TNFα receptor II was also more abundant in the sera from DHF versus DF patients, and its levels showed a positive correlation with the extent of plasma leakage and corresponding outcome of DSS versus DHF without shock (Hober, *et al.*, 1996; Bethell, *et al.*, 1998; Green, *et al.*, 1999b; Libraty, *et al.*, 2002a). These molecules implicated a role for immune cell activation in dengue pathogenesis, although their production could have originated from a variety of immune cell types. However, levels of soluble CD4 were elevated in the sera of DENV-infected individuals, implicating a specific role for $CD4^+$ T cells (Kurane, *et al.*, 1991). Soluble CD8 was significantly higher in those experiencing DHF compared to those with DF or those with other, non-dengue, febrile illnesses (Kurane, *et al.*, 1991; Green, *et al.*, 1999b). This suggested an expressly pathogenic function for $CD8^+$ T cells. A higher frequency of activated T cells ($CD69^+CD8^+$) was detected in patients with DHF versus DF (Green, *et al.*, 1999a). Taken together, the timing of these responses, with their peak coincident with viral clearance and one or two days before the onset of plasma leakage, implicated the immune response, and that specifically of T cells, in contributing to dengue pathology (Kurane and Ennis, 1992).

How T cells contribute to DHF pathogenesis is still a matter of debate, but investigators suggested that 'original antigenic sin' plays a role in secondary heterologous DENV infection (Mongkolsapaya, *et al.*, 2003; Welsh and Rothman, 2003). This concept, as it refers to T cells, implies that the DENV-specific memory T cell repertoire generated in response to a primary infection contains T cells with a range of avidities for the closely-related, heterologous DENV serotypes. Upon secondary

heterologous DENV infection, serotype-cross-reactive memory T cells are activated and mount an effector response. This response, however, is inadequate as it is dominated by presumably low avidity, serotype-cross-reactive T cells instead of high avidity serotype-specific T cells. An inadequate T cell response would result in an altered cytokine profile (e.g. unusually high TNFα production) that could contribute to dengue pathology.

To test the role of T cells in DENV infection, analysis of blood samples from DENV-immune individuals detected circulating T cells which proliferated and produced cytokines upon *in vitro* DENV stimulation (Kurane*, et al.*, 1989a). Importantly, these cells could also lyse target cells presenting DENV antigen from homologous or heterologous serotypes; this was seen at the bulk population and T cell clonal level, revealing the serotype-cross-reactive potential of both $CD4^+$ and $CD8^+$ T cells (Bukowski*, et al.*, 1989; Kurane*, et al.*, 1989b; Green*, et al.*, 1993; Dharakul*, et al.*, 1994; Livingston*, et al.*, 1995; Gagnon*, et al.*, 1996). These studies used peripheral blood mononuclear cells (PBMC) from subjects vaccinated with experimental live-attenuated DENV strains and noted that the response to stimulation with whole viral antigens of the homologous DENV serotype (i.e. the serotype with which the individual was immunized) was generally greater than the response to stimulation with heterologous serotypes. One report analyzed PBMC from subjects with naturally-acquired primary DENV infections and found that DENV-2 immune T cells responded best to DENV-2 with some cross-reactivity toward the other serotypes, whereas DENV-1 immune T cells proliferated equally well to DENV-1 and DENV-2 with some response toward DENV-3 and -4 (Sierra*, et al.*, 2002). This study highlighted the influence of distinct DENV serotypes on

the response of T cells and suggested that there may also be differences in the T cell response generated by subjects naturally exposed to DENV versus those exposed through vaccination.

Advancing technology allowed for the identification of multiple T cell epitopes in DENV (Livingston, *et al.*, 1995; Gagnon, *et al.*, 1996; Mathew, *et al.*, 1996; Mathew, *et al.*, 1998; Zivny, *et al.*, 1999; Appanna, *et al.*, 2007). Studies on PBMC from individuals immunized with experimental DENV vaccines or patients with natural secondary infection indicated that cytotoxic T lymphocyte responses were mainly directed against the more conserved non-structural viral proteins, in particular NS3 and NS1-2a, although some responses were detected against E and preM (Mathew, *et al.*, 1996; Mathew, *et al.*, 1998). A more recent study assessing responses to the entire DENV genome in PBMC from subjects with naturally-acquired secondary infections confirmed that the majority of T cell responses were directed at NS3 (Duangchinda, *et al.*, 2010). Epitope identification enabled investigators to determine the breadth and magnitude of particular T cell subsets that contribute to the global anti-DENV T cell response. Functional analyses of epitope-specific T cells confirmed the presence of serotype-specific and serotype-cross-reactive subsets (Livingston, *et al.*, 1995; Gagnon, *et al.*, 1996; Zivny, *et al.*, 1999), and one study suggested that the TCRs for these different epitope-specific T cell subsets may use distinct variable region gene segments (Livingston, *et al.*, 1995).

Supporting a role for altered cytokine responses in dengue pathology, a study using PBMC obtained after naturally-acquired primary DENV infections demonstrated that the production of TNFα after *in vitro* stimulation only occurred in those individuals

who were subsequently hospitalized during a secondary DENV infection (Mangada*, et al.*, 2002). On the other hand, individuals with broadly serotype-cross-reactive IFNγ responses tended to experience sub-clinical secondary infections. Whether such an alteration in effector response could occur due to the stimulation of epitope-specific T cells by an epitope variant from a heterologous serotype (i.e. naturally-occurring APL) prompted experiments that evaluated multiple effector functions in response to epitope variants. The ratio of IFNγ/TNFα production by $CD4^+$ T cells in subjects that received a live-attenuated DENV vaccine favored TNFα production when stimulated by heterologous serotypes compared to stimulation with the homologous serotype (Mangada and Rothman, 2005). The hierarchy of T cell effector responses (proliferation, cytokine production, degranulation and/or cytotoxicity) directed toward a particular epitope was found to differ depending on the individual, the serotype from which the epitope sequence was derived, and the epitope itself (Zivny*, et al.*, 1999; Bashyam*, et al.*, 2006; Mongkolsapaya*, et al.*, 2006; Imrie*, et al.*, 2007). These data supported studies assessing *ex vivo* effector responses of epitope-specific T cells in early convalescence, which demonstrated higher cytokine production than degranulation in serotype-cross-reactive versus serotype-specific T cells (Mongkolsapaya*, et al.*, 2006) and in T cells from subjects with DHF versus DF (Duangchinda*, et al.*, 2010). A similar pattern was observed in virus-stimulated PBMC from subjects who had primary DENV infection several years prior, which showed greater induction of regulatory cytokines (TGFβ and IL-10) after homologous stimulation versus an increased pro-inflammatory response (TNFα and IFNγ) after heterologous stimulation (Sierra*, et al.*, 2010). Such analyses

indicate that DENV-specific T cells exhibit a balanced response to re-challenge with the same serotype, but a skewed response to heterologous DENV infection, favoring excessive pro-inflammatory cytokine production, and may be a major source of circulating TNFα, or other supposed detrimental responses, in DHF.

The development of soluble peptide-MHC tetrameric complexes made possible the detection of epitope-specific T cells without reliance on a functional readout (Altman, *et al.*, 1996). As functional responses of DENV-specific T cells change according to the stimulus, staining with pMHC tetramers specific for epitopes encoded by the different DENV serotypes enabled assessment of serotype-cross-reactivity unrelated to function. Staining DENV-immune PBMC with multiple pMHC tetramer variants simultaneously demonstrated that, in some subjects with secondary infection, the majority of epitope-specific T cells bound tetramers containing peptides from DENV serotypes other than the currently-infecting serotype (Mongkolsapaya, *et al.*, 2003; Mongkolsapaya, *et al.*, 2006; Dong, *et al.*, 2007). These results were interpreted to indicate that the T cells which bound to heterologous pMHC tetramer variants were reactivated memory T cells generated during a previous DENV infection. This, together with the finding that DENV-specific T cell responses can differ according to the serotype used for stimulation, fit with the model of original antigenic sin.

To have a better understanding of how T cell responses relate to the onset of clinical symptoms, recent studies have used pMHC tetramer technology to investigate the kinetics of expansion and activation of DENV-specific T cells during acute infection and convalescence. Mongkolsapaya, *et al.* reported that the frequency of T cells specific for

an immunodominant HLA-A*1101-restricted epitope on the NS3 protein ($NS3_{133\text{-}142}$) peaked two weeks after the conclusion of fever (defervescence) in Thai children with secondary DENV infections (Mongkolsapaya, *et al.*, 2003). Similar findings were later reported by this group for a different DENV epitope as well (Mongkolsapaya, *et al.*, 2006). The peak frequency of A11-$NS3_{133}$-specific T cells occurred earlier (within days of defervescence) in a study of Vietnamese infants (Chau, *et al.*, 2008), raising the possibility of a difference in the kinetics of this response between different age groups or between primary and secondary infections. Differences in timing of sample collection might also obscure the time at which true T cell frequencies peak. Regardless, some groups noted an association between the magnitude of epitope-specific T cell responses and the severity of dengue disease (Zivna, *et al.*, 2002; Mongkolsapaya, *et al.*, 2003; Mongkolsapaya, *et al.*, 2006), while others did not (Simmons, *et al.*, 2005b; Chau, *et al.*, 2008; Dung, *et al.*, 2010). One study reported that neither epitope-specific nor total activated $CD8^+$ T cells were detected at all prior to the onset of clinical features of severe dengue disease (Dung, *et al.*, 2010), which led them to question the role of T cells in the induction of DHF. Studies with samples from more time points and greater numbers of subjects, including those differentiated by serology (i.e. primary versus secondary infection) and serotype of infection, will help to clarify any correlation of T cell responses with disease outcome.

While evidence points to the involvement of T cells in DENV infection, their role in disease pathogenesis remains unclear and many questions linger. Few studies have compared T cell responses in natural primary and secondary infections; only recently

Duangchinda, *et al.* distinguished between these two groups and found higher cytokine responses in secondary infection (Duangchinda*, et al.*, 2010). Studies have yet to address whether T cells are more activated or have a different phenotype in primary versus secondary infection. In addition, no study has evaluated T cell serotype-cross-reactivity in primary infection to determine whether heterologous tetramer binding is the result of original antigenic sin. Furthermore, in-depth characterization of DENV-specific T cell lines has been largely limited to those isolated from vaccine donors or subjects with secondary infections. T cells from subjects with natural primary infection, however, are those in position to respond in secondary heterologous infection (a risk factor for severe disease). Therefore, detailed analysis of these cells will help us to understand their potential role in dengue disease.

N. Thesis objectives

Due to their low threshold for activation and rapid response time relative to naïve T cells, memory $CD8^+$ T cells generated by a primary DENV infection are primed to respond during a subsequent DENV infection. This thesis sought to characterize this ‘first generation’ DENV-specific T cell response to natural infection and to reveal its capacity for serotype-cross-reactivity upon subsequent DENV exposure. This work helps to identify the differences in the phenotype and function of T cells generated in primary infection to those generated in secondary infection and provides insight into the mechanism(s) of T cell involvement in the development of severe dengue disease.

We hypothesized that:

a) **DENV-specific memory T cells generated after primary DENV infection differ from those detected after secondary infection by way of frequency, extent of cross-reactivity, and phenotype (e.g. activation, effector/memory)**
b) **serotype-cross-reactive memory T cells detected after primary infection respond differently to heterologous compared to homologous stimulation (e.g. cytokine responses versus cytotoxicity, qualitatively different TCR-mediated signaling events)**
c) **qualitatively distinct T cell subsets contribute to an immunopathological outcome**

This work is presented in two parts:

CHAPTER III: Striking Cross-reactivity and Expansion of Dengue Virus-specific $CD8^+$ T cells During Acute Primary and Secondary Infections in Humans

Questions:

- Are T cell responses to primary DENV infection predominantly serotype-specific, in contrast to secondary DENV infection?
- Do T cell responses to different epitopes follow the same kinetics within individual subjects?
- How do the characteristics of the DENV-specific T cell response (e.g. magnitude, cross-reactivity, or activation) correlate with disease severity?

Approach:

- Stain PBMC from DENV-infected subjects (obtained throughout acute infection and convalescence) with multiple DENV serotype-specific epitope variants together with a panel of activation and phenotypic markers
- Perform *ex vivo* stimulation of DENV-immune PBMC with epitope variants to measure degranulation and cytokine production by T cells

CHAPTER IV: Memory $CD8^+$ T cells from Subjects with Naturally-acquired Primary Dengue Virus Infection are Functionally Cross-reactive

Questions:

- What types of T cells comprise the A11-$NS3_{133}$ epitope-specific T cell repertoire?
- Do different epitope variants induce variable effector responses from T cell clones?
- Do signaling events downstream of peptide stimulation correlate with variable effector responses?

Approach:

- Stimulate PBMC with A11-$NS3_{133}$ peptide variants and isolate epitope-specific $CD8^+$ T cell clones
- Assess serotype-cross-reactivity by pMHC tetramer binding, cytotoxicity, and cytokine responses
- Compare T cell signaling and effector function of T cells after stimulation with the different serotype-specific epitope variants

CHAPTER II

MATERIALS AND METHODS

A. Patient recruitment and collection of blood samples

Blood samples were collected from subjects enrolled in an ongoing clinical study of acute DENV infections in Thailand; the study design has been reported in detail elsewhere (Kalayanarooj, *et al.*, 1997; Vaughn, *et al.*, 1997). Briefly, the subjects enrolled in the study were Thai children 6 months to 14 years of age with acute febrile illnesses. Serology and virus isolation from acute phase plasma were used to confirm acute DENV infections, and primary and secondary infections were distinguished based on serologic responses (primary infection = IgM:IgG ratio ≥1.8:1 and hemagglutination-inhibition [HAI] antibody titer ≤1:1280; secondary infection = IgM:IgG ratio <1.8:1 and HAI antibody titer >1:1280). The serotype of infection was determined by ELISA, using monoclonal antibodies specific for E protein from the different DENV serotypes and supernatants from C6/36 cells infected with virus isolated from acute patient samples (Vaughn, *et al.,* 1997). Blood samples were obtained daily during acute illness, once in early convalescence (approximately 10 days after enrollment, corresponding to 5-7 days after defervescence), and at 6-12 month intervals during late convalescence (6 months to 3 years after study entry). Time points are reported relative to the day of defervescence (i.e. the day at which fever dissipated and the patient subsequently maintained a temperature below 38°C), which was termed fever day 0 (d0). Fever days -1, -2, *etc.* occurred before defervescence, and day +1, *etc.* occurred after defervescence. Written

informed consent was obtained from each subject and/or his/her parent or guardian and the study design was approved by the Institutional Review Boards of the Thai Ministry of Public Health, the Office of the U.S. Army General and the University of Massachusetts Medical School. PBMC were isolated by density gradient centrifugation, cryopreserved, and stored in liquid nitrogen. Frozen PBMC were shipped on dry ice to the University of Massachusetts Medical School for analysis. HLA typing was performed on blood obtained 6 months or more after acute illness at the University of Massachusetts Medical School or the Department of Transfusion Medicine, Siriraj Hospital, as previously described (Mathew, *et al.*, 1998; Stephens, *et al.*, 2002). We also used blood samples from a U.S. volunteer obtained 8 months after vaccination with an experimental live-attenuated monovalent DENV vaccine (DEN-1 45AZ5), as previously described (Green, *et al.*, 1993).

B. Peptides and generation of peptide-MHC tetramers

Peptides specific for three variants of the HLA-A*1101-restricted DENV $NS3_{133-142}$ epitope (Mongkolsapaya, *et al.*, 2003) were synthesized at >90% purity from AnaSpec, Inc. (San Jose, CA, USA). Peptide-MHC (pMHC) tetramers of these epitope variants were generated at the University of Massachusetts Medical School Tetramer Core as previously described (Luzuriaga, *et al.*, 2000). Biotinylated pMHC monomers were mixed with the respective avidin-conjugated fluorochrome at a molar ratio of 4:1 to form tetramers. Multimers were also made (mixed at molar ratios of 25:1 and 50:1, respectively) for the HLA-A*0201-restricted $M1_{58-66}$ influenza epitope

Table 2.1. Summary of epitope sequence information.

Epitope	HLA restriction	Serotype	Sequence	Designation[a]
DENV $NS3_{133\text{-}142}$[b]	A*1101	DENV-1	GTSGSPIVNR	pD1
		DENV-2	GTSGSPIVDR	pD2
		DENV-3, -4	GTSGSPIINR	pD3/4
DENV $NS3_{222\text{-}231}$[c]	B*07	DENV-2, -3, -4	APTRVVAAEM	B7-DENV
Influenza $M1_{58\text{-}66}$[d]	A*0201	n/a	GILGFVFTL	A2-Flu

[a]*As used in this paper*

[b]*Identified by Mongkolsapaya, et al., 2003*

[c]*Identified by Mathew, et al., 1998*

[d]*Identified by Bednarek, et al., 1991 and Morrison, et al., 1992*

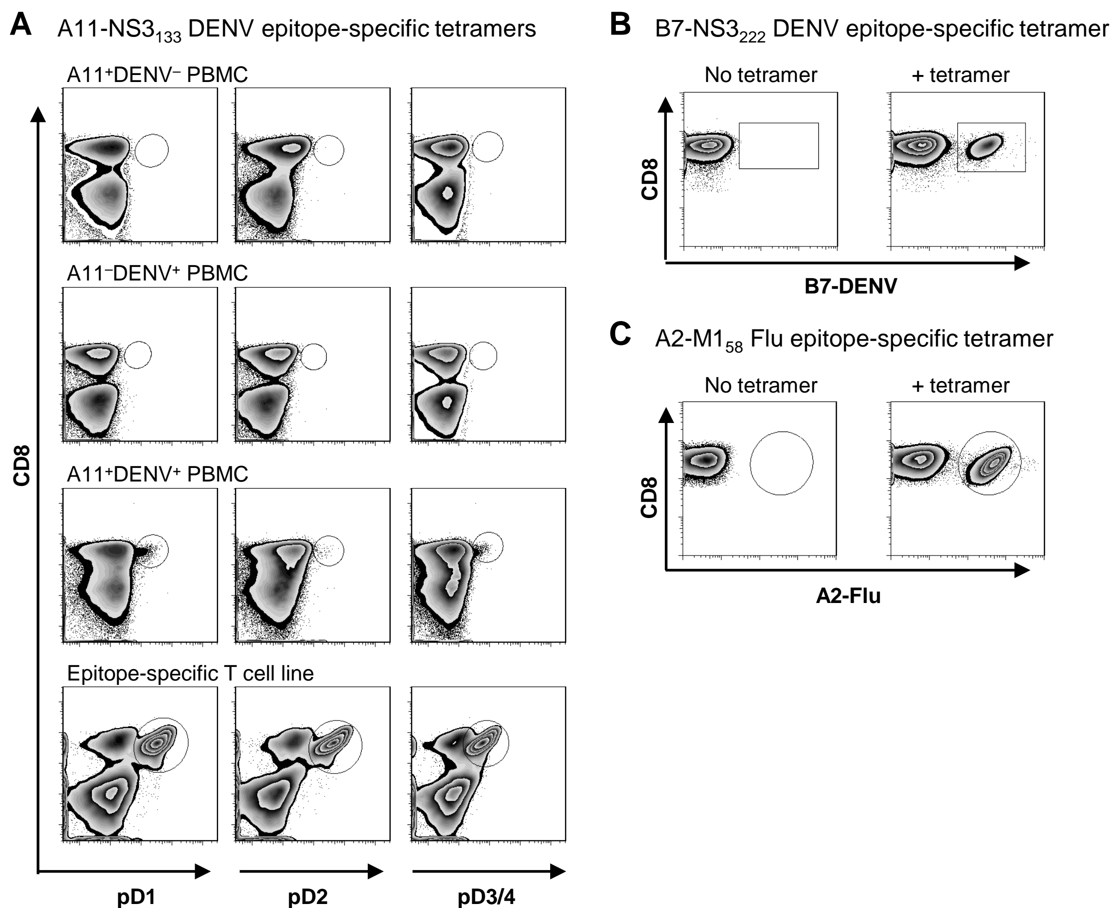

FIGURE 2.1. Specificity of pMHC tetramers generated for this study.

(A) Peptide-MHC tetramers specific for each of three A11-$NS3_{133}$ DENV epitope variants were generated. Tetramer specificity was verified by staining PBMC from HLA-A*1101^{+} DENV-naïve, HLA-A*1101^{-} DENV-immune, and HLA-A*1101^{+} DENV-immune subjects as well as an epitope-specific T cell line. Cells were gated on live, $CD3^{+}$ singlet lymphocytes. (B) A B7-$NS3_{222}$ DENV epitope-specific T cell line was established previously (Zivna, *et al.,* 2002) and was spiked into a non-specific T cell line to ensure pMHC tetramer specificity. (C) An A2-$M1_{58}$ Flu epitope-specific T cell clone was established previously (Jameson*, et al.,* 1998) and was spiked into a non-specific T cell line to ensure pMHC tetramer specificity. Cells shown in (B) and (C) were gated on live, $CD3^{+}CD8^{+}$ singlet lymphocytes.

(Bednarek*, et al.*, 1991; Morrison*, et al.*, 1992) and the HLA-B*07-restricted DENV $NS3_{222\text{-}231}$ epitope (Mathew*, et al.*, 1998). The different pMHC complexes were conjugated to distinct fluorochromes to allow for staining with multiple epitopes/variants simultaneously (APC-A11-$NS3_{133}$-pD1, PE-Cy7-A11-$NS3_{133}$-pD2, PE-A11-$NS3_{133}$-pD3/4, Qdot-605-A2-Flu, Qdot-605-B7-$NS3_{222}$). Peptide sequences are shown in Table 2.1; the specificity of each pMHC multimer (from here on all are referred to as tetramers, for simplicity) was confirmed as shown in Figure 2.1.

C. Tetramer staining and phenotypic characterization of PBMC

Cryopreserved PBMC were thawed and washed in RPMI before resting in RPMI/10% FBS at 37°C for approximately 2 hours. Cells were washed in PBS and stained with the dead cell marker LIVE/DEAD Aqua (Molecular Probes, Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were then washed and incubated with 0.5-2μL pMHC tetramer for 20 minutes at 4°C. Monoclonal antibodies (mAbs) specific for CD3 (clone UCHT1, Alexa Fluor 700 or clone SK7, PerCP-Cy5.5; BD Biosciences, San Jose, CA, USA), CD8 (clone SK1, PerCP-Cy5.5 from BD Biosciences or clone 3B5, Qdot-655 from Invitrogen Corp. or clone RAP-T8, Alexa Fluor 700 from BD Biosciences), CD45RA (clone HI100, FITC; BD Biosciences), CCR7 (clone 3D12, APC-eFluor 780; eBioscience, San Diego, CA, USA), CD127 (clone eBioRDR5, Pacific Blue from eBioscience), CD38 (clone HIT2 conjugated to Qdot-655 from Invitrogen Corp. or to PerCP-Cy5.5 from BD Biosciences) and/or CD69 (clone FN50, APC-Cy7; BD Biosciences) were then added to the cells and incubated at 4°C for

Table 2.2. List of antibodies/markers used in 10 (or 8)-color staining panels for *ex vivo* analysis of DENV-immune PBMC.

Fluorochrome /channel	Panel 1A	Panel 1B	Panel 2	Panel 3
APC	pD1 tetramer	pD1 tetramer	pD1 tetramer	pD1 tetramer
PE-Cy7	pD2 tetramer	pD2 tetramer	pD2 tetramer	--
PE	pD3/4 tetramer	pD3/4 tetramer	pD3/4 tetramer	--
LIVE/DEAD Aqua	Dead cells	Dead cells	Dead cells	Dead cells
AlexaFluor 700	CD3	CD3	Granzyme B	CD8
PerCP-Cy5.5	CD8	CD38	CD3	CD38
FITC	CD45RA	CD45RA	Ki-67	CD45RA
APC-eFluor 780	CCR7	CCR7	CD69[a]	CCR7
Pacific Blue	CD127	CD127	Bcl-2[b]	CD127
Qdot-655	CD38	CD8	CD8	pMHC multimer[c]

[a]*The antibody for CD69 was conjugated to APC-Cy7*

[b]*The antibody for Bcl-2 was conjugated to AlexaFluor 405*

[c]*The B7-NS3*$_{222}$ *DENV and A2-M1*$_{58}$ *Flu pMHC multimers were conjugated to Qdot-605*

an additional 30 minutes. Cells were washed and either fixed with BD Stabilizing Fixative (BD Biosciences) or permeabilized with BD Cytofix/Cytoperm (BD Biosciences) for 20 minutes at 4°C. After permeabilization, cells were washed and incubated with mAbs against Ki-67 (sc-23900, FITC; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bcl-2 (sc-7382, Alexa Fluor 405; Santa Cruz Biotechnology), and granzyme B (clone GB11, Alexa Fluor 700; BD Biosciences) for 30 minutes at 4°C. Finally, cells were washed and fixed as above. Data were collected on a BD FACSAria and analyzed using FlowJo version 7.5.5. Table 2.2 lists the antibodies/markers used for each staining panel.

D. Bulk culture and cloning of PBMC and maintenance of T cell lines

Cryopreserved PBMC were thawed and stimulated *in vitro* with 10μg/mL peptide in complete RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 5ng/mL IL-7. Cells were fed with RPMI/10% FBS medium containing 50U/mL IL-2 on day 3 and every 2-3 days thereafter. After 2 to 3 weeks, T cells were positively selected using the tetramer specific for the DENV-1 variant of the A11-$NS3_{133}$ epitope together with MACS α-allophycocyanin (APC) microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). T cell lines were then generated by limiting dilution (3 or 10 cells/well) and maintained by biweekly re-stimulation with α-CD3, allogeneic irradiated PBMC, and 50U/mL IL-2.

E. Tetramer staining of T cell lines

Approximately 0.1–1x10^6 cells were stained with a dead cell marker (LIVE/DEAD Aqua; Molecular Probes, Invitrogen Corp.) according to the manufacturer's instructions. After washing, cells were stained with 0.5-2μL of each tetramer for 20 minutes at 4°C before addition of mAb directed at the surface markers CD3 (clone UCHT1, Alexa Fluor 700; BD Biosciences), CD8 (clone SK1, PerCP-Cy5.5; BD Biosciences), and CD4 (clone OKT4, Pacific Blue; eBioscience) for 30 minutes at 4°C. Cells were washed and placed in BD Cytofix or BD Stabilizing Fixative until analysis. Data were collected on a BD FACSAria and analyzed using FlowJo version 7.2.2 or 7.2.4.

F. Cytotoxicity assay

Cytotoxicity was assessed as previously described (Mathew*, et al.*, 1998). Briefly, HLA-A*1101$^+$ B-lymphoblastoid cell line (BLCL) targets were labeled with ^{51}Cr for 1 hour, washed, and pulsed with 0.01 – 10μg/mL of the indicated peptide. Target cells were cultured with T cells at an effector-to-target ratio of 10:1. Wells containing targets with no effectors were used to determine background ('minimum') ^{51}Cr release; wells containing targets with no effectors but with detergent (to lyse the target cells) were used to determine 'maximum' ^{51}Cr release. After 4 hours, supernatants were harvested and ^{51}Cr content was measured in a gamma counter. Percent specific lysis was calculated by the following formula: % lysis = (experimental ^{51}Cr release – minimum ^{51}Cr release) / (maximum ^{51}Cr release – minimum ^{51}Cr release) x 100.

G. Intracellular cytokine staining assay

PBMC or T cells ($2x10^5$) and HLA-A*1101$^+$ BLCL ($2x10^4$) were cultured with 0.01 – 10μg/mL peptide, anti-CD107a (clone H4A3, FITC; BD Biosciences), brefeldin A and monensin (BD GolgiPlug and BD GolgiStop; BD Biosciences) for 6 hours. Cells were washed and stained with the viability marker LIVE/DEAD Aqua before subsequent staining with mAbs specific for CD3 (clone SK7, APC-H7; BD Biosciences), CD14 (clone SJ25C1, PE-Cy7; BD Biosciences), CD19 (clone M5E2, PE-Cy7; BD Biosciences), CD8 (clone SK1, PerCP-Cy5.5; BD Biosciences), and CD4 (clone OKT4, Pacific Blue; eBioscience) at 4°C for 30 minutes. After washing, the cells were fixed and permeabilized (BD Cytofix/Cytoperm; BD Biosciences) before staining with mAbs specific for IFNγ (clone B27, Alexa Fluor 700; BD Biosciences), MIP-1β (clone D21-1351, PE; BD Biosciences), and TNFα (clone Mab11, APC; BD Biosciences). Cells were washed again and placed in fixative until data collection.

H. Drug inhibition studies

Intracellular cytokine staining assays were performed as above but included pre-treatment of T cells with cyclosporine A (CSA). Prior to stimulation, T cells (together with BLCL) were incubated with 0.01-1μM CSA for 30 minutes at 37°C. Cells were then washed and incubated in the presence of peptide, anti-CD107a and protein transport inhibitors as above.

I. Phosphoflow signaling assay

T cell receptor-induced signaling events were analyzed by flow cytometry as described (Perez, *et al.*, 2005) and modifying the methods detailed by BD Biosciences (Techniques for Phospho Protein Analysis Application Handbook, First edition, <www.bdbiosciences.com>). Briefly, $4x10^5$ HLA-A*1101$^+$ BLCL and $2x10^5$ T cells were pulsed with 0.01 – 10μg/mL peptide for 30 minutes on ice. Cells were then washed and placed in 1% FBS in PBS for 3 or 10 minutes in a 37ºC water bath. Cells were immediately fixed (Phosflow Fix Buffer I; BD Biosciences), washed, and then permeabilized (Phosflow Perm Buffer III; BD Biosciences) on ice for 30 minutes. After washing, cells were blocked with mouse IgG for 10 minutes prior to addition of mAbs specific for CD3 (clone SK7, PerCP-Cy5.5; BD Biosciences), CD4 (clone RPA-T4, APC; BD Biosciences), CD8 (clone RPA-T8, PE; BD Biosciences), and phosphorylated CD3ζ (clone K25-407.69, Alexa Fluor 488; BD Biosciences) or phosphorylated ERK1/2 (clone 20A, Alexa Fluor 488; BD Biosciences) for an additional 30 minutes at room temperature. Cells were washed and placed in fixative until data collection.

J. V_β analysis

Flow cytometric analysis (surface staining) of V_β usage for epitope-specific TCRs was performed using the IOTest Beta Mark TCR V_β Repertoire kit (Beckman Coulter, Brea, CA, USA). Briefly, the protocol was the same as that detailed above for tetramer staining of T cell lines but included the addition of 20μL of the respective V_β staining

vial along with the mAbs against CD3, CD8 and CD4 before incubating at 4°C for 30 minutes.

K. Peptide-MHC binding prediction analysis

MHC class I binding prediction scores were calculated for the three A11-$NS3_{133}$ epitope variants used in this study according to three prediction models provided by the Immune Epitope Database and Analysis Resource website (<http://tools.immuneepitope.org/main/html/tcell_tools.html>).

L. CD137 expression analysis

Approximately $2x10^5$ T cells were cultured together with $2x10^4$ HLA-A*1101$^+$ BLCL and 10μg/mL peptide in RPMI/10% FBS at 37°C for 0-24 hours. At T-30min, cells were washed in PBS and stained with LIVE/DEAD Aqua as above, then fixed (Phosflow Fix Buffer I; BD Biosciences) and placed at 4°C until all time points were collected. At the end of the time course, all of the cells were washed and stained with mAbs against CD3 (clone UCHT1, V450; BD Biosciences), CD8 (clone RPA-T8, Alexa Fluor 700; BD Biosciences), CD4 (clone RPA-T4, APC; BD Biosciences), and CD137 (clone 4B4-1, PE; BD Biosciences) for 30 minutes at room temperature. Finally, cells were washed and placed in fixative until data collection.

M. Statistical analysis

For variables with a normal distribution, t-tests were used to compare continuous outcomes at a single time point between two groups and random intercept models were used to make comparisons between multiple groups across time points with the assumption that each individual within a group follows the same slope. For these models, missing values in the CD38 and CD45RA data sets were imputed with the means of all donors at each time point. For variables that were not normally distributed, the Mann-Whitney rank sum test was used to make comparisons between two groups and Friedman's test was used to make comparisons between three or more groups. Correlations between skewed variables were determined using Spearman's rank correlation. Fischer's exact test was used to compare categorical data. P values of ≤ 0.05 were considered significant; p values of ≤ 0.1 but >0.05 were considered indicative of non-significant trends. All statistical analyses were performed with help from James Potts, using Stata Intercooled version 9 (Stata Corporation, College Station, TX, USA) and GraphPad Prism (La Jolla, CA, USA) software packages.

CHAPTER III

STRIKING CROSS-REACTIVITY AND EXPANSION OF DENGUE VIRUS-SPECIFIC $CD8^+$ T CELLS DURING ACUTE PRIMARY AND SECONDARY INFECTIONS IN HUMANS

Various studies have explored the immunopathogenic role of cross-reactive memory T and B cells in DENV infection utilizing blood samples from DENV-infected human subjects. A high percentage of DENV-specific T cells recognize multiple DENV serotypes (Bashyam, *et al.,* 2006; Dong, *et al.,* 2007; Imrie, *et al.,* 2007; Mangada and Rothman, 2005; Mongkolsapaya, *et al.,* 2003; Mongkolsapaya, *et al.,* 2006; Moran, *et al.,* 2008; Sierra, *et al.,* 2002; Zivny, *et al.,* 1999), and patients with more severe disease (DHF) have been shown to have higher overall levels of T cell activation *in vivo* than patients with milder disease (DF) (Green, *et al.,* 1999a; Green, *et al.,* 1999b; Green, *et al.,* 1999c; Kurane, *et al.,* 1991; Libraty, *et al.,* 2002a; Perez, *et al.,* 2004). A few studies have reported a higher frequency of DENV epitope-specific T cells in patients with DHF (Mongkolsapaya, *et al.,* 2003; Mongkolsapaya, *et al.,* 2006; Zivna, *et al.,* 2002), but other studies have found no association and have questioned the timing of T cell activation (Chau, *et al.,* 2008; Dung, *et al.,* 2010). Comparison of these divergent studies is complicated by differences in methods, study populations, infecting viruses and the T cell epitopes studied. However, none of the previous reports have incorporated a comparison of responses to multiple epitopes in cohorts with naturally-acquired primary and secondary infection in association with data on outcome of infection.

We therefore took advantage of a long-standing collaboration between our laboratory, the Queen Sirikit National Institute of Child Health, and the Armed Forces Research Institute of Medical Sciences, both of which are in Thailand, which established a longitudinal study to follow children with naturally-acquired primary and secondary DENV infections (Kalayanarooj*, et al.*, 1997; Vaughn*, et al.*, 1997). Blood samples were obtained from patients throughout acute infection and up to three years post-defervescence. We selected subjects who were positive for HLA-A11 and/or HLA-B7 to analyze the frequency, serotype-cross-reactivity and phenotype of T cells specific for two previously identified immunodominant DENV epitopes.

A. T cells specific for an immunodominant HLA-A11-restricted DENV epitope expand during acute infection

To compare the magnitude and kinetics of epitope-specific $CD8^+$ T cells in patients with primary or secondary DENV infection, we initially focused on subjects with HLA-A11, an allele common in the Thai population (Chandanayingyong*, et al.*, 1997), for which an immunodominant epitope ($NS3_{133-142}$) had been previously reported (Mongkolsapaya*, et al.*, 2003). Our study cohort included 44 $HLA\text{-}A^*1101^+$ Thai children including 17 with primary DENV infection and 27 with secondary DENV infection (Table 3.1). The cohort included cases of infection with all four DENV serotypes; however, there was only 1 subject with DENV-4 infection (a secondary infection) and only 7 with DENV-2 infection (5 of which were secondary infections). To distinguish serotype-cross-reactive T cells, we generated three A11-$NS3_{133}$ pMHC

Table 3.1. List of HLA-A*1101$^+$ and HLA-B*07$^+$ subjects and clinical information.

Subject	Serology[a]	Serotype[b]	Diagnosis[c]	MHC Class I Haplotype A	MHC Class I Haplotype B	MHC Class I Haplotype C	Peak Tet$^+$ Frequency[d] (%)	PEI[e]	Change in Hematocrit[f] (%)	Max. AST[g] (IU/L)	Min. Platelet[h] (cells/mm^3)
C94127	1°	DENV-1	DF	11,33	44,61	7	0.19	0	17.65	49	104
C95017	1°	DENV-1	DF	11,24	38,75	7,1	0.18	0	17.14	32	157
C95031	1°	DENV-1	DF	11,33	58,60	3,7	nd[i]	0	13.51	172	146
C95039	1°	DENV-1	DF	1,11	56,57	6,7	19.13	0	40.74	146	240
C96040	1°	DENV-1	DF	11	62	6	0.37	0	18.92	41	250
C00015	1°	DENV-1	DF	11,24	7,60	nd	nd	0	15.79	55	114
C96041	1°	DENV-1	DHF II	11	51,62	4	0.52	20	59.38	1253	17
C00002	1°	DENV-2	DF	2,11	13,61	nd	0.68	0	11.43	38	243
C01030	1°	DENV-2	DF	11,33	13,58	nd	0.18	0	14.29	37	238
C95059	1°	DENV-3	DF	11,24	27,54	1,7	0.91	0	11.11	41	179
C96062	1°	DENV-3	DF	2,11	62,46	1,6	0.35	0	22.06	56	142
C96106	1°	DENV-3	DF	11,33	7,51	nd	0.74	na[j]	na	na	na
C97035	1°	DENV-3	DF	11,24	39,51	nd	1.22	0	42.42	36	80
C99091	1°	DENV-3	DF	2,11	18,62	nd	1.29	0	25	322	91
C95054	1°	DENV-3	DHF I	2,11	13,38	7	1.34	2.12	15.63	51	152
C95089	1°	DENV-3	DHF II	11,24	18,52	7,1	2.27	2.88	20	58	173
C96025	1°	DENV-3	DHF II	11,26	51,60	3	12.77	na	na	na	na
C95090	2°	DENV-1	DF	2,11	46	1	1.56	0	17.65	33	157
C96089	2°	DENV-1	DF	11,24	7,60	7	2.32	0	13.16	55	174
C97025	2°	DENV-1	DF	11	70,75	1	17.32	0	17.65	109	71
C97047	2°	DENV-1	DF	11	46	nd	0.67	0	10.81	141	76
C97141	2°	DENV-1	DF	2,11	61,75	nd	nd	0	18.42	56	95
C96069	2°	DENV-1	DHF II	11,24	18,55	1,7	0.25	25	32.35	160	11
C97024	2°	DENV-1	DHF III	11	7,38	nd	6.95	16.66	21.21	347	49

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Table 3.1. List of HLA-A*1101$^+$ and HLA-B*07$^+$ subjects and clinical information. (*cont'd*)

Subject	Serology[a]	Serotype[b]	Diagnosis[c]	MHC Class I Haplotype A	B	C	Peak Tet$^+$ Frequency[d] (%)	PEI[e]	Change in Hematocrit[f] (%)	Max. AST[g] (IU/L)	Min. Platelet[h] (cells/mm^3)
K95011	2°	DENV-1	DHF I	11	13	3	0.15	21.05	34.55	176	45
C01017	2°	DENV-1	DHF II	11,29	7,75	nd	0.57	4.67	17.65	109	46
C96088	2°	DENV-1	DHF II	2,11	38,55	1,7	0.79	21.69	20.51	623	20
C96094	2°	DENV-1	DHF II	11,24	54,63	1,7	0.64	12.26	25	91	93
C01010	2°	DENV-2	DF	11,11	7,27	nd	0.83	0	8.57	363	176
C02066	2°	DENV-2	DF	11,24	13,27	nd	1.09	2.15	14.29	44	146
C00001	2°	DENV-2	DHF II	11,24	48,75	nd	0.62	19.98	17.95	56	29
C01078	2°	DENV-2	DHF II	1,11	37,58	nd	nd	nd	21.62	21	82
C02063	2°	DENV-2	DHF III	11,24	35,51	nd	nd	61.38	27.5	627	36
C95075	2°	DENV-3	DF	11,30	13	3	0.57	na	na	na	na
C96016	2°	DENV-3	DF	11,30	13,46	1,6	2.15	0	13.51	81	93
C99014	2°	DENV-3	DF	11,33	7,44	nd	nd	0	16.22	131	72
C00026	2°	DENV-3	DF	11,33	7,60	nd	0.51	0	18.42	97	70
C96045	2°	DENV-3	DHF II	11,24	13,62	3,6	2.19	24.56	32.5	187	7
C97019	2°	DENV-3	DHF III	2,11	35,51	4	0.14	37.35	27.78	709	22
C97027	2°	DENV-3	DHF II	11,26	60	nd	0.41	2.08	19.44	138	64
C97036	2°	DENV-3	DHF II	11,33	7,75	6	nd	1.03	72.41	438	46
C97044	2°	DENV-3	DHF II	2,11	13,75	nd	0.45	9.3	16.67	107	43
K95005	2°	DENV-3	DHF I	2,11	35,38	4,7	2.30	3.96	9.59	95	164
C00025	2°	DENV-4	DF	11	75	nd	1.55	0	16.22	69	57
Subjects on whom only B7-NS3$_{222}$ tetramer analysis was done:											
C95029	1°	DENV-3	DF	24,34	7,75	1,6	0.23	0	40.63	50	186
C95098	2°	DENV-1	DHF II	29	7,61	3	7.35	0	27.03	301	48

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Table 3.1. List of HLA-A*1101⁺ and HLA-B*07⁺ subjects and clinical information. (*cont'd*)

Subject	Serology[a]	Serotype[b]	Diagnosis[c]	MHC Class I Haplotype A	B	C	Peak Tet⁺ Frequency[d] (%)	PEI[e]	Change in Hematocrit[f] (%)	Max. AST[g] (IU/L)	Min. Platelet[h] (cells/mm³)
K94024	2º	DENV-2	DHF III	2,24	7,46	1,7	8.12	16.26	43.66	130	28
C94115	2º	DENV-3	DF	2,29	7,60	3	1.10	0	5.71	44	149
K97093	2º	DENV-3	DF	24,33	7,44	7,7	1.17	0	19.44	180	70
C97014	2º	DENV-3	DHF III	3,11	7,13	4,6	0.33	27.84	48.57	177	40
C94094	2º	DENV-4	DHF I	2,11	7,13	3,7	7.01	2.5	10.26	94	140
C94132	2º	DENV-4	DHF II	11,29	7,75	8	1.14	3.26	2.7	101	200

[a]*Primary (1º) or secondary (2º) infection*

[b]*Of current infection*

[c]*According to WHO guidelines; DF = dengue fever, DHF I-IV = dengue hemorrhagic fever, grades I-IV*

[d]*A11-NS3*$_{133}$ *or B7-NS3*$_{222}$ *tetramer-positive T cells detected <1 year post-defervescence*

[e]*PEI = pleural effusion index*

[f]*As calculated using measurements taken over the course of hospitalization: max-min/min X 100*

[g]*AST = aspartate aminotransferase; IU/L=international units per liter*

[h]*As measured throughout hospitalization; values shown are divided by 1000*

[i]*nd = not determined*

[j]*na = not available*

tetramers, each containing a peptide variant corresponding to a different DENV serotype. The epitope sequences for DENV-3 and DENV-4 are identical (designated as pD3/4) and differ, as does the DENV-2 sequence (pD2), by a single amino acid from the DENV-1 sequence (pD1; refer to Table 2.1).

Figure 3.1A details the gating strategy employed to identify A11-$NS3_{133}$ tetramer-positive T cells. Each experiment included PBMC from a healthy DENV-naïve negative control donor ($A11^{+}DENV^{-}$) as well as healthy PBMC spiked with an epitope-specific $CD8^{+}$ T cell line as a positive control to help place tetramer-positive gates (Figure 3.1A). Figure 3.1B shows flow cytometry plots comparing pD1 versus pD2 and pD1 versus pD3/4 tetramer staining of T cells in PBMC of a single donor obtained at multiple time points from acute infection until 1 year post-defervescence. The mean±SD background tetramer staining for 12 healthy, $A11^{+}$ DENV-naïve subjects was 0.095±0.079%. A positive cutoff for tetramer frequencies was defined as any frequency greater than the tetramer frequency measured for 11 of the 12 $A11^{+}DENV^{-}$ subjects (92nd percentile, >0.14%). A11-$NS3_{133}$ tetramer-positive $CD8^{+}$ T cells were detected at one or more time points and increased over time to a distinct peak in 36 DENV-infected subjects (Figure 3.1C and D). Peak frequencies in these 36 subjects ranged from 0.15% to 19% with a median of 0.74% (mean = 2.33%; Figure 3.1E). Of the 8 subjects without a detectable response above the cutoff, 3 subjects had data from no or one sample during the acute infection and 3 subjects were missing data for one of the tetramer variants; only 2 subjects with adequate data failed to show an epitope-specific T cell response (one of whom had a peak frequency of 0.14%). In some individuals, the tetramer-positive $CD8^{+}$

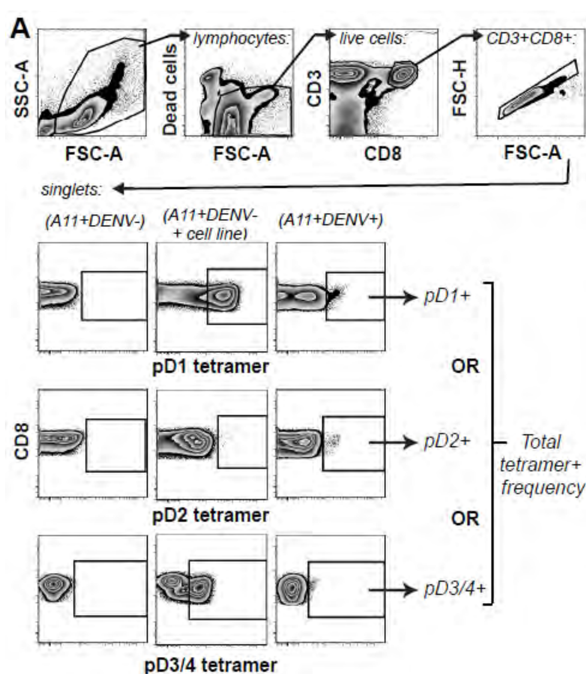

Figure 3.1. A11-NS3$_{133}$-specific T cells expand in acute infection.

(A) The gating strategy used to identify tetramer^{+}CD8^{+} T cells started by selecting cells within the lymphocyte gate as defined by forward and side scatter profiles. Live cells were next selected by exclusion of the viability marker LIVE/DEAD Aqua. T cells were identified by dual CD3 and CD8 expression followed by gating for singlet cells. (B) Tetramer staining of PBMC from an individual subject over the course of acute illness and convalescence. Days are relative to the day of defervescence (d0). (C) Tetramer^{+}CD8^{+} T cell frequencies in PBMC from subjects with primary (*n=17*) or (D) secondary (*n=22*) DENV infection. Each colored line represents a single individual tracked over time. The black line represents the frequency of background tetramer staining measured for 11/12 A11^{+}DENV-naïve subjects (=0.14%).

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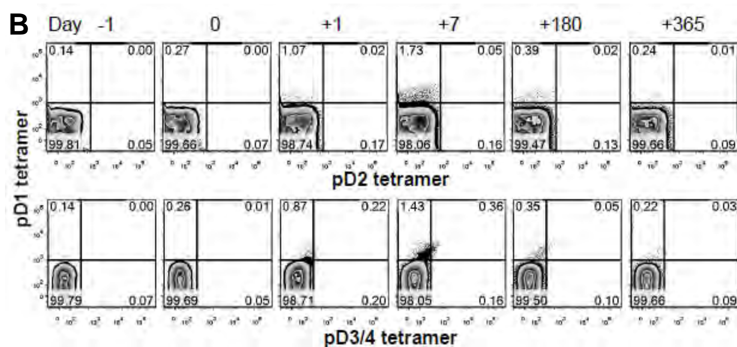

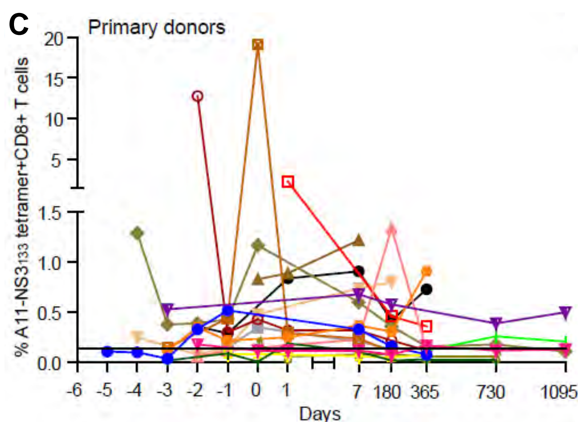

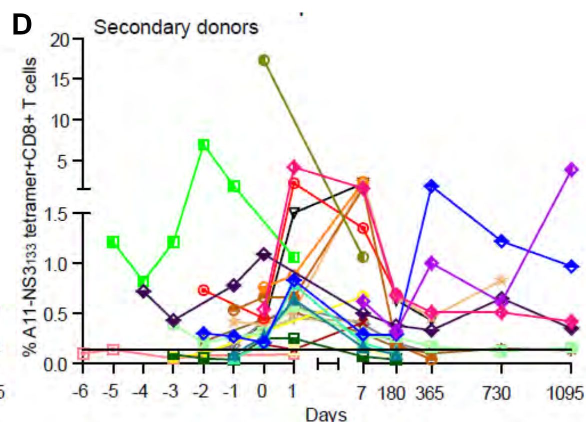

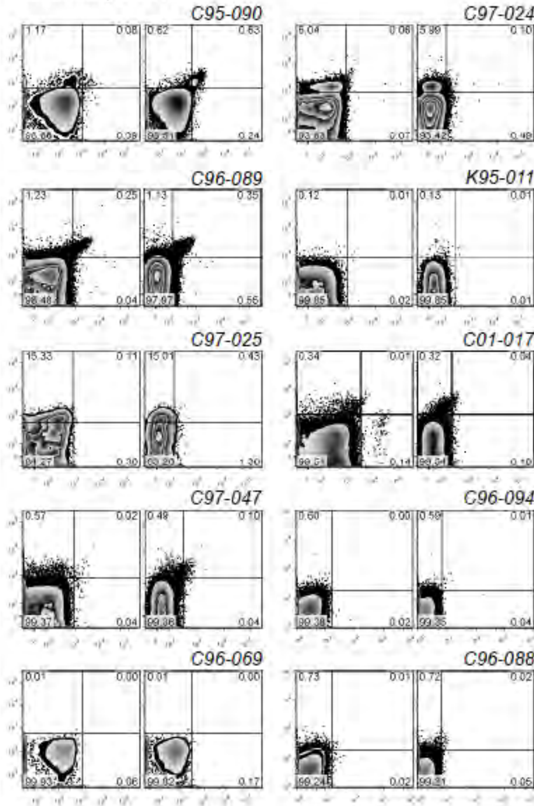

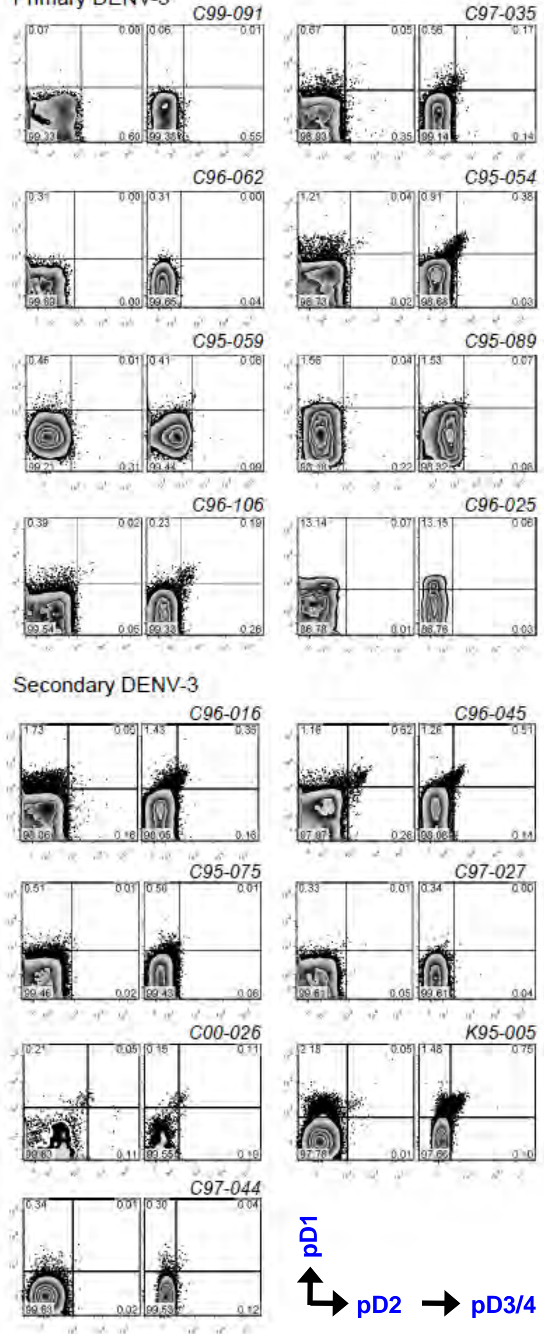

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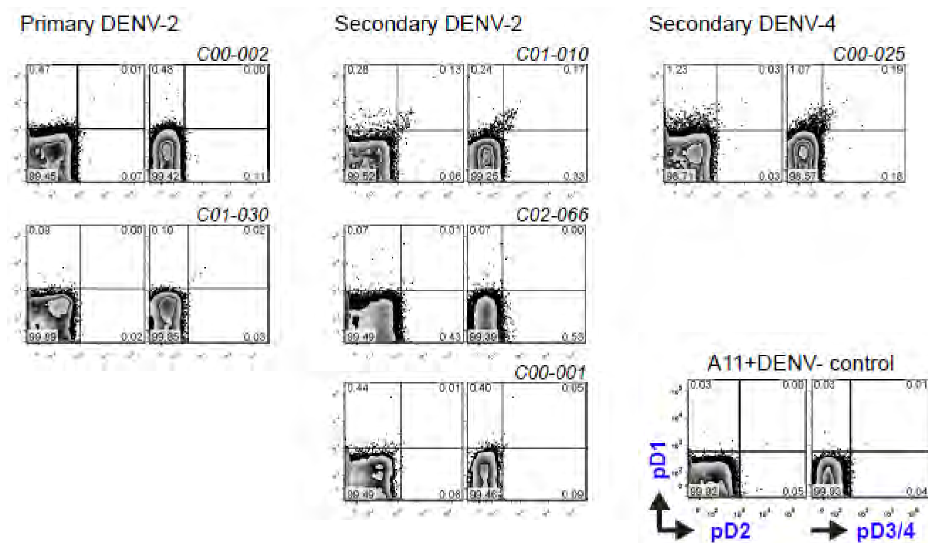

Figure 3.1. A11-NS3$_{133}$-specific T cells expand in acute infection. (*cont'd*)
...(E) Zebra plots show the peak frequency of A11-NS3$_{133}$ epitope-specific T cells for 36 subjects, arranged according to the serotype of infection and whether the subject had a primary or secondary infection. Tetramer staining of PBMC from an A11^{+}DENV-naïve subject is also shown. PBMC from each subject was stained simultaneously with all three tetramer variants; the two plots show pD1 versus pD2 and pD1 versus pD3/4 tetramer staining. Numbers represent the frequency of total CD8^{+} T cells. Subject ID numbers are included on top and to the right of the respective flow plots. Flow plots are shown with large-sized dots.

T cell frequency changed rapidly, with large increases and decreases even in consecutive (daily) blood samples. Although pre-infection PBMC samples were not available for this cohort, tetramer-positive $CD8^+$ T cell frequencies were higher in acute infection and early convalescence than in late convalescence (≥6 months post-infection). Sixteen of 20 subjects with samples available from 1 year post-infection (and a distinct peak frequency during acute infection) showed an average contraction of 76% of A11-$NS3_{133}$ tetramer-positive T cells at the 1 year time point. Four subjects had the same or higher tetramer-positive $CD8^+$ T cell frequencies at 1-3 years post-infection (compared to their peak frequency during acute infection) but had 16-65% contraction of epitope-specific T cells at 6 months post-infection; the possibility of subsequent DENV infection in these cases cannot be excluded.

B. T cell expansion in acute DENV infection is antigen-specific

We considered that conclusions based on the analysis of a single DENV epitope might not be generalizable or could reflect non-specific rather than antigen-specific T cell expansion. To address the first possibility, we sought to analyze the frequency of T cells specific to another DENV epitope. While a significant number of DENV T cell epitopes have been identified (Dung*, et al.*, 2010; Vaughan*, et al.*, 2010), lack of definition and low population frequencies of the restricting HLA alleles limit their utility; fortunately, we had pMHC tetramers available for an HLA-B7-restricted epitope ($NS3_{222-231}$) we previously defined as immunodominant in HLA-$B7^+$ Thai subjects (Mathew*, et al.*, 1998).

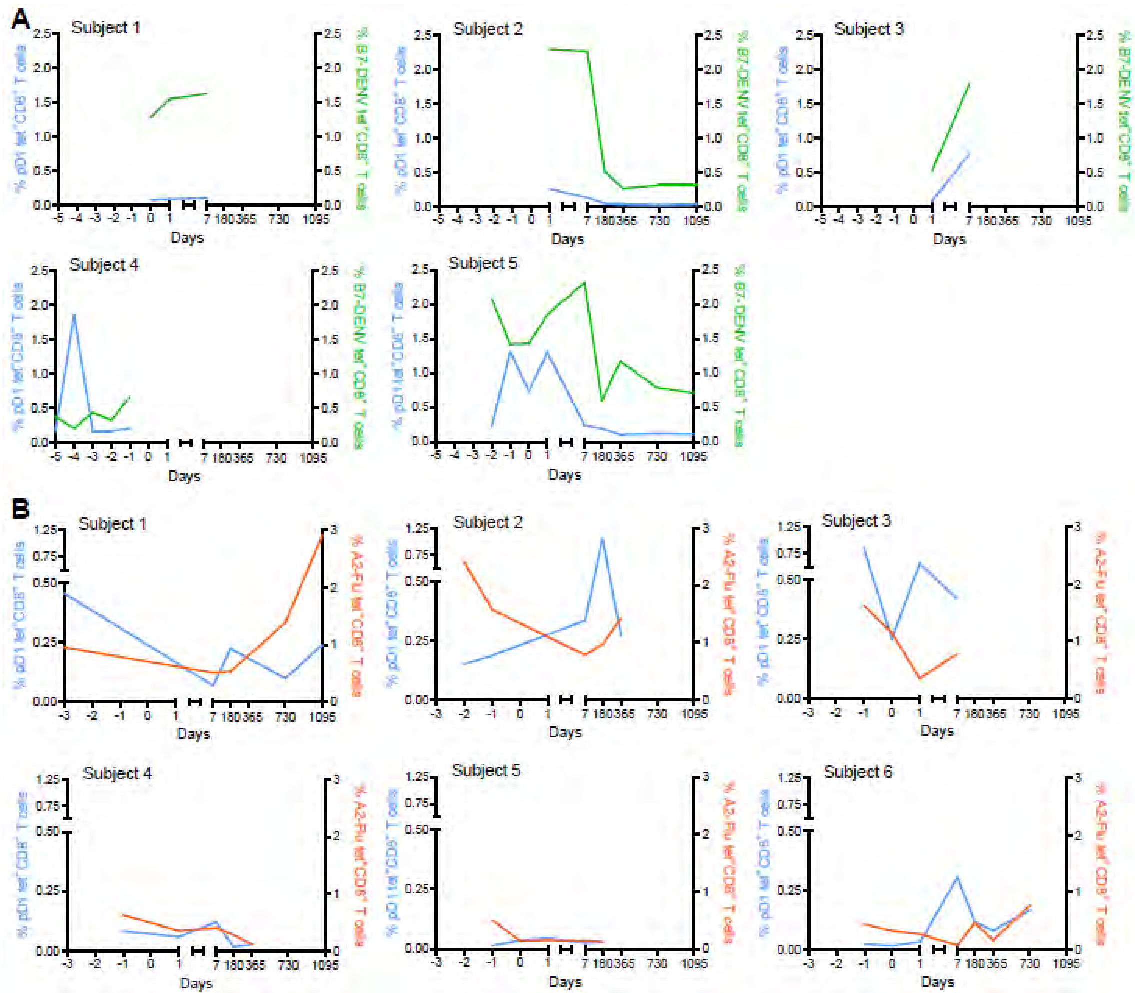

Figure 3.2. T cell expansion in acute DENV infection is antigen-specific.

(A) Line graphs show frequencies of A11-NS3$_{133}$ tetramer (pD1; blue) and B7-NS3$_{222}$ multimer (B7-DENV; green) positive T cells from 5 HLA-A11^{+}B7^{+} individuals throughout acute illness and convalescence. (B) Line graphs show frequencies of pD1 tetramer (blue) and A2-Flu M1$_{58}$ multimer (A2-Flu; orange) positive T cells from 6 HLA-A11^{+}A2^{+} individuals throughout acute illness and convalescence. Days are relative to the day of defervescence (d0).

We therefore compared the frequencies of A11-NS3$_{133}$ (pD1$^+$) and B7-NS3$_{222}$ tetramer-positive T cells in a subset of A11$^+$B7$^+$ subjects (n=5) in order to determine their relative contributions to the overall anti-viral CD8$^+$ T cell response (Figure 3.2A). These frequencies were similar in magnitude (each tetramer population was <2.5% of total CD8$^+$ T cells), although the frequencies of B7-NS3$_{222}$-specific CD8$^+$ T cells were consistently higher in 4 of the 5 subjects studied. Both populations of epitope-specific T cells were present at higher frequency during acute infection than in late convalescence.

To investigate whether DENV epitope-specific T cells were selectively expanded, we compared the frequency of A11-NS3$_{133}$-specific T cells to that of T cells specific for the immunodominant HLA-A*0201-restricted influenza virus (Flu) M1$_{58-66}$ epitope (Figure 3.2B) (Bednarek*, et al.*, 1991; Morrison*, et al.*, 1992). A2-Flu M1$_{58}$ tetramer-positive CD8$^+$ T cells were present at high frequency, consistent with previous findings (Gianfrani*, et al.*, 2000), but decreased in frequency during acute DENV infection coincident with the increase in frequency of DENV epitope-specific CD8$^+$ T cells. This demonstrates selective expansion of antigen-specific T cells during acute DENV infection and implies that there was minimal contribution of bystander cells to the observed T cell response.

C. Epitope-specific T cell frequencies peak earlier in primary than in secondary DENV infection

Previous studies have yielded conflicting conclusions regarding the timing of expansion of DENV-specific T cells. In this cohort, epitope-specific T cells generally

Table 3.2. A11-NS3$_{133}$ tetramer frequencies peak earlier in primary DENV infection[a].

	No. subjects	
	Primary	***Secondary***
Fever day ≤0[b]	4	1
Fever day +1	1	8[d]
Fever day +7[c]	1	6

[a]*This analysis only considered those subjects for whom we had blood samples from all three of these time points (n=21)*
[b]*One primary subject had peak tetramer frequency at fever day -2*
[c]*The actual day post-defervescence differed for each donor (range=4-12, mean=7)*
[d]*The difference between day 0 and day +1 is significant by Fisher's exact test (p=0.02)*

peaked in frequency around defervescence or early convalescence before declining (Figure 3.1B-D), corresponding to the usual kinetics of viral clearance in acute DENV infections (Vaughn*, et al.*, 1997; Vaughn*, et al.*, 2000; Libraty*, et al.*, 2002a). Among 21 subjects with samples available from fever days 0, +1 and +7, tetramer-positive $CD8^+$ T cell frequencies were highest on the day of defervescence (fever day 0) or earlier in 5 subjects, on fever day +1 in 9 subjects, and at the 1 week follow-up visit in 7 subjects (Table 3.2). To determine whether prior DENV infection influenced the kinetics of the T cell response to acute infection, we determined when the peak frequency of A11-$NS3_{133}$ tetramer-positive $CD8^+$ T cells occurred for individuals experiencing either a primary or secondary infection (Table 3.2). Epitope-specific T cell frequencies in patients with primary infections peaked during acute infection, with the majority of peak responses detected on the day of defervescence (fever day 0). Surprisingly, tetramer-positive T cell frequencies peaked later in patients with secondary DENV infection, typically one day or within one week after defervescence (p=0.02 for the difference in timing of peak frequencies between primary and secondary subjects at fever days 0 and +1, Table 3.2).

D. The frequency of A11-$NS3_{133}$ tetramer-positive T cells does not correlate with clinical findings

Some previous studies reported that the magnitude of the DENV-specific T cell response correlates with disease severity (Zivna*, et al.*, 2002; Mongkolsapaya*, et al.*, 2003; Mongkolsapaya*, et al.*, 2006). We examined this question in the present cohort, which included both primary and secondary donors with varying disease severity. As

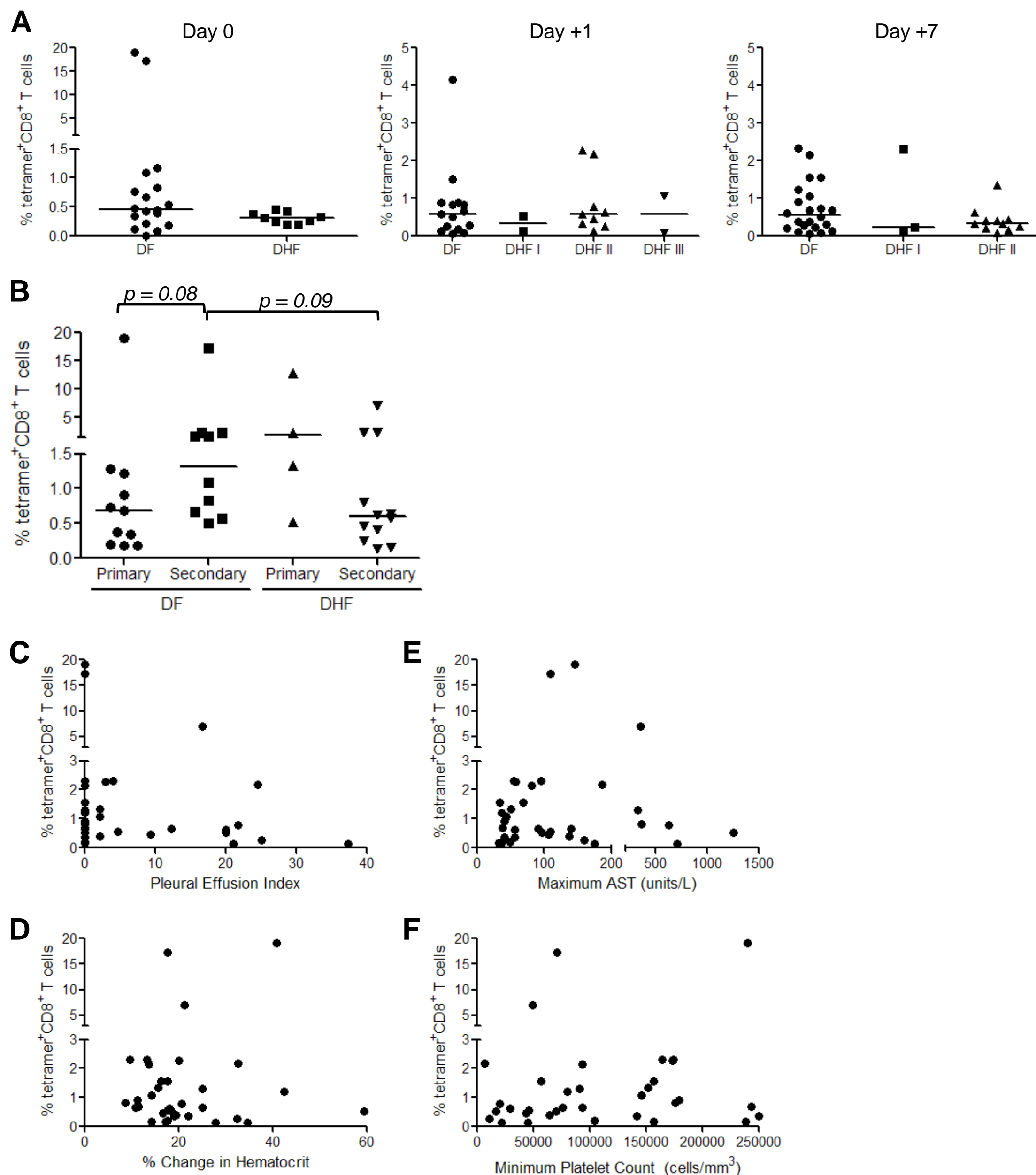

Figure 3.3. The magnitude of A11-NS3$_{133}$ epitope-specific T cells does not correlate with disease severity.

(A) A11-NS3$_{133}$ tetramer^{+}CD8^{+} T cell frequencies are shown for individuals at fever day 0, +1 or +7. Data are classified according to disease severity: DF=dengue fever, DHFI-III=dengue hemorrhagic fever grades I, II, or III. Peak A11-NS3$_{133}$ tetramer^{+}CD8^{+} T cell frequencies are plotted versus (B) clinical diagnosis (DF versus DHF) and type of infection (primary versus secondary), (C) pleural effusion index, (D) % change in hematocrit, (E) maximum aspartate aminotransferase (AST) value and (F) minimum platelet count. No significant correlations were detected by Mann-Whitney (B) or Spearman's test (C-F).

symptoms of severe disease typically occur around defervescence (fever day 0) we compared the frequency of A11-NS3$_{133}$ tetramer-positive T cells and disease severity at fever day 0, fever day +1 and 1 week after defervescence (Figure 3.3A). We detected no correlation between tetramer-positive T cell frequency and clinical diagnosis (DF versus DHF) at any of these time points. Among subjects with DF, peak tetramer-positive T cell frequencies were slightly higher in secondary infection than primary infection, but this difference did not reach statistical significance (p=0.08; Figure 3.3B). Among subjects with secondary DENV infections, peak tetramer frequencies were slightly higher in subjects with DF than those with DHF, but this difference was also not statistically significant (p=0.09, Figure 3.3B). No significant differences were found between subjects with primary DF and either primary DHF or secondary DHF. We also tested whether peak tetramer-positive T cell frequencies correlated with measures of disease severity such as pleural effusion index (Figure 3.3C), percent change in hematocrit (Figure 3.3D), maximum aspartate aminotransferase values (Figure 3.3E) and minimum platelet count (Figure 3.3F), and found no significant correlations.

To investigate whether other DENV epitope-specific T cells correlated with clinical diagnosis, we assessed the frequency of B7-NS3$_{222}$-specific T cells in subjects with DF or DHF. No significant differences were detected among HLA-A11^{+}B7^{+} subjects with regard to diagnosis (Figure 3.4). Among HLA-A11^{-}B7^{+} subjects, those with DHF had higher frequencies of B7-NS3$_{222}$ tetramer-positive CD8^{+} T cells than subjects with DF at all time points analyzed (Figure 3.4). However, the number of subjects for this analysis was too small for statistical analysis (2 with DHF and 3 with

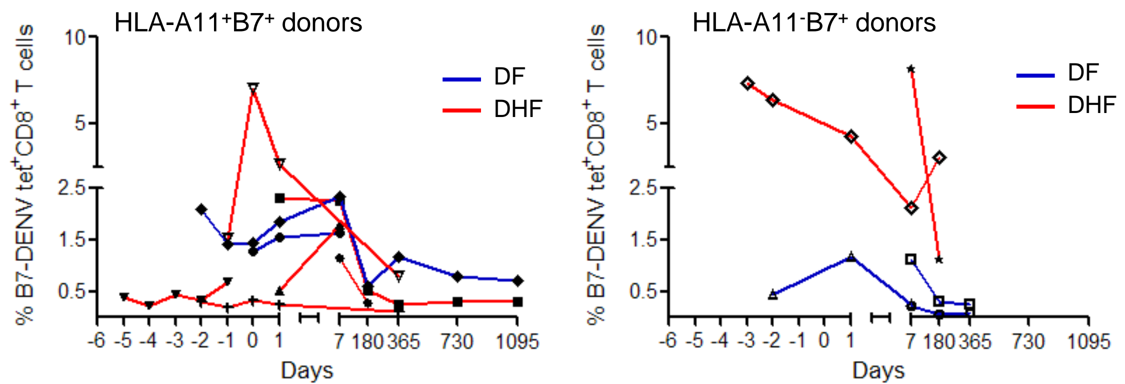

Figure 3.4. HLA-A11 may influence the expansion of B7-NS3$_{222}$ epitope-specific T cells. B7-NS3$_{222}$ tetramer frequencies for HLA-A11^{+}B7^{+} or HLA-A11^{-}B7^{+} donors with a final diagnosis of DF (blue lines) or DHF (red lines) are plotted over time. Days are relative to the day of defervescence (d0).

DF). These findings suggest that the HLA-A*1101 allele may influence the expansion of HLA-B*07-restricted $NS3_{222}$ epitope-specific T cells in DENV-infected individuals.

E. A11-$NS3_{133}$-specific T cells are cross-reactive to multiple serotypic variants in both primary and secondary DENV infection

Previous studies found that PBMC from subjects with secondary DENV infection preferentially bound to A11-$NS3_{133}$ pMHC tetramers specific for heterologous DENV serotypes, suggesting that the T cell repertoire reflected “original antigenic sin” related to the prior infection (Mongkolsapaya*, et al.*, 2003). We reasoned that the effect of prior infections would only be evident by comparing the patterns of tetramer staining in PBMC from subjects with primary and secondary infection with different DENV serotypes. Based on the model of original antigenic sin, we expected that PBMC from primary donors would primarily bind homotypic tetramers whereas secondary donor PBMC would bind both homotypic and heterotypic tetramer variants.

Patterns of triple tetramer staining were analyzed according to the Boolean gating strategy outlined in Figure 3.5. The distribution of tetramer staining among epitope-specific T cells varied less across time points from the same individual than between individuals, but PBMC from subjects with primary infection (Figure 3.6A-C) displayed similar levels of heterotypic tetramer binding as PBMC from subjects with secondary infection (Figure 3.6D-G). Of tetramer-positive T cells detected at 1 week post-defervescence, median values of 67% and 72% ($p=0.44$) bound tetramers specific for heterologous serotypes in primary and secondary infection, respectively.

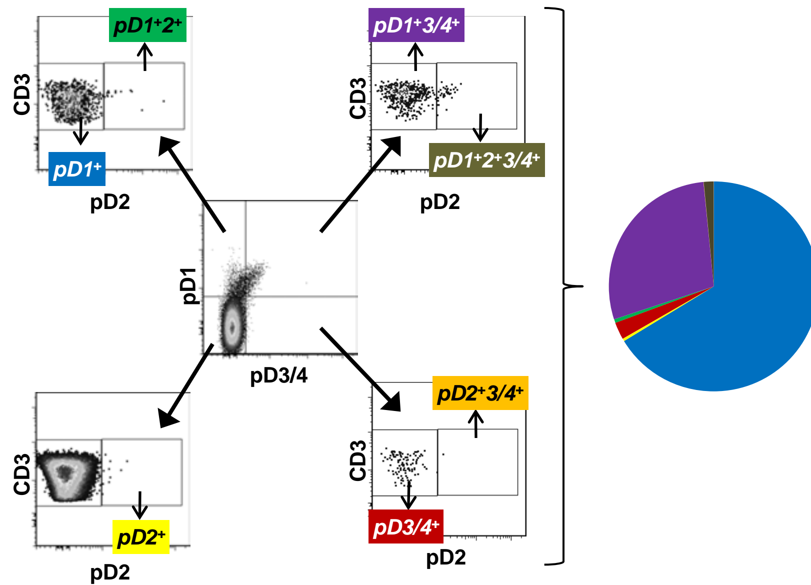

Figure 3.5. Outline of the gating strategy to identify tetramer-positive T cell subsets.

The Boolean gating strategy used to identify the distribution of sub-populations of tetramer^{+} T cells within the live, CD3^{+}CD8^{+} singlet lymphocyte population (refer to Figure 3.1A) and the resulting pie chart. The four outer dot plots are shown with large-sized dots.

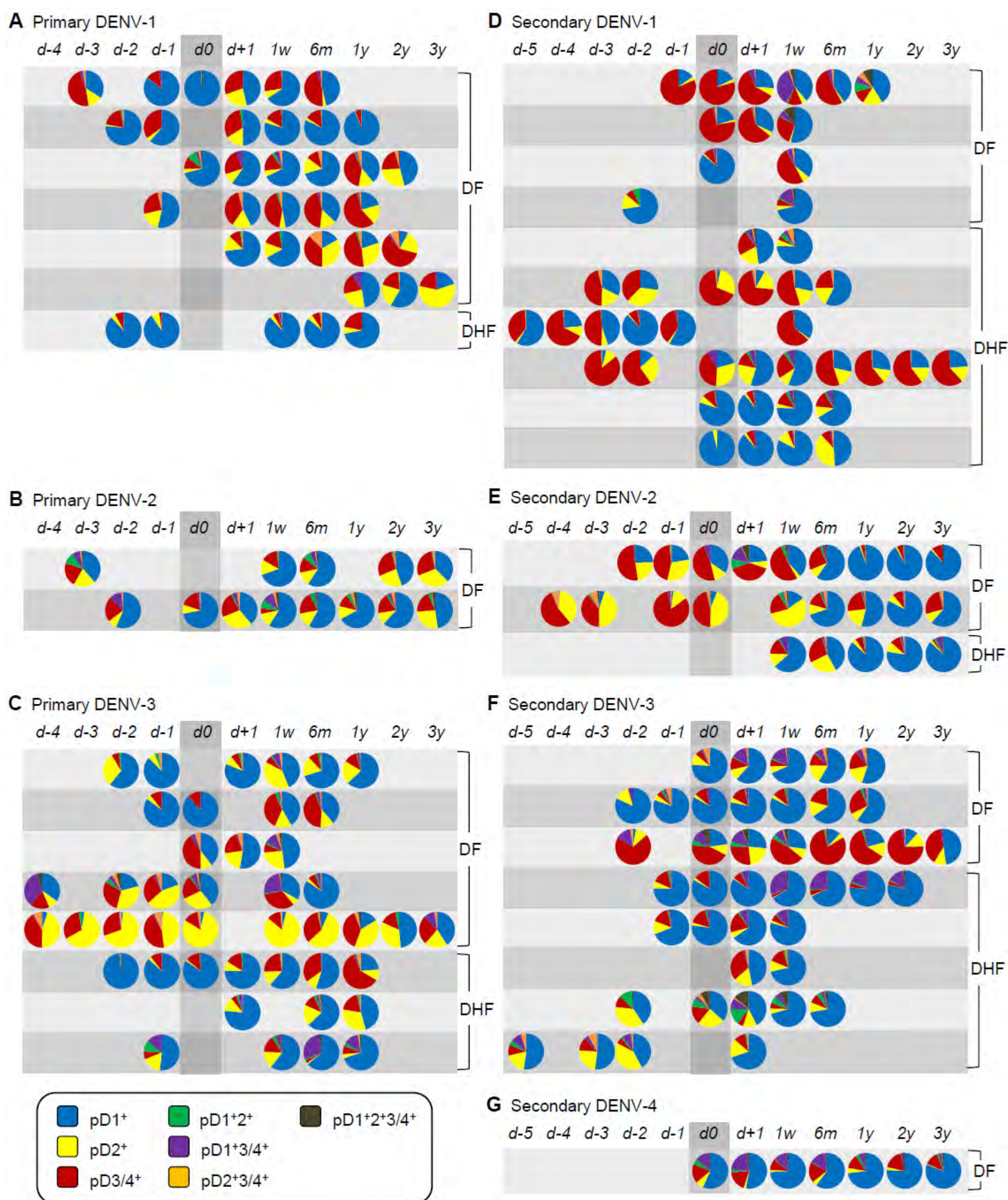

Figure 3.6. Tetramer-positive T cells are highly serotype-cross-reactive in subjects with primary or secondary infection.

The distribution of sub-populations within the whole population of A11-$NS3_{133}$ tetramer$^+$CD8$^+$ T cells is depicted using pie charts. Subjects with a (A-C) primary or (D-G) secondary infection with (A,D) DENV-1, (B,E) DENV-2, (C,F) DENV-3 or (G) DENV-4 are shown. No subjects with primary DENV-4 infection were available. Pie charts are shown for those time points with data from at least 20 tetramer-positive T cells (mean and median number of tetramer$^+$CD8$^+$ T cells per sample = 435 and 164, respectively). Each row represents data from a single donor over time. Time points shown are relative to the day of defervescence (d0), d = day, w = week, m = month, y = year.

The pMHC tetramer staining did not follow a consistent pattern according to the serotype of the infecting virus or clinical diagnosis. However, there were some similarities among subjects within a particular donor group. For example, there was a greater percentage of $pD2^+$ cells (shown in yellow) in subjects with primary DENV-3 infections compared to those with secondary DENV-3 or DENV-1 infections, whether primary or secondary, at 1 week post-infection (median = 15% versus 5% and 6%, respectively; $p<0.01$). Additionally, subjects infected with DENV-1(whether during primary or secondary infection) had a greater percentage of $pD3/4^+$ cells (shown in red) than those infected with DENV-3 (median = 30% versus 13%; p=0.04) at fever day +1. Relatively few T cells bound to two or more tetramers at once (shown in green, purple, orange and brown) and were more often found in subjects with secondary infections than those with primary infections (median = 6% for primary versus 11% for secondary at 1 week, p=0.07). These observations support previous indications that the sequence of infection influences the DENV-specific memory $CD8^+$ T cell pool (Bashyam*, et al.*, 2006; Beaumier*, et al.*, 2008).

This analysis also revealed a prevalence of T cells that bound the pD1 tetramer (shown in blue). To exclude the possibility that this was an artifact of our staining conditions, we compared staining with all three tetramer variants at once to staining with each variant individually (Figure 3.7A and B). The frequency of tetramer-positive T cells was slightly different between the two staining conditions, indicating some competition amongst the variants. Nevertheless, the relative staining pattern with particular tetramer variants was comparable. We also tested whether decreasing the concentration of one

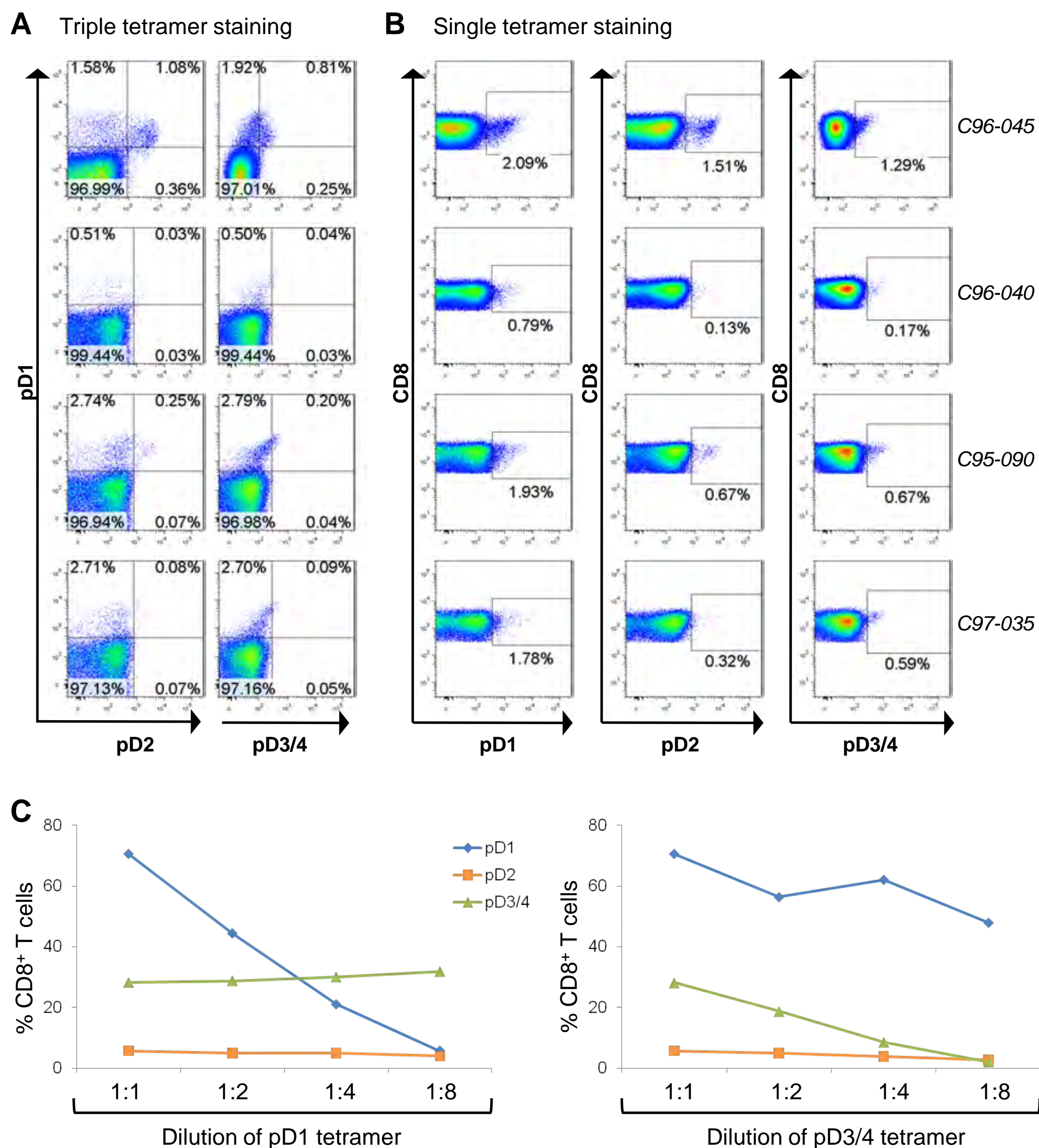

Figure 3.7. The prevalence of pD1 tetramer binding by patient PBMC is not an artifact of staining conditions.

Flow plots show repeated tetramer staining of PBMC from four subjects which were stained with each of the three A11-$NS3_{133}$ tetramer variants (A) together ("triple tetramer staining") or (B) alone ("single tetramer staining"). Numbers inside each plot indicate the frequency of total $CD8^+$ T cells. Subject ID numbers are included to the right of each row of respective flow plots. (C) An epitope-specific T cell line which stains with pD1 and pD3/4 tetramers was subject to triple tetramer staining conditions in which the concentration of pD1 or pD3/4 tetramer was decreased while the concentration of the other two tetramers remained constant.

tetramer while keeping the other tetramer concentrations constant would influence the frequency of T cells that bound to particular tetramer variants. Using an A11-$NS3_{133}$ epitope-specific T cell line which binds the pD1 and pD3/4, but not pD2, tetramers, we reduced the concentration of pD1 or pD3/4 by nearly 10-fold but detected no differences in the frequency of cells that bound to the other tetramer variants (Figure 3.7C).

The pie chart analysis, while informative, does have limitations. For example, quadrant gates were placed based on positive and negative controls, which do not take into account subject-to-subject variation. The flow plots shown in Figure 3.1E demonstrate that for some subjects (e.g. subject C96-045), pD1 tetramer-positive T cells seem to 'lean' toward the pD3/4 tetramer-positive gates, but do not meet the threshold used to count them as pD3/4 tetramer-positive ($pD1^{+}3/4^{+}$); these cells would thus show up as $pD1^{+}$ (blue) in the respective pie chart (if we do not consider pD2 tetramer staining). To account for this, the intensity of pD3/4 tetramer staining of all pD1 tetramer-positive $CD8^{+}$ T cells, relative to total tetramer-negative $CD8^{+}$ T cells, was compared across all time points for subjects with primary versus secondary DENV-3 infections (Figure 3.8). No differences were detected between these subject groups, further supporting our finding that the extent of serotype-cross-reactivity does not differ according to DENV infection history.

Another limitation of the pie chart analysis regards the use of the Mann-Whitney rank sum test to assess differences in the presence of particular epitope-specific T cell subsets (e.g. $pD1^{+}$, $pD1^{+}3/4^{+}$) between groups of subjects (e.g. primary/secondary, serotype of infection). This is a non-parametric test that accounts for the large amount of

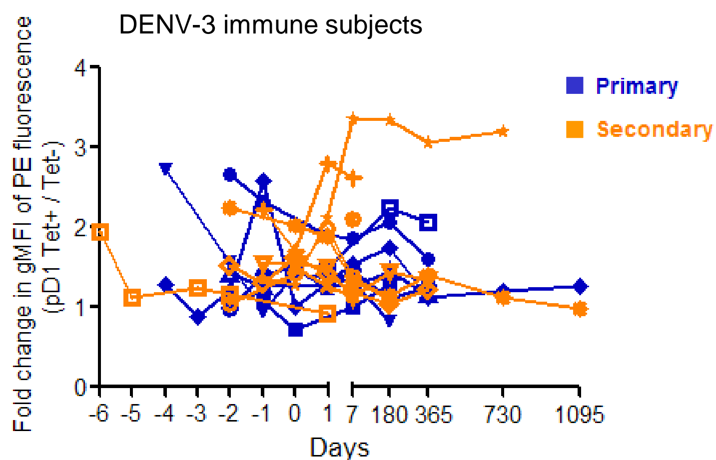

Figure 3.8. The intensity of pD3/4 tetramer staining of pD1 tetramer-positive T cells does not differ in subjects with primary versus secondary infection.

Shown is the gMFI of PE (pD3/4) fluorescence for all pD1^{+}CD8^{+} T cells, relative to total tetramer-negative CD8^{+} T cells, for subjects with a primary (blue) or secondary (orange) DENV-3 infection. Each line represents a single subject over time. Days are relative to the day of defervescence (d0).

variation within our samples; however, the test is based on the ranks of the data and not the actual values. Additionally, our study was not powered to detect specific size effects between subject groups. Future studies should be powered to appropriately test the differences seen in our study. A further limitation of our analysis was the exclusion of subjects that did not have a viable sample for the time point chosen for comparison. The explanations for not having a sample at this time point include failure to obtain a sample, insufficient sample size, and/or viability issues pertaining to that particular sample. For these reasons, we cannot exclude the possibility of selection bias in our results.

To support the finding of preferential binding of T cells to heterologous tetramers in primary DENV infections, we assessed cytokine responses in a subset of PBMC samples by intracellular cytokine staining (ICS) after peptide stimulation. We detected variable responses in PBMC from children with primary or secondary DENV-3 infections (Figure 3.9). Flow plots show the A11-$NS3_{133}$ epitope-specific T cell response for a representative subject with secondary infection (Figure 3.9A). PBMC from some subjects with primary DENV-3 infections responded to stimulation with heterologous epitope variants (pD1 and pD2) more than to the homologous peptide (pD3/4; Figure 3.9B); these responses seemed to change with time, consistent with the temporal evolution of tetramer binding seen for some subjects (Figure 3.6). The patterns of responses were similar in PBMC from subjects with secondary DENV-3 infections, though pD3/4-specific responses appeared higher than in subjects with primary infection (Figure 3.9C). These functional data, while limited, support the findings based on

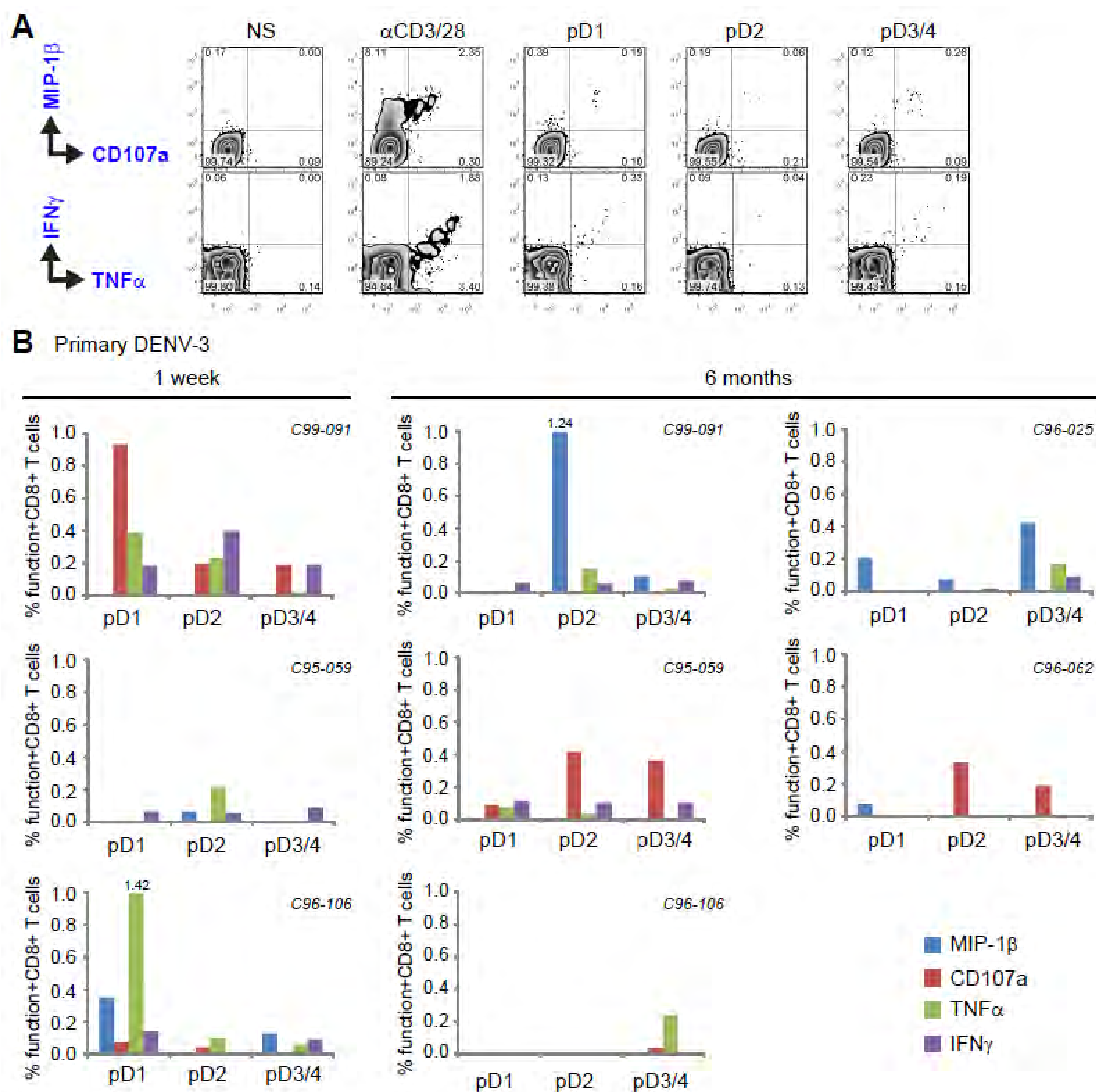

Figure 3.9. Peptide-stimulated PBMC from subjects with primary or secondary infection demonstrate serotype-cross-reactive T cell functional responses.

(A) Representative flow plots show degranulation (CD107a) and production of cytokines (MIP-1β, TNFα, IFNγ) by DENV-immune PBMC (subject C96-016) after no stimulation (NS) or stimulation with anti-CD3/28 (positive control) or 10μg/mL of pD1, pD2, or pD3/4 for 6 hours in an intracellular cytokine staining (ICS) assay. Flow plots are shown with large-sized dots. Bar graphs show the response detected by ICS from PBMC from subjects with (B) primary DENV-3 infections obtained 1 week or 6 months post-defervescence, or (C) secondary DENV-3 infections obtained 1 week post-defervescence.

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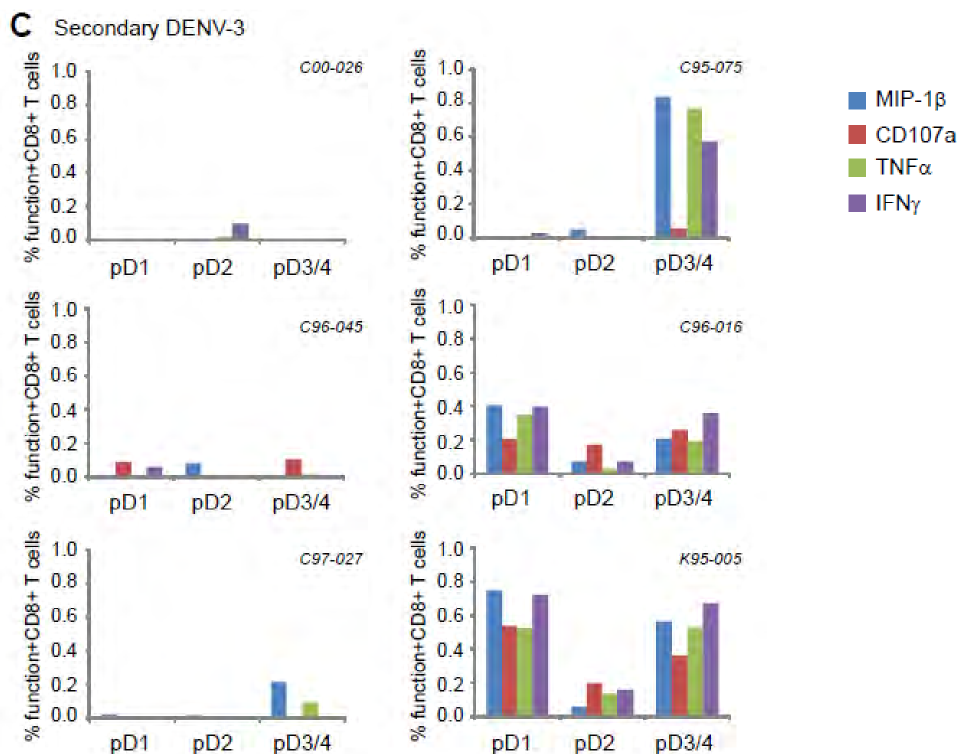

Figure 3.9. Peptide-stimulated PBMC from subjects with primary or secondary infections demonstrate serotype-cross-reactive T cell functional responses. (*cont'd*)

...Shown are frequencies (relative to NS) of total CD8+ T cells that responded to each peptide variant by degranulation (CD107a; red) or production of MIP-1β (blue), TNFα (green), or IFNγ (purple). Subject ID numbers are included in the top-right-hand corner of each bar graph.

patterns of tetramer binding, indicating striking cross-reactivity in primary DENV infections.

F. Antigen-specific CD8^{+} T cells are highly activated during acute DENV infection

Previous data suggested that T cells were more highly activated in severe dengue disease (Green*, et al.*, 1999a; Chau*, et al.*, 2008). However, a recent report concluded that CD38-expressing CD8^{+} T cells did not appear until after viral clearance and suggested that DENV-specific T cells could therefore not contribute to severe disease (Dung*, et al.*, 2010). Since our study showed high frequencies of A11-NS3$_{133}$-specific T cells during acute infection, we examined CD38 expression to assess whether antigen-specific T cells were differently activated in primary versus secondary infection and if levels of T cell activation correlated with outcome.

We detected high CD38 expression in total CD8^{+} T cells and even higher expression in epitope-specific T cells during acute infection and shortly after defervescence (Figure 3.10A). While levels of CD38 expression varied across individuals (Figure 3.10B), the average geometric mean fluorescence intensity (gMFI) of tetramer-positive T cells in PBMC from the four subject groups (primary DF, primary DHF, secondary DF and secondary DHF) showed similar patterns over time (Figure 3.10C). CD38 expression was slightly higher in subjects with secondary versus primary infection during acute illness; however, this difference did not achieve statistical significance (Figure 3.10D).

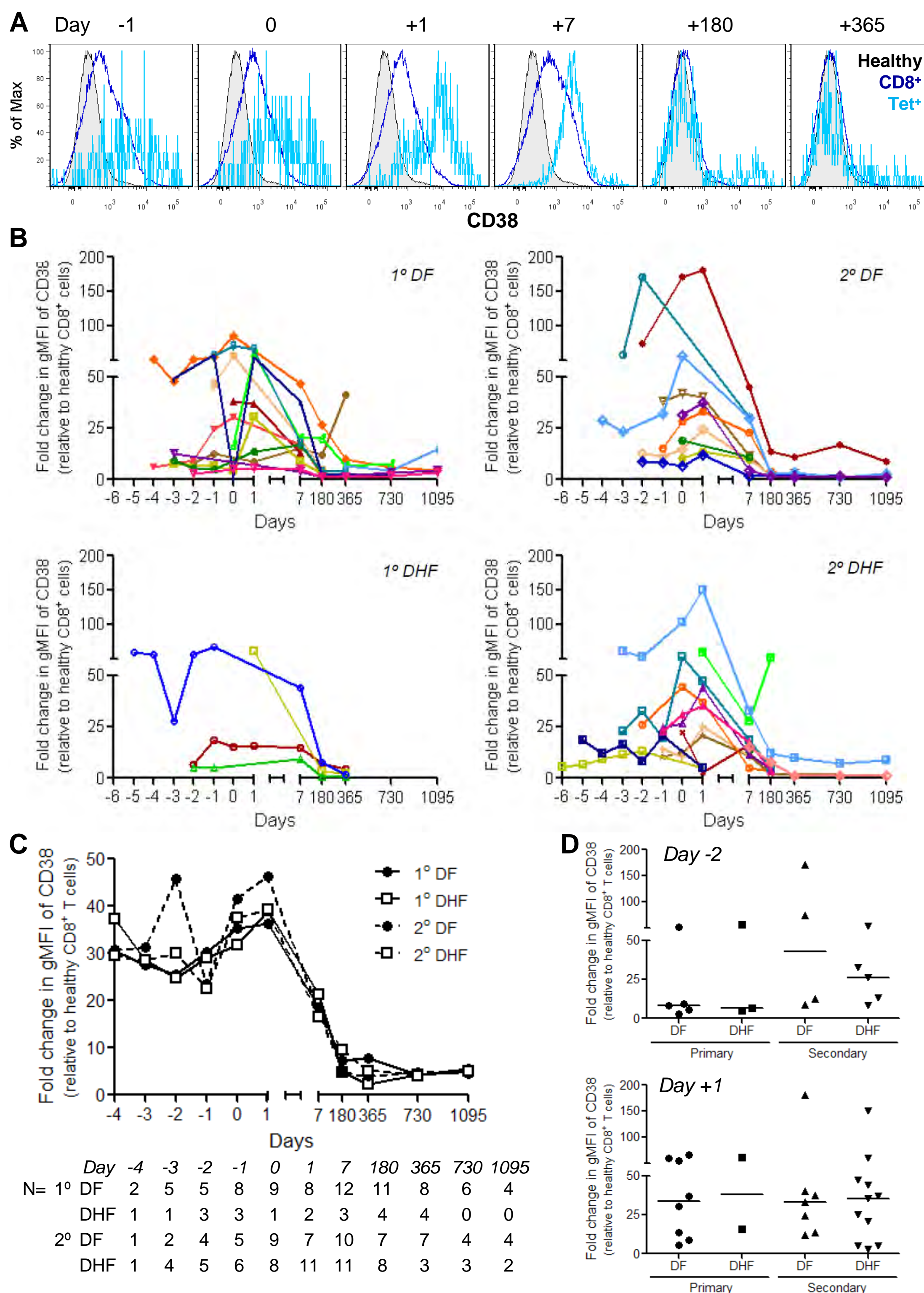

Figure 3.10. Antigen-specific T cells are highly activated during acute DENV infection and early convalescence.

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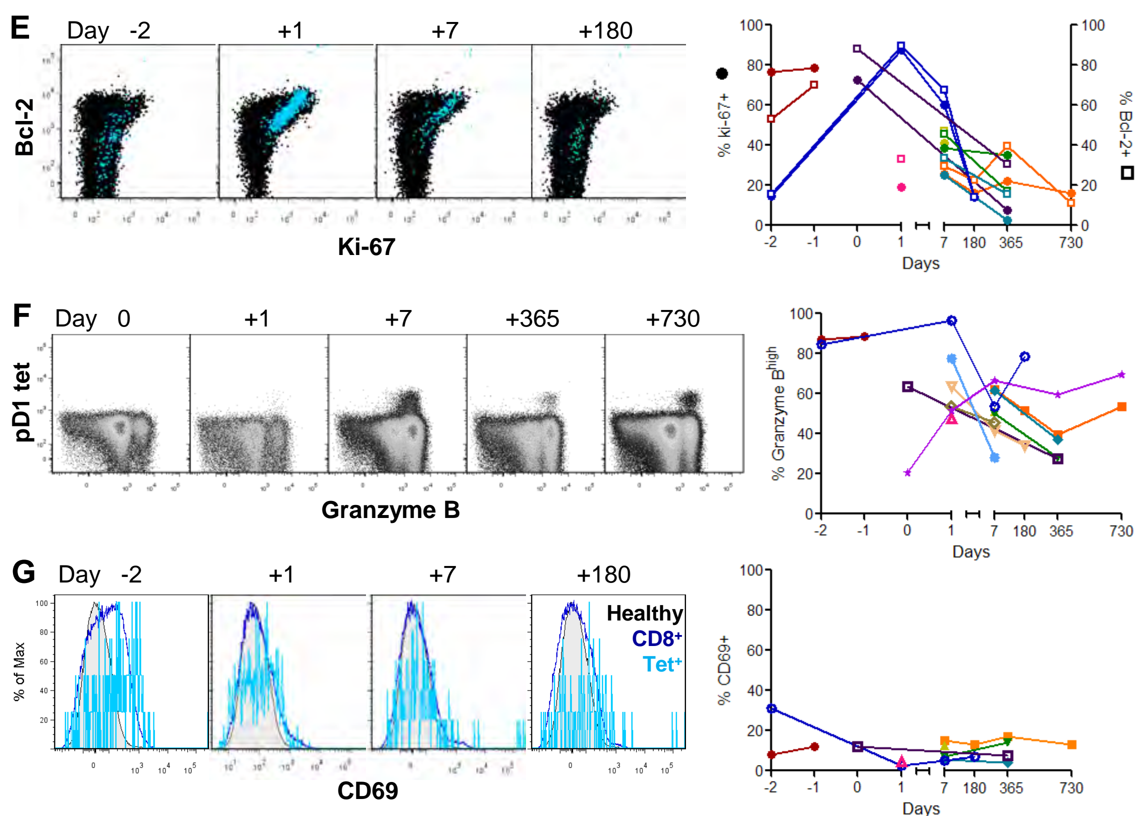

Figure 3.10. Antigen-specific T cells are highly activated during acute DENV infection and early convalescence. (*cont'd*)

(A) Representative histograms show the expression of CD38 over time in total $CD8^+$ T cells from an $A11^+$ DENV-infected donor (dark blue line) as well as A11-$NS3_{133}$ $tetramer^+CD8^+$ T cells from the same DENV-infected donor (light blue line). Also shown is CD38 expression of total $CD8^+$ T cells from a healthy $A11^+$ donor (shaded). (B) The fold change in geometric mean fluorescence intensity (gMFI) of CD38 expression in $tetramer^+CD8^+$ T cells from individuals experiencing either a primary (1º) or secondary (2º) DENV infection with DF or DHF. Each colored line represents a single subject over time. Numbers are relative to total $CD8^+$ T cells from a healthy control donor included in each experiment. (C) The mean fold change in gMFI of CD38 staining in A11-$NS3_{133}$ $tetramer^+CD8^+$ T cells from subjects with 1º (solid lines) or 2º (dotted lines) DENV infections with either DF (closed circles) or DHF (open squares) over time. Data are relative to the gMFI of CD38 of total $CD8^+$ T cells from a healthy control donor included in each experiment. (D) Fold change in gMFI of CD38 is shown for 1º DF, 1º DHF, 2º DF and 2º DHF donors at fever days -2 and +1. Bars indicate median values for each group. Differences were not statistically significant by Mann-Whitney test. (E) Representative dot plots from a DENV-infected individual show the expression of Ki-67 and Bcl-2 in $tetramer^+CD8^+$ T cells (blue) compared to total $CD8^+$ T cells (black). Dot plots are shown with large-sized dots. Each pair of colored lines on the graph represents $tetramer^+CD8^+$ T cells from a single individual over time and their expression of Ki-67 (filled circles) or Bcl-2 (open squares). (F) Dot plots show the expression of granzyme B in live, $CD3^+CD8^+$ singlet T cells over time for a DENV-infected individual. Each line on the graph represents granzyme B expression by $tetramer^+CD8^+$ T cells from an individual patient over time. (G) Overlaid histograms show the expression of CD69 over time of $tetramer^+CD8^+$ T cells (light blue line) and total $CD8^+$ T cells (dark blue line) from a DENV-infected individual relative to total $CD8^+$ T cells from a healthy control donor included in the experiment (shaded histogram). Each line on the graph represents the percentage of $CD69^+tetramer^+CD8^+$ T cells from a single subject over time. Days are relative to the day of defervescence (d0).

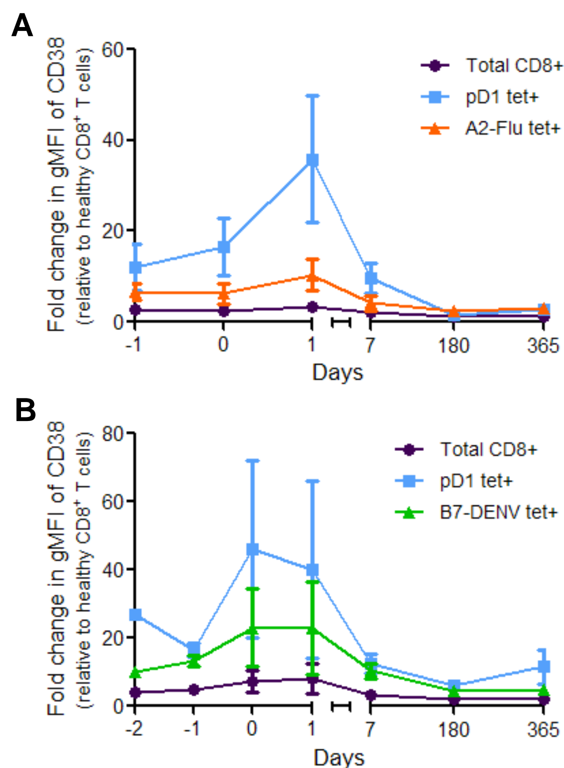

Figure 3.11. Antigen-specific T cells are preferentially activated during acute DENV infection and early convalescence.

(A) The mean fold change in gMFI of CD38 expression in $pD1^{+}CD8^{+}$ T cells (blue), $A2\text{-}Flu^{+}CD8^{+}$ T cells (orange) and total $CD8^{+}$ T cells (purple) over time in $HLA\text{-}A2^{+}A11^{+}$ subjects (*n=6*). Bars indicate standard deviation at each time point. (B) The mean fold change in gMFI of CD38 expression in $pD1^{+}CD8^{+}$ T cells (blue), $B7\text{-}DENV^{+}CD8^{+}$ T cells (green) and total $CD8^{+}$ T cells (purple) over time in $HLA\text{-}A11^{+}B7^{+}$ subjects (*n=5*). Bars indicate standard deviation at each time point. Days are relative to the day of defervescence (d0).

Decreasing CD38 expression one week post-defervescence coincided with a decrease in Ki-67 (a marker of proliferation) and Bcl-2 (an anti-apoptotic marker) expression (Figure 3.10E). Granzyme B, on the other hand, remained highly expressed in tetramer-positive T cells as well as a subset of tetramer-negative $CD8^+$ T cells late into convalescence (Figure 3.10F). CD69 expression was only detected prior to defervescence (Figure 3.10G), consistent with a previous study (Green*, et al.*, 1999a).

As the majority of $CD8^+$ T cells (both tetramer-positive and tetramer-negative) appeared to up-regulate CD38 expression, we compared the relative activation of DENV-specific T cells and T cells specific for other non-dengue antigens in order to assess the selectivity of this effect. We found that CD38 expression on A2-Flu $M1_{58}$-specific $CD8^+$ T cells was slightly increased during acute DENV infection (Figure 3.11A); however, the average increase in gMFI on these cells was much lower than on DENV-specific T cells detected by either A11-$NS3_{133}$ or B7-$NS3_{222}$ pMHC tetramers (Figure 3.11B). These data suggest that, while T cells specific for other antigens are activated to an extent, there is a preferential activation of DENV-specific T cells.

G. Evolution of T cell phenotypes follows different kinetics in primary versus secondary DENV infection

The phenotypic classification of naïve, effector and memory $CD8^+$ T cell subsets and the paradigm of their evolution continue to be refined. Nevertheless, the expression of CD45RA, CCR7, and/or CD127 has been used previously and provides a point of reference for comparison to studies of other human viral infections

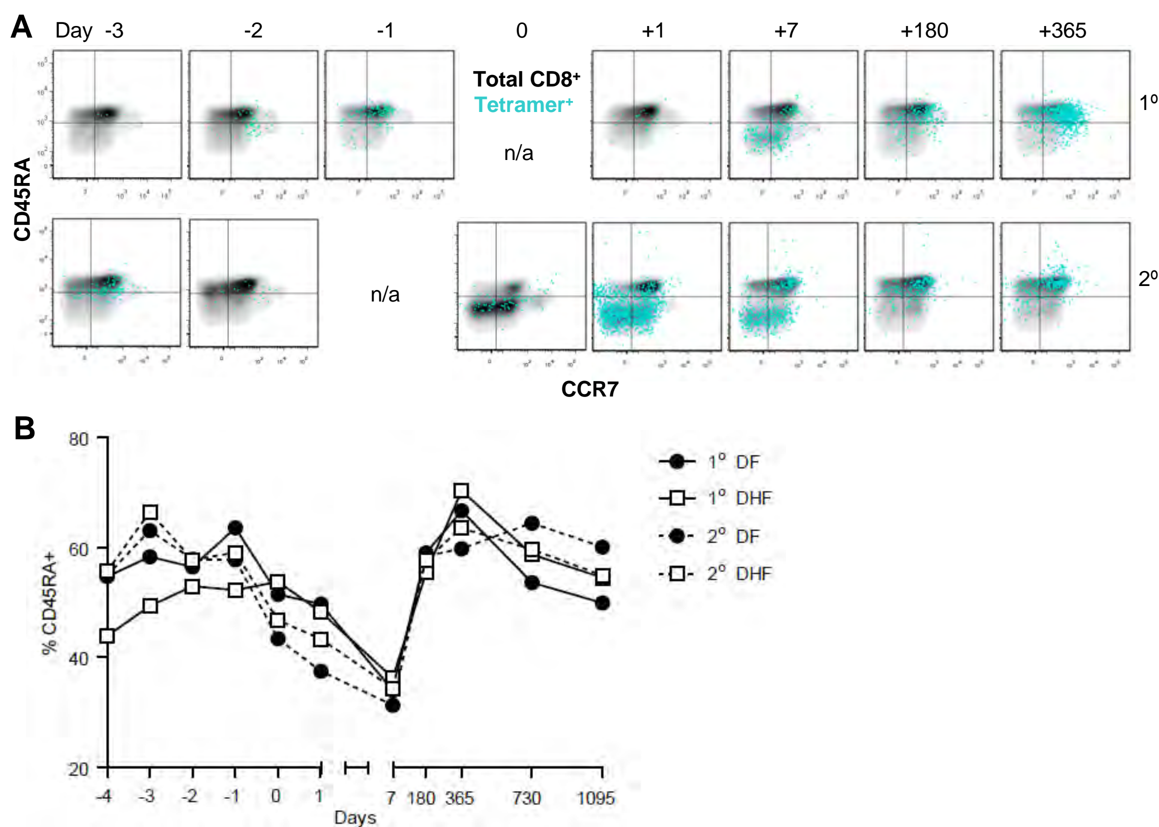

Figure 3.12. Effector-to-memory phenotypic evolution of epitope-specific T cells in DENV-infected subjects.

(A) Overlaid flow plots show the expression of CD45RA versus CCR7 of total $CD8^+$ T cells (black; density plots) and A11-$NS3_{133}$ $tetramer^+CD8^+$ T cells (blue; dot plots shown with large-sized dots) over time in PBMC from representative subjects with primary (1º) or secondary (2º) infections. (B) The percentage of $CD45RA^+tetramer^+CD8^+$ T cells from subjects with primary (solid lines) and secondary (dotted lines) DENV infections with either DF (closed circles) or DHF (open squares) over time. Data are presented as means within each subject group.

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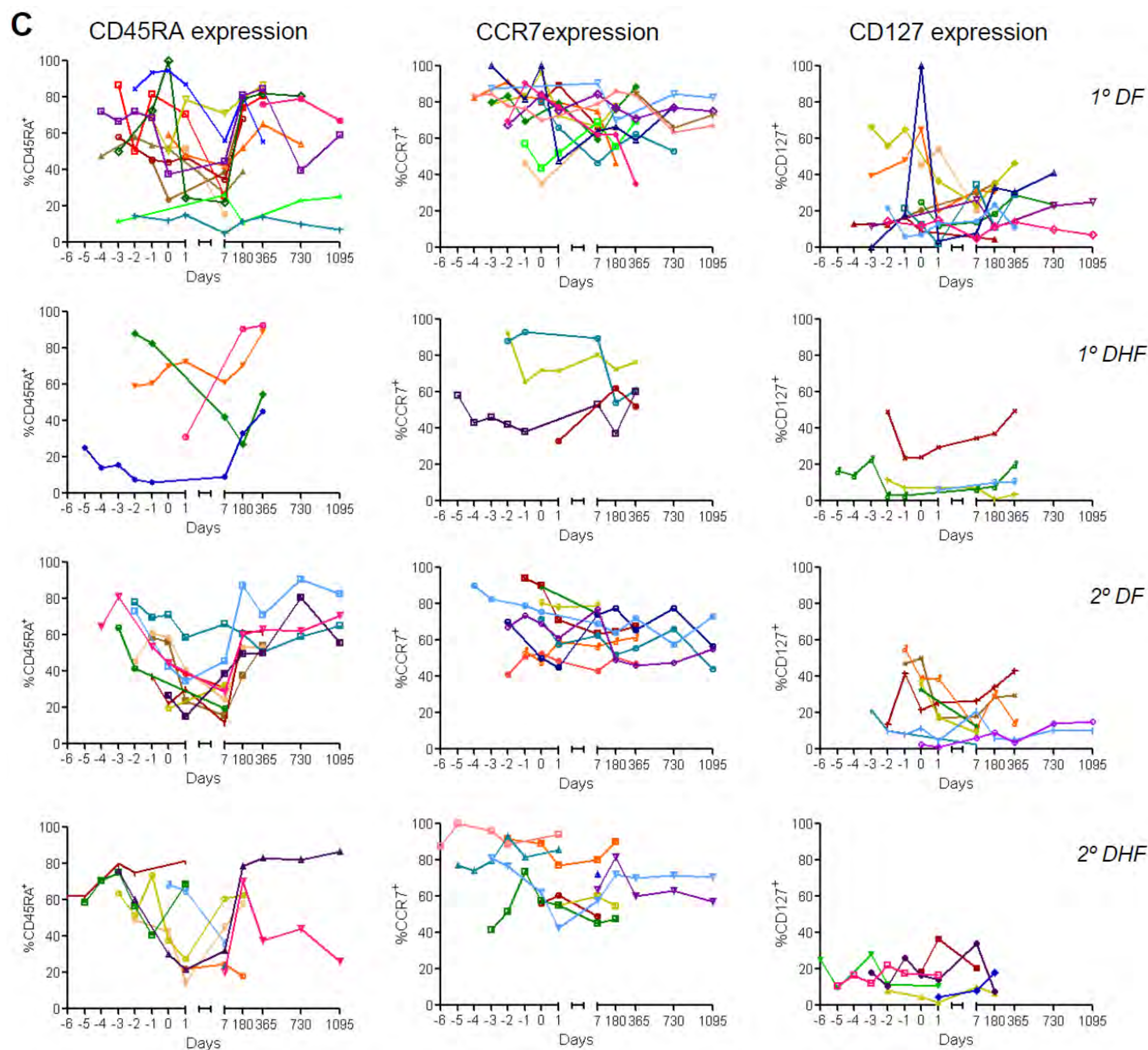

Figure 3.12. Effector-to-memory phenotypic evolution of epitope-specific T cells in DENV-infected subjects. (*cont'd*)

...(C) Line graphs show frequencies of tetramer+CD8+ T cells expressing CD45RA, CCR7 or CD127 over time. Each line represents an individual subject. The donors are grouped according to serologic response (primary [1º] or secondary [2º]) and clinical diagnosis (DF or DHF). Days are relative to the day of defervescence (d0).

(Precopio*, et al.*, 2007; Miller*, et al.*, 2008; Akondy*, et al.*, 2009). We therefore assessed the expression of these markers by DENV-specific T cells to compare the evolution of T cell phenotype over time in primary and secondary infections.

The expression of CD45RA and CCR7 declined on both tetramer-positive and total $CD8^+$ T cells during acute infection followed by re-expression of both markers in convalescence (Figure 3.12A). The decline of the frequency of $CD45RA^+$ tetramer-positive T cells during acute infection was consistent across all four subject groups (primary DF, primary DHF, secondary DF, secondary DHF), reaching its nadir in early convalescence; these changes occurred slightly earlier and to a greater extent in subjects with secondary infection compared to those with primary infection (Figure 3.12B). CD45RA expression rebounded with similar kinetics on tetramer-positive T cells from all subjects by 6 months to 1 year post-infection. A relatively low frequency of tetramer positive T cells expressed CD127 during acute infection, which appeared to start increasing late into convalescence (Figure 3.12C).

H. Chapter summary

Serotype-cross-reactive memory T cells reactivated during a secondary heterologous DENV infection have been hypothesized to help trigger severe manifestations of dengue disease. We compared the frequency, kinetics and phenotype of DENV epitope-specific T cells in a pediatric Thai cohort undergoing naturally-acquired primary or secondary DENV infections. Using peptide-MHC tetramers specific for two previously identified immunodominant DENV epitopes, together with activation

and phenotypic markers, we performed a longitudinal analysis of epitope-specific $CD8^+$ T cells in PBMC of a large cohort of patients during, immediately following and up to three years after acute infection. We detected robust expansion of HLA-A*1101-restricted $NS3_{133-142}$ and HLA-B*07-restricted $NS3_{222-231}$-specific T cells with an activated phenotype during and shortly after the febrile phase in PBMC of patients with both mild and severe disease. No correlation was found between A11-$NS3_{133}$ T cell frequency and disease severity. Contrary to our expectations, patients with primary infection had high frequencies of serotype-cross-reactive T cells, indicating that heterotypic tetramer binding is not only a result of secondary infections. Our study demonstrates that activated T cells are present during acute DENV infection, supporting the potential for them to contribute to the pathogenesis of dengue disease; however, the frequency of T cells specific for any single epitope is not the principal determinant of disease severity.

CHAPTER IV

MEMORY $CD8^+$ T CELLS FROM SUBJECTS WITH NATURALLY-ACQUIRED PRIMARY DENGUE VIRUS INFECTION ARE HIGHLY CROSS-REACTIVE

Our laboratory and others have demonstrated the ability of DENV-specific T cells to recognize multiple DENV serotypes. Most of these studies analyzed PBMC either from donors that received candidate live-attenuated monovalent vaccines or naturally-infected patients who experienced a secondary DENV infection. One recent study reported cytokine secretion and degranulation from epitope-specific T cells directly *ex vivo* from the PBMC of naturally-infected Thai children (Duangchinda*, et al.*, 2010). Our data in Chapter III also demonstrated cytokine secretion directly *ex vivo* in Thai children with natural primary infections. We found that these T cell responses occurred after stimulation with peptide variants specific for heterologous serotypes to a greater extent than after homologous stimulation. T cells from subjects with primary infection are primed to respond in secondary DENV infection, so it is of particular importance to analyze the mechanisms that naturally-generated memory DENV-specific T cells use to respond to homologous versus heterologous serotypes.

We wanted to extend our *ex vivo* observations of serotype-cross-reactivity by investigating the breadth of $CD8^+$ T cells that comprise the A11-$NS3_{133}$ epitope-specific response. We also investigated whether the three A11-$NS3_{133}$ epitope variants induce different signals that result in different functional outcomes. We studied homologous and heterologous T cell responses in primary DENV-immune PBMC after short-term bulk

culture and isolated epitope-specific $CD8^+$ T cell lines to determine functional serotype-cross-reactivity at the T cell clonal level.

A. Cross-reactive T cells expand after homologous and heterologous stimulation of PBMC from primary DENV-immune subjects

Since frequencies of virus-specific T cells are low *ex vivo*, we wanted to assess functional responses after short-term bulk culture. HLA-A11 is a prevalent haplotype in DENV-endemic regions (in particular, Thailand) so we decided to use serotype-specific variants of the A11-$NS3_{133}$ DENV epitope (Mongkolsapaya*, et al.*, 2003) to expand epitope-specific T cells *in vitro*. The DENV-2 variant (pD2) as well as the DENV-3 and -4 variant (pD3/4) each differ from the DENV-1 variant (pD1) by a single amino acid (refer to Table 2.1). We used convalescent PBMC from four DENV-immune HLA-A*1101^+ individuals (Table 4.1). Donor 1 was immunized with a live-attenuated, experimental DENV-1 vaccine and was included due to a well-defined DENV infection history and the availability of large sample volumes. We also had access to large sample volumes from donor 2, who experienced a naturally-acquired DENV infection several years prior; the details of the subject's serotype of infection or DENV infection history were unknown. Donors 3 and 4 were Thai subjects with natural primary DENV-3 infections for whom we also had data from the *ex vivo* study (Chapter III). For this study, PBMC from each subject were stimulated with each of the three epitope variants.

After approximately two weeks in culture, pMHC tetramer staining of bulk cultures from all four subjects revealed a modest enrichment of epitope-specific $CD8^+$ T

Table 4.1. Summary of HLA-A*1101^{+} primary DENV-immune subjects and clinical information.

Donor	Serology	Serotype (Exposure)	Diagnosis[a]	Age Range[b]	Country of Origin	MHC Class I			Time point of PBMC[c]
						A	B	C	
1	Primary	DENV-1 (vaccine)	n/a[d]	Adult	U.S.A.	2,11	27,40	1,3	8 months
2	Unknown	Unknown (natural)	DF	Child	Philippines	11,24	35,40	7,7	Years
3	Primary	DENV-3 (natural)	DHF I	Child	Thailand	2,11	13,38	7	6 months
4	Primary	DENV-3 (natural)	DHF II	Child	Thailand	11,24	18,54	7,8	1 week

[a]*according to WHO guidelines; DF = dengue fever, DHF = dengue hemorrhagic fever*

[b]*at time of infection*

[c]*post-defervescence*

[d]*subject experienced mild dengue-like illness post-vaccination; see Green, et al., 1993*

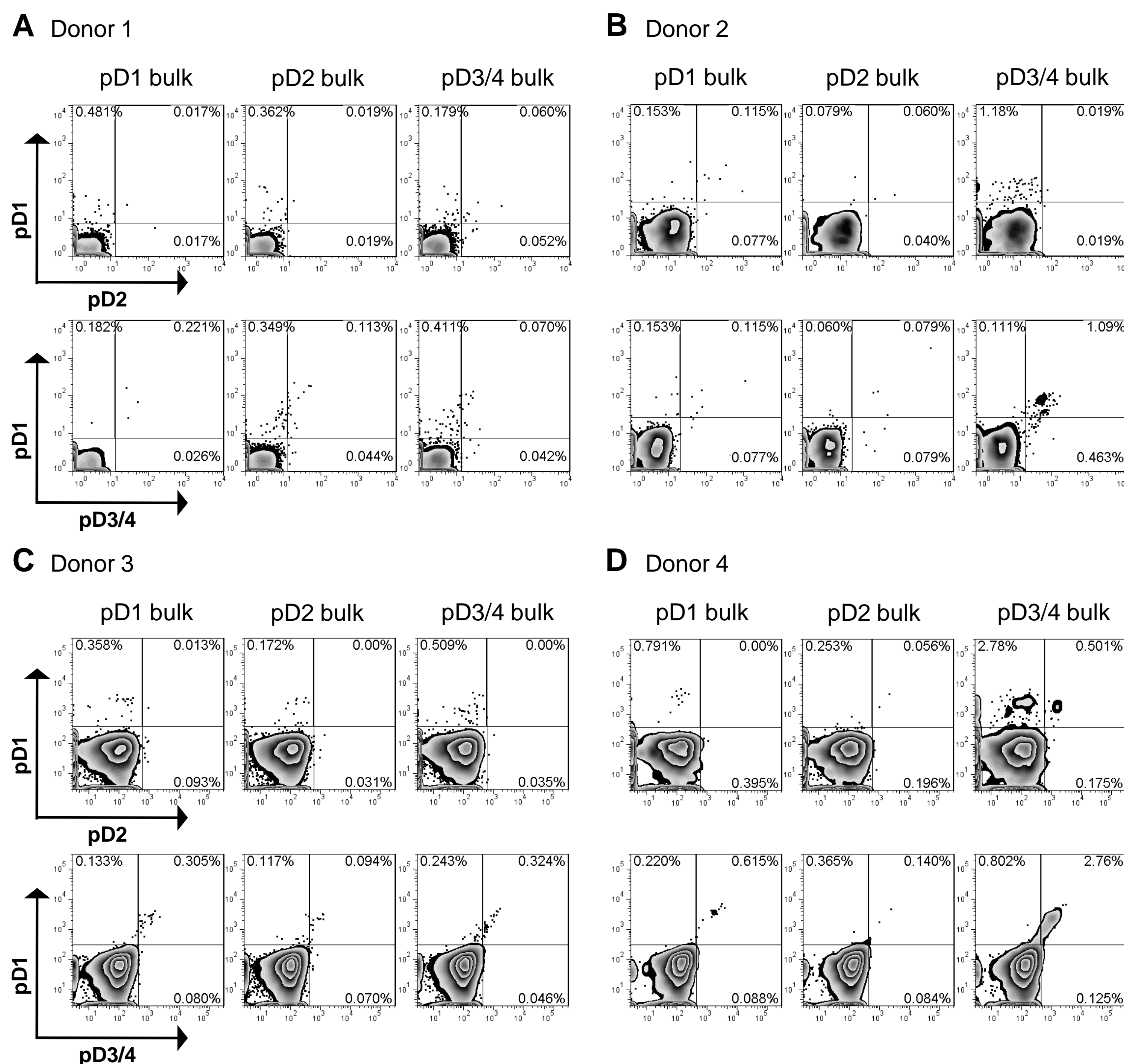

FIGURE 4.1. A11-NS3$_{133}$ peptide-stimulated bulk cultures from four A11^{+} DENV-immune subjects demonstrate serotype-cross-reactivity with preference for the DENV-1 variant pMHC tetramer.

After approximately two weeks, peptide-stimulated bulk cultures from (A) donor 1, (B) donor 2, (C) donor 3, or (D) donor 4 were stained with epitope-specific pMHC tetramers. Shown is dual tetramer staining (pD1/pD2 or pD1/pD3/4) for donor 1 bulk cultures and triple tetramer staining (pD1/pD2/pD3/4) for donors 2, 3, and 4 bulk cultures.

cells (Figure 4.1). Regardless of the subject or peptide variant used for stimulation, nearly all of the expanded tetramer-positive T cells bound the DENV-1 variant tetramer. Therefore, to increase the frequency of epitope-specific T cells, we magnetically sorted for pD1 tetramer-positive cells. For donor 1 (1° DENV-1), we tetramer-sorted half of each bulk culture (followed by non-specific anti-CD3 stimulation) while we re-stimulated the other half with the same peptide variant originally used. Subsequent tetramer staining revealed complex patterns of serotype-cross-reactive T cells, which were similar regardless of whether the bulk culture was tetramer-sorted or unsorted (though epitope-specific T cell frequencies were higher in the sorted bulk cultures; Figure 4.2a and b). T cells expanded by pD1 stimulation had $pD1^+$ and $pD1^+2^+$ or $pD1^+3/4^+$ populations, as determined by dual tetramer staining (pD1/pD2 tetramer staining or pD1/pD3/4 tetramer staining). T cells expanded by pD2 or pD3/4 stimulation also showed $pD1^+$ T cells when stained with pD1/pD2 tetramers, but the majority of these appeared to be $pD1^+3/4^+$ when stained with pD1/pD3/4 tetramers. These data suggested that only pD1 stimulation could expand pD1 serotype-specific ($pD1^+$) T cells, in addition to serotype-cross-reactive ($pD1^+2^+$ and $pD1^+3^+$) T cells, whereas pD2 and pD3/4 stimulation mostly expanded serotype-cross-reactive T cells. However, single tetramer staining of the sorted bulk cultures with the pD3/4 tetramer indicated (by comparison of frequencies) that the "pD1 serotype-specific" ($pD1^+$) T cells were actually serotype-cross-reactive but of lower avidity for the pD2 and pD3/4 tetramers and preferentially bound the pD1 tetramer when it was present.

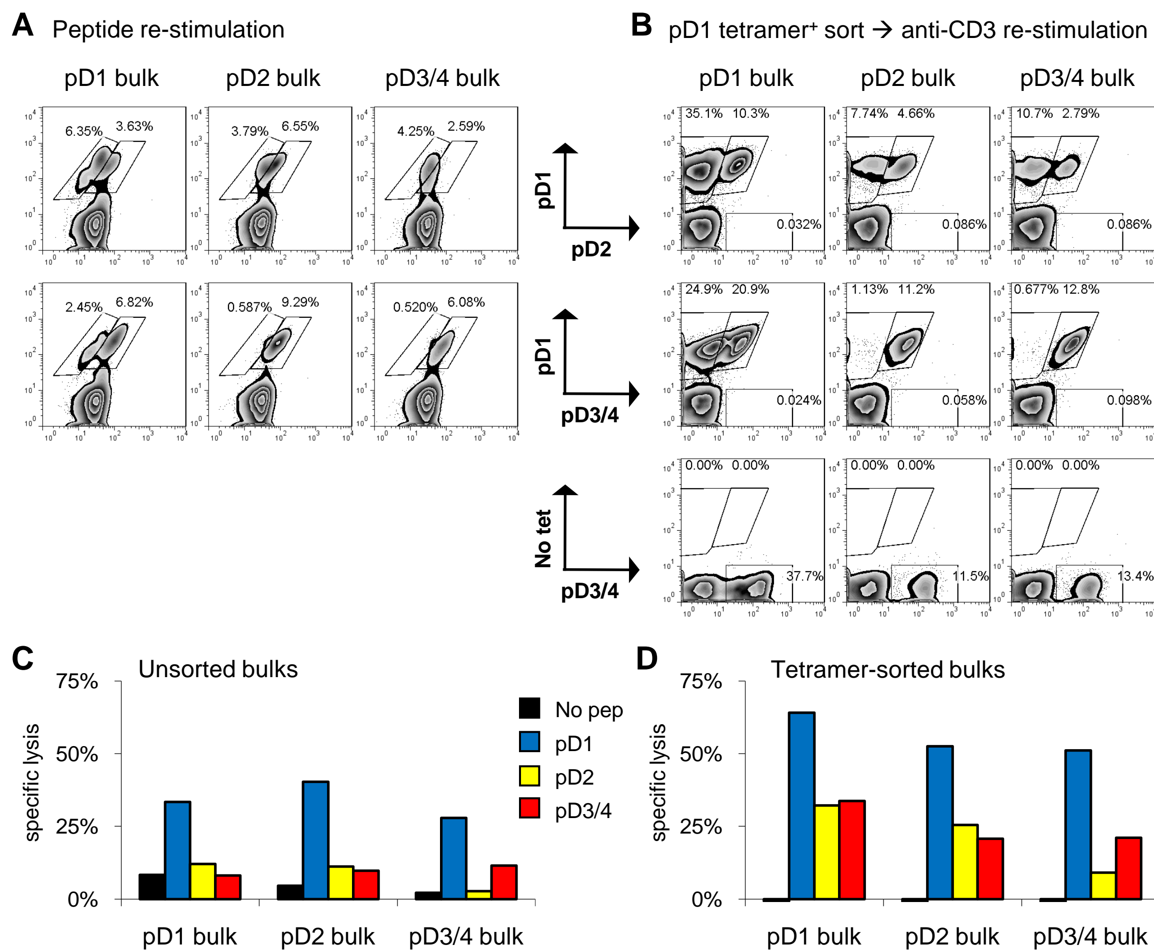

FIGURE 4.2. A11-NS3$_{133}$ peptide-stimulated bulk cultures from donor 1 show marked serotype-cross-reactivity with preference for DENV-1.

After two weeks, peptide-stimulated bulk cultures from donor 1 were (A) re-stimulated with peptide or (B) magnetically sorted for pD1 tetramer^{+} cells before non-specific re-stimulation. Shown is dual or single tetramer staining for each bulk culture. (C) Unsorted or (D) tetramer-sorted bulk culture cells were incubated for 4 hours with ^{51}Cr-labeled autologous BLCL coated with pD1 (blue), pD2 (yellow), pD3/4 (red), or no peptide (black) to induce peptide-specific lysis. This assay used an effector-to-target ratio of 25:1.

We also wanted to determine whether serotype-cross-reactive tetramer binding reflected cross-reactive functional responses (Figure 4.2c and d). Tetramer-sorted bulk cultures lysed autologous B-lymphoblastoid cell line (BLCL) target cells coated with any of the three epitope variants, with the highest response to $pD1^{+}$ BLCL. Unsorted bulk culture cells were only able to lyse BLCL coated with pD1, whereas lysis of $pD2^{+}$ or $pD3/4^{+}$ BLCL was minimal (even in pD2 and pD3/4 stimulated bulk cultures). Cytolysis of $pD1^{+}$ target cells was lower in unsorted bulk cultures compared to tetramer-sorted bulk cultures at the same effector-to-target ratio, which may reflect the frequency of epitope-specific T cells (which was substantially higher in the sorted bulks). These findings suggest that sorting A11-$NS3_{133}$ epitope-specific bulk cultures using the pD1 tetramer enriched for epitope-specific T cells and maintained the same pattern of tetramer staining and function as unsorted cells.

B. $CD8^{+}$ T cell lines isolated from subjects who had primary DENV infection demonstrate variable degrees of serotype-cross-reactivity

We next wanted to isolate individual epitope-specific T cell clones to assess the breadth of cell types that compose the A11-$NS3_{133}$-specific T cell repertoire. We used the bulk cultures generated above to seed limiting dilution assay plates at 10 or 3 cells per well (Figure 4.3). Epitope-specific T cell lines were chosen on the basis of their ability to selectively lyse peptide-coated HLA-A*1101^{+} BLCL target cells and were subsequently characterized with regard to peptide dose-dependent cytotoxicity as well as pMHC tetramer staining.

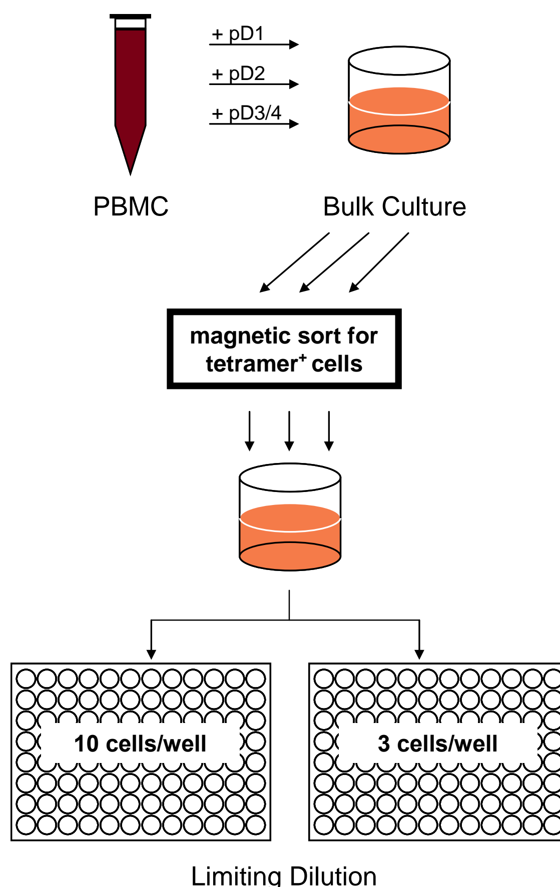

FIGURE 4.3. Generation of A11-NS3$_{133}$ epitope-specific T cell lines.

Protocol used to generate and isolate epitope-specific CD8^{+} T cells lines from the PBMC of four DENV-immune donors. PBMC were stimulated with 10µg/mL of one of the three A11-NS3$_{133}$ epitope variants to generate three peptide-specific bulk cultures. Each bulk culture was magnetically-sorted for pMHC tetramer-positive T cells before seeding at 10 or 3 cells/well in limiting dilution assays. Epitope-specific T cell lines were selected for further analysis on the basis of specific lysis of HLA-A*1101^{+} BLCL coated with A11-NS3$_{133}$ peptide.

We isolated three types of epitope-specific T cell lines: pD1 serotype-specific, pD1-3/4 cross-reactive, and pD1-2-3/4 cross reactive. Data from representative cell lines are shown in Figure 4.4. Each T cell line was stained with each individual tetramer in order to assess its ability to recognize the three peptide variants. In general, pMHC tetramer binding reflected the ability to lyse target cells coated with the same peptide in ^{51}Cr release assays.

Of the thirty-three cell lines that were established, the majority demonstrated serotype-cross-reactivity by pMHC tetramer staining regardless of the donor or bulk culture from which they originated (Table 4.2). The extent of pMHC tetramer binding did not always predict the magnitude of the cytolytic response. For example, the cell line 10D6 from donor 3 demonstrated greatest binding to the pD1 tetramer while its cytolytic response was comparable toward targets coated with any of the three peptides. In addition, the lack of binding to a given tetramer did not necessarily indicate that the cell line could not mount a functional response against that particular peptide variant. For example, the cell line 10D7 did not bind the pD2 or pD3/4 tetramers yet demonstrated robust cytolysis of pD2 and pD3/4 peptide-coated target cells. Notably, there were no cases in which a cell line bound a given tetramer and did not respond in ^{51}Cr release assays towards BLCL coated with that particular peptide variant. Thus, discrepancies between tetramer binding and cytolytic responses likely reflect lower avidity of a soluble pMHC tetramer compared to the pMHC complex presented on the surface of an antigen presenting cell.

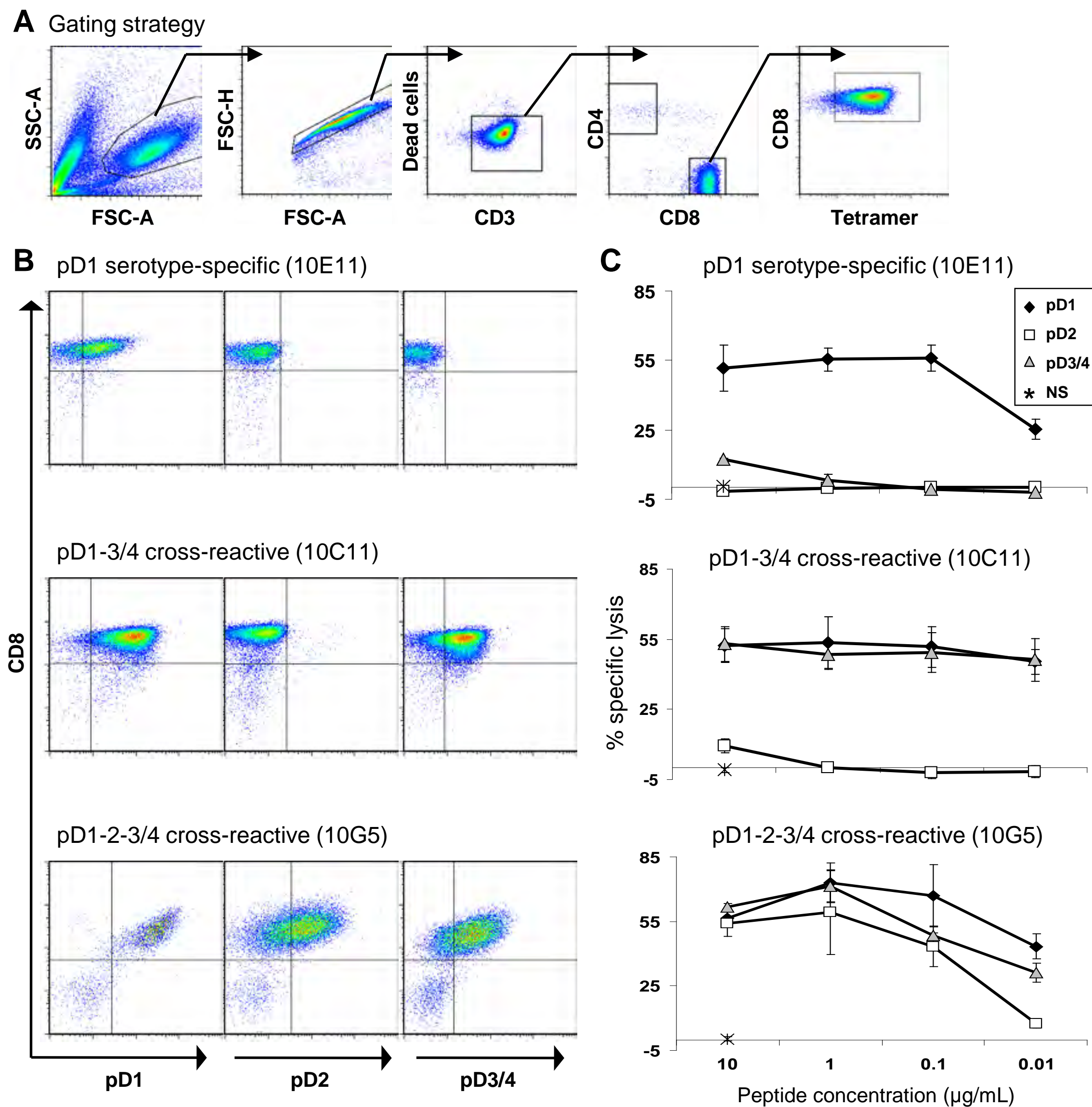

FIGURE 4.4. Three predominant patterns of serotype-cross-reactivity amongst A11-NS3$_{133}$-specific T cell lines.

(A) Gating strategy used to identify tetramer^{+}CD8^{+} T cells. (B) Cell lines were stained with each of the three tetramer variants and reveal three types of serotype-cross-reactivity: pD1 serotype-specific, pD1-3/4 cross-reactive, pD1-2-3/4 cross-reactive. Cells were gated on live, CD3^{+} singlet lymphocytes. (C) ^{51}Cr release assays demonstrate peptide dose-dependent cytolytic activity of representative T cell lines at an effector-to-target ratio of 10:1. NS = no stimulation.

Table 4.2. Lytic activity and tetramer specificity of A11-$NS3_{133}$ epitope-specific T cell lines.

Donor	Peptide stimulation (bulk)	Cell line	Tetramer specificity[a]			% Specific Lysis[b]											
			pD1	pD2	pD3/4	[pD1]				[pD2]				[pD3/4]			
1	pD1[c]	10B4[d]	+++	--	++	71	32	<0	<0	<0	<0	<0	<0	29	39	29	<0
		10B8	++	++	++	59	---	---	---	49	48	40	16	55	45	61	42
		10D6[d]	+++	--	++	58	34	5	1	1	0	0	1	40	37	28	3
		10D12	+++	--	++	53	54	57	38	24	10	2	1	53	51	47	45
		10E11	++	--	--	51	55	55	25	<0	<0	<0	<0	12	3	<0	<0
		10H11[d]	+++	++	++	61	30	4	2	49	49	65	47	49	40	36	51
	pD2	10A1[d]	+++	--	++	49	35	13	9	37	28	27	1	44	44	41	50
		10A3	+++	+/-	+	49	56	70	60	44	44	9	2	57	84	59	87
		10D7	++	--	--	50	55	62	37	37	27	8	1	49	78	45	65
		10E12	+++	+/-	+++	76	66	57	43	61	57	24	3	97	86	86	81
		10F9	+++	--	+/-	49	58	62	47	36	38	19	1	44	73	44	50
		10H3	+++	+/-	+++	72	64	59	68	83	76	55	23	89	82	84	76
	pD3/4	10A7[d]	+++	--	++	13	0	<0	2	0	<0	1	<0	20	22	22	20
		10D5[d]	+++	--	++	57	48	16	5	31	25	36	10	41	34	37	41
		10D9[d]	+++	--	++	38	23	<0	<0	16	13	10	<0	18	20	22	30
		10E5[d]	+++	--	++	50	24	0	4	12	1	3	<0	38	34	36	41
		10H7[d]	+++	--	++	42	15	9	7	6	4	5	2	42	49	39	38
		10H9[d]	+++	--	--	41	34	18	5	32	31	25	8	29	27	10	5

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Table 4.2. Lytic activity and tetramer specificity of A11-NS3$_{133}$ epitope-specific T cell lines (*cont'd*)

Donor	Peptide stimulation (bulk)	Cell line	Tetramer specificity[a]			% Specific Lysis[b] [pD1]				[pD2]				[pD3/4]			
			pD1	pD2	pD3/4												
2	pD1[c]	10D4[d]	+	--	+++	18	1	2	0	0	0	<0	1	26	27	19	2
	pD2	3B3[d]	++	--	+++	29	1	2	<0	1	2	<0	1	39	41	31	25
		3C7[d]	++	--	+++	27	0	<0	0	<0	<0	<0	<0	48	45	35	23
	pD3/4	3D10[d]	+++	--	+++	19	0	<0	<0	<0	<0	<0	<0	24	21	6	<0
		3G10[d]	+++	--	+++	24	1	1	3	<0	<0	<0	1	20	18	5	<0
		3G11[d]	+++	--	+++	32	4	<0	<0	<0	<0	<0	2	27	19	14	2
3	pD3/4	10A6	+++	++	+	54	61	45	71	66	38	42	5	37	62	39	23
		10C6	+++	+	++	65	58	65	72	70	42	31	4	56	72	48	23
		10D6	+++	+/-	+	33	41	43	70	50	46	40	4	48	48	61	36
		10F5	+++	+	++	68	71	60	92	48	31	28	5	56	52	50	29
		10G5	+++	+	++	57	73	67	43	54	59	43	8	62	71	48	31
		10H5	+++	+	++	62	64	47	66	48	23	31	4	54	45	50	28
4	pD3/4	10C1[d]	+	--	+	44	17	<0	<0	<0	1	0	<0	32	38	46	56
		10C11	+++	--	+++	53	54	53	46	10	1	<0	<0	54	49	50	47
		10F12	+++	--	+++	59	60	60	35	<0	0	0	1	55	57	55	56

[a]*based on staining with each tetramer variant individually; --, did not bind tetramer; +/-, <%30 bound tetramer; +, <%60 bound tetramer; ++, <%90 bound tetramer; +++, ≥%90 bound tetramer*

[b]*[peptide] = 10, 1, 0.1, 0.01 μg/mL; numbers indicate average % specific lysis relative to negative (no peptide) control, n ≥ 3*

[c]*This bulk culture was generated using the 11mer variant of the pD1 peptide, GTSGSPIVNR**E**, which was originally identified in Mongkolsapaya, et al., 2003*

[d]*Data generated for this cell line using pD1 was performed with the 11mer variant of the pD1 peptide*

C. TCRs on A11-NS3$_{133}$ epitope-specific T cells comprise select V$_{\beta}$ chains regardless of serotype specificity

We wanted to analyze the V$_{\beta}$ gene segments that compose A11-NS3$_{133}$ epitope-specific TCRs in order to determine whether particular V$_{\beta}$ chains were associated with varying degrees of serotype-cross-reactivity, as had been suggested previously for a different DENV epitope (Livingston*, et al.*, 1995). Surface staining on selected T cell lines showed preference for three V$_{\beta}$ chain families: 8, 21.3 and 23 (Table 4.3). No correlation was observed between particular V$_{\beta}$ chains and serotype-cross-reactivity profiles.

D. The three A11-NS3$_{133}$ epitope variants have different predicted peptide-MHC binding affinities

To ascertain potential differences in the ability of these epitope variants to bind HLA-A*1101, we employed three peptide-MHC binding prediction algorithms provided by the Immune Epitope Database and Analysis Resource website. The three epitope variants were calculated to have comparable binding scores, though a consistent hierarchy of pD3/4 > pD1 >> pD2 did emerge (Table 4.4). The differences between these peptides largely reflected the hierarchy of potency seen for them in ^{51}Cr release assays with our epitope-specific CD8^{+} T cell lines.

Table 4.3. A11-NS3$_{133}$-specific TCRs show preference for select V_β chains.

Serotype-cross-reactive profile[a]	**Cell line**[b]	**V_β chain**[c]
pD1 serotype-specific	10E11	8
pD1-3/4 cross-reactive	10B4	8
	10D12	21.3
	10D4	21.3
	3D10	23
	3G11	23
	10C1	21.3
	10C11	21.3
	10F12	8
pD1-2-3/4 cross-reactive	10B8	none[d]
	10D5	21.3

[a]*As determined by patterns of tetramer staining and cytotoxicity*

[b]*8/11 cell lines stained ≥ 97% with a single V_β chain-specific antibody; 10B4 was ≥ 93% specific; 10D5 was ~86% specific*

[c]*The V_β nomenclature system used here follows that by Wei, et al., 1994*

[d]*This cell line was negative for the 24 V_β chains included in the staining kit*

Table 4.4. Peptide-MHC binding predictions of A11-NS3$_{133}$ epitope variants.

Peptide variant	MHC-I Binding Prediction (nM)[a]		
	ann[b]	smm	arb
pD1	87.5	102.1	52.0
pD2	185.2	222.5	136.3
pD3/4	52.8	71.1	37.4

[a]*Determined by the Immune Epitope Database and Analysis Resource T cell epitope prediction tool for peptide binding to HLA-A*1101*

[b]*MHC class I binding prediction methods: ann=Artificial neural network, smm=Stabilized matrix method, arb=Average relative binding*

E. A11-NS3$_{133}$ peptide variants elicit a hierarchy of functional responses from epitope-specific CD8$^+$ T cells

We next wanted to determine whether cytokine response profiles of individual CD8$^+$ T cell lines correlated with lytic activity. Therefore, we assessed degranulation and cytokine expression following stimulation with different concentrations of homologous and heterologous peptides in intracellular cytokine staining (ICS) assays (Figures 4.5 and 4.6). ICS of the pD1 serotype-specific cell line 10E11 revealed a robust response to high concentrations of homologous peptide; most cells up-regulated all four effector functions studied: degranulation (CD107a staining) and production of MIP-1β, TNFα, and IFNγ (Figure 4.5B). Conversely, stimulation with pD2 resulted in no detectable response. Stimulation with pD3/4 caused a low percentage of CD8$^+$ T cells to respond. Of the responding T cells, most only produced MIP-1β, fewer cells were MIP-1β$^+$CD107a$^+$, and even fewer were MIP-1β$^+$CD107a$^+$TNFα$^+$; no cells produced IFNγ.

Stimulation of pD1-3/4 cross-reactive cell lines (as shown for the representative line 10C11 in Figure 4.5C) with either pD1 or pD3/4 induced nearly all CD3$^+$CD8$^+$ cells to respond, and a majority of cells in the culture expressed all four effector functions. On the other hand, pD2 stimulation induced a low percentage of cells to respond, most of which only produced MIP-1β. Akin to the cytolytic response, most cells of pD1-2-3/4 cross-reactive cell lines (represented by 10B8 in Figure 4.5D) responded with all four effector functions upon stimulation with any of the three peptide variants.

Regardless of a cell line's recognition of different peptide variants, its response to stimulation exhibited a hierarchical induction of MIP-1β > degranulation > TNFα >

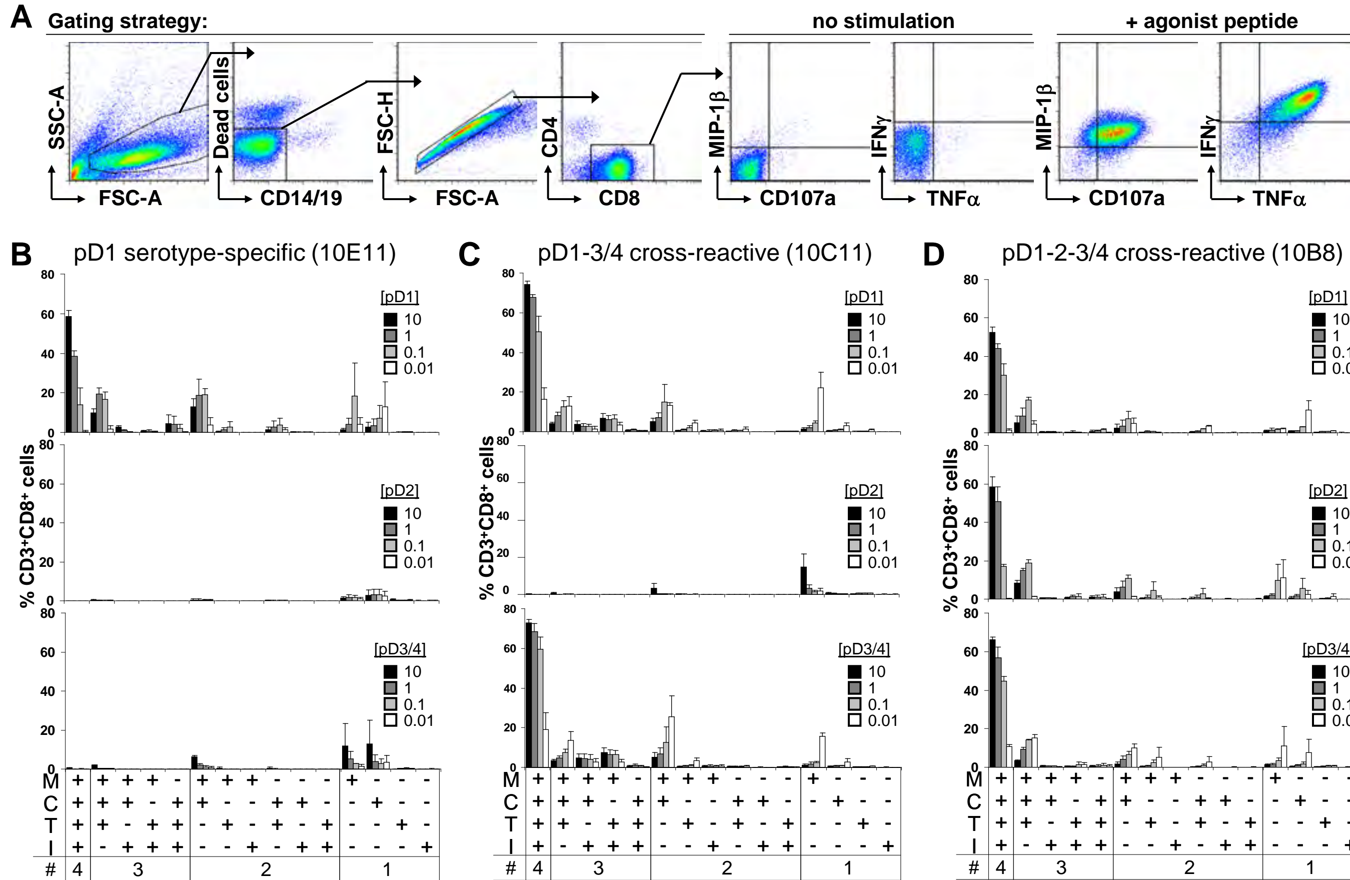

FIGURE 4.5. Hierarchical response of effector functions in epitope-specific T cells.

(A) Gating strategy and representative flow plots of a cell line in response to the absence or presence of agonist peptide. (B) pD1 serotype-specific, (C) pD1-3/4 cross-reactive, and (D) pD1-2-3/4 cross-reactive cell lines were stimulated with each of the A11-NS3$_{133}$ peptide variants in intracellular cytokine staining assays. All possible combinations of the four effector functions (MIP-1β [M], TNFα [T], and IFNγ [I] production as well as degranulation [C], as determined by CD107a staining) are displayed across the *x* axis and grouped according to the number (#) of effector functions exhibited. Mean frequencies of responding CD8^{+} T cells of 10E11 (n = 2), 10C11 (n = 4), and 10B8 (n = 2) are shown. Peptide concentrations are shown in μg/mL.

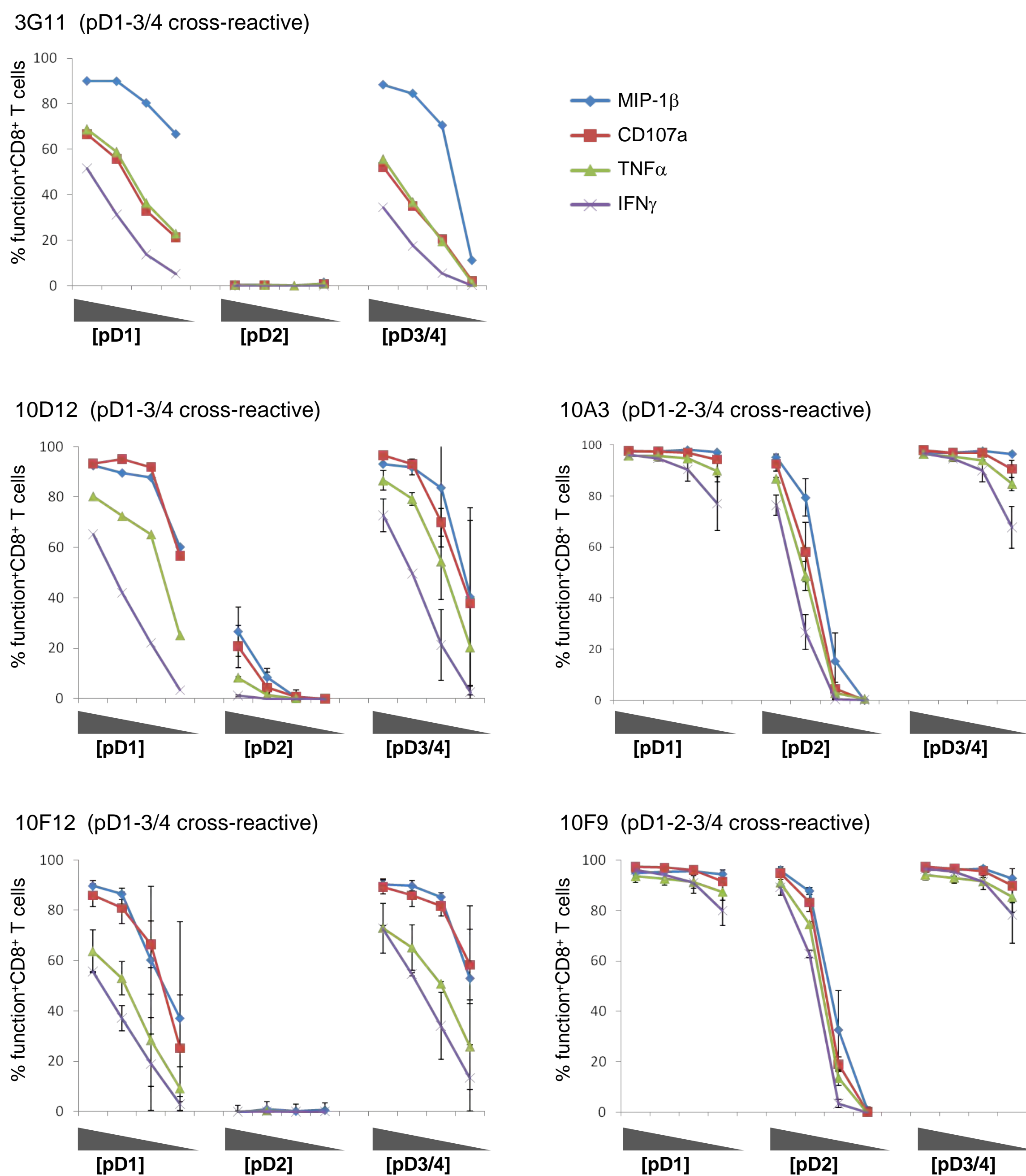

Figure 4.6. Different epitope-specific T cell lines show similar effector responses.

Line graphs show the frequency of $CD8^+$ T cells from five A11-$NS3_{133}$-specific T cell lines that responded to peptide stimulation in ICS assays by degranulation (CD107a; red) or production of MIP-1β (blue), TNFα (green), or IFNγ (purple). Data is shown as mean standard error and is relative to responses detected with no stimulation. Peptide concentrations were 10, 1, 0.1, and 0.01μg/mL. The name of each cell line is located to the top-left-hand side of the respective graph.

IFNγ, as evidenced by its peptide-dose response curve. With decreasing concentrations of agonist peptide (for example, pD1 and pD3/4 for the cell line 10C11), the effector response changed. As the overall frequency of responding cells decreased, their distribution shifted toward exhibiting fewer effector functions, in particular loss of IFNγ and TNFα expression to primarily producing MIP-1β. Below a critical concentration of agonistic peptide MIP-1β production also dropped off, as demonstrated by low concentrations of a partial agonist peptide (for example, pD2 for the cell line 10C11). Data were consistent for multiple epitope-specific T cell lines (Figure 4.6) and suggested that a signal threshold is required to generate a polyfunctional effector response.

F. Cytokine responses induced by all three A11-NS3$_{133}$ epitope variants are susceptible to cyclosporine A.

To initiate an effector response, peptide-MHC complexes engage the TCR, which then initiates a series of protein phosphorylation events. The TCR is a part of the CD3 complex which also includes the ζ chain, one of the first proteins to become phosphorylated after pMHC-TCR engagement (refer to Introduction and Figure 1.1). This allows Zap70 to become activated and recruit other proteins which continue the phosphorylation cascade. Two major pathways are involved (calcium and MAP kinase) which ultimately activate transcription factors (NFκB, NFAT and AP-1) that initiate the transcription of immune response genes. Due to the different responses induced by pD2, compared to pD1 or pD3/4 (in pD1-3/4 cross-reactive T cell lines), we wanted to determine whether pD2 signaled via a pathway distinct from the other variants. To do

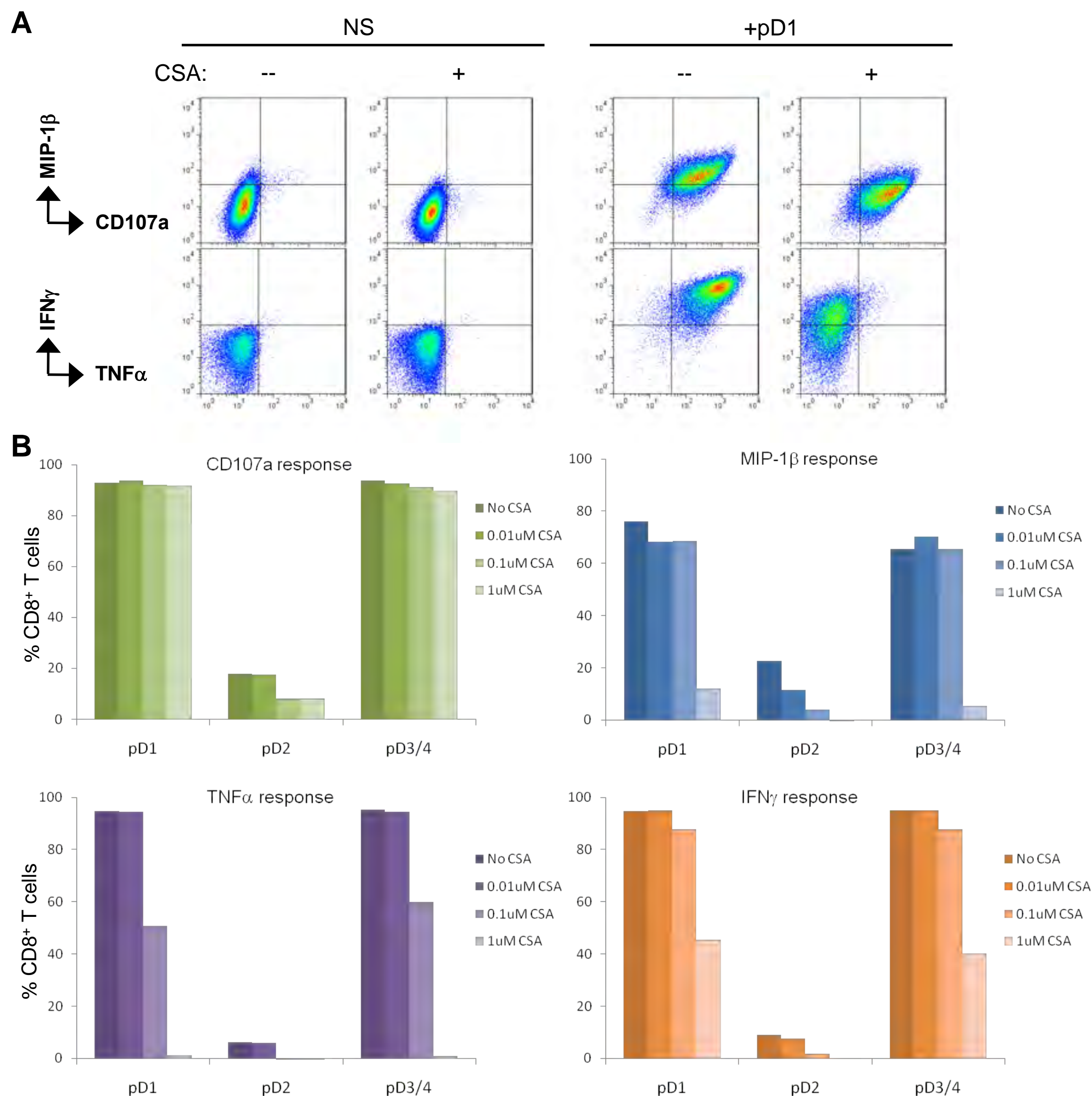

FIGURE 4.7. Cytokine responses, but not degranulation, elicited by all three A11-NS3$_{133}$ epitope variants are susceptible to cyclosporine A.

(A) Representative flow plots show the response of the cell line 10C11 to no stimulation (NS) or stimulation with pD1 in the presence or absence of cyclosporine A (CSA). (B) Bar graphs show the dose-dependent effect of CSA on degranulation (CD107a; green) and the production of MIP-1β (blue), TNFα (purple), and IFNγ (orange) by 10C11. The concentration of CSA is shown as μM.

this, we pre-treated T cells with cyclosporine A (CSA), a drug known to inhibit the calcium pathway by binding calcineurin and preventing it from activating NFAT (Shaw, *et al.*, 1995). CSA-treated T cells (10C11, pD1-3/4 cross-reactive) showed a dose-dependent decrease in their production of IFNγ, TNFα, and MIP-1β in ICS assays, whereas degranulation was minimally affected (Figure 4.7). These data indicate that cytokine responses elicited by these three dengue epitope variants all require NFAT activation, while the signal for degranulation is largely NFAT-independent.

G. Phosphorylation of ERK1/2 correlates with cytolysis, degranulation, and induction of TNFα and IFNγ but not MIP-1β production

To determine whether other pathways are involved in signaling after stimulation with each of the peptide variants, we utilized phosphoflow, a technique developed by Perez, *et al.* (Perez, *et al.*, 2005). Phosphoflow allows for the identification of phosphorylated proteins downstream of TCR signaling by flow cytometry. We first needed to optimize protocol conditions to accommodate stimulation of T cell lines by viral peptides (Figure 4.8).

We began by testing whether antigen presenting cells were necessary to help present exogenous peptide and induce robust phosphorylation of ERK1/2, a MAP kinase family member (refer to Figure 1.1). We used different concentrations of peptide in the presence or absence of antigen presenting cells using a previously established T cell clone specific for the influenza virus (Flu) $M1_{58-66}$ peptide. Levels of phosphorylated ERK1/2 (pERK1/2) were higher in T cells that were incubated with BLCL than in those

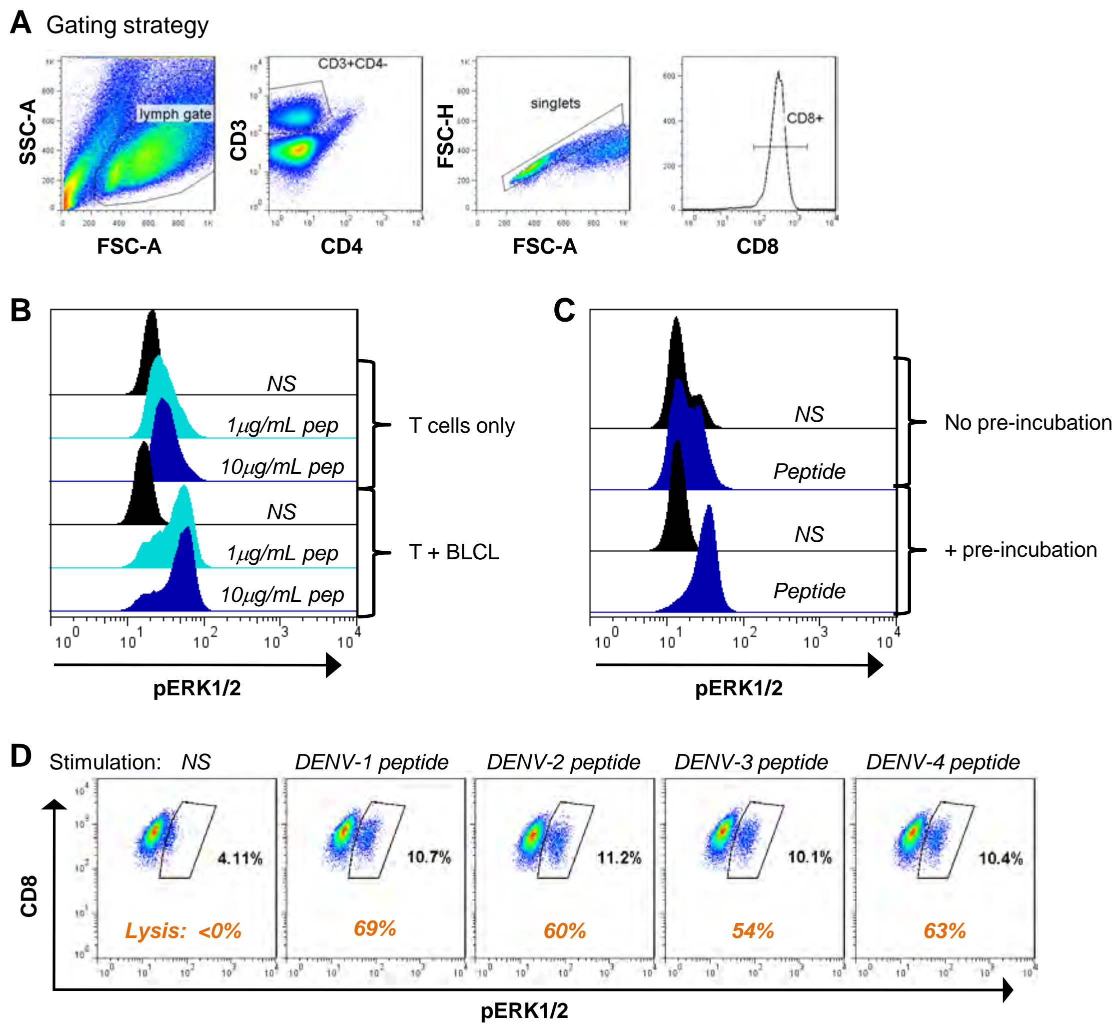

FIGURE 4.8. Optimization of the phosphoflow protocol.

(A) Gating strategy used to identifiy T cells for their expression of pERK1/2. (B) T cells with or without BLCL were stimulated in the presence (blue) or absence (black) of peptide for 10 minutes in a 37 C water bath to induce the phosphorylation of ERK1/2. (C) The Flu $M1_{58}$-specific T cell clone together with autologous BLCL and peptide was pre-incubated, or not, on ice for 30 minutes prior to being placed in the water bath as above. (D) The DENV $NS3_{529}$-specific T cell line 1C2 up-regulates pERK1/2 upon stimulation with peptide variants derived from all four DENV serotypes. Numbers in black indicate the frequency of pERK1/2^{+}CD8^{+} T cells. Percent specific lysis as determined by ^{51}Cr-release assay at an effector-to-target ratio of 25:1 is also shown (orange numbers). NS = no stimulation.

that were not, demonstrating the requirement of antigen presenting cells for robust peptide stimulation of a T cell line (Figure 4.8B). Because of the short incubation period required for detection of pERK1/2 (10 minutes), we questioned whether pre-incubation of T cells, antigen presenting cells, and peptide (on ice) would enhance the pERK1/2 signal by allowing cell-cell contacts to form before placing them into a 37°C water bath to enable kinase activity. T cells that were pre-incubated with BLCL and peptide on ice for 30 minutes had higher levels of pERK1/2 than those that were not pre-incubated (Figure 4.8C). We also tested whether cytokine- or serum-starvation of T cells would ensure low background levels of pERK1/2 but found no differences regardless of T cell culture conditions (data not shown). We next tested our protocol on a $CD8^+$ T cell line previously established in our lab (1C2; generated by Pra-On Supradish, unpublished data) and specific for a DENV epitope ($NS3_{529-537}$) and found that we could measure DENV-specific, peptide-induced pERK1/2 (Figure 4.8D). Similar to its ability to lyse peptide-coated BLCL, 1C2 demonstrated comparable induction of pERK1/2 after stimulation with epitope variants derived from all four DENV serotypes.

Stimulation of three representative A11-$NS3_{133}$-specific T cell lines with increasing concentrations of agonist peptide resulted in a dose-dependent increase of pERK1/2 (Figure 4.9). The phosphorylation of ERK1/2 correlated with the induction of cytolysis, degranulation, and TNFα and IFNγ production detected in ICS assays. This was not the case for MIP-1β production, however, which was observed under conditions that failed to demonstrate ERK1/2 phosphorylation. For instance, pD1 or pD3/4 stimulation of the pD1-3/4 cross-reactive cell line 10C11 resulted in increased pERK1/2.

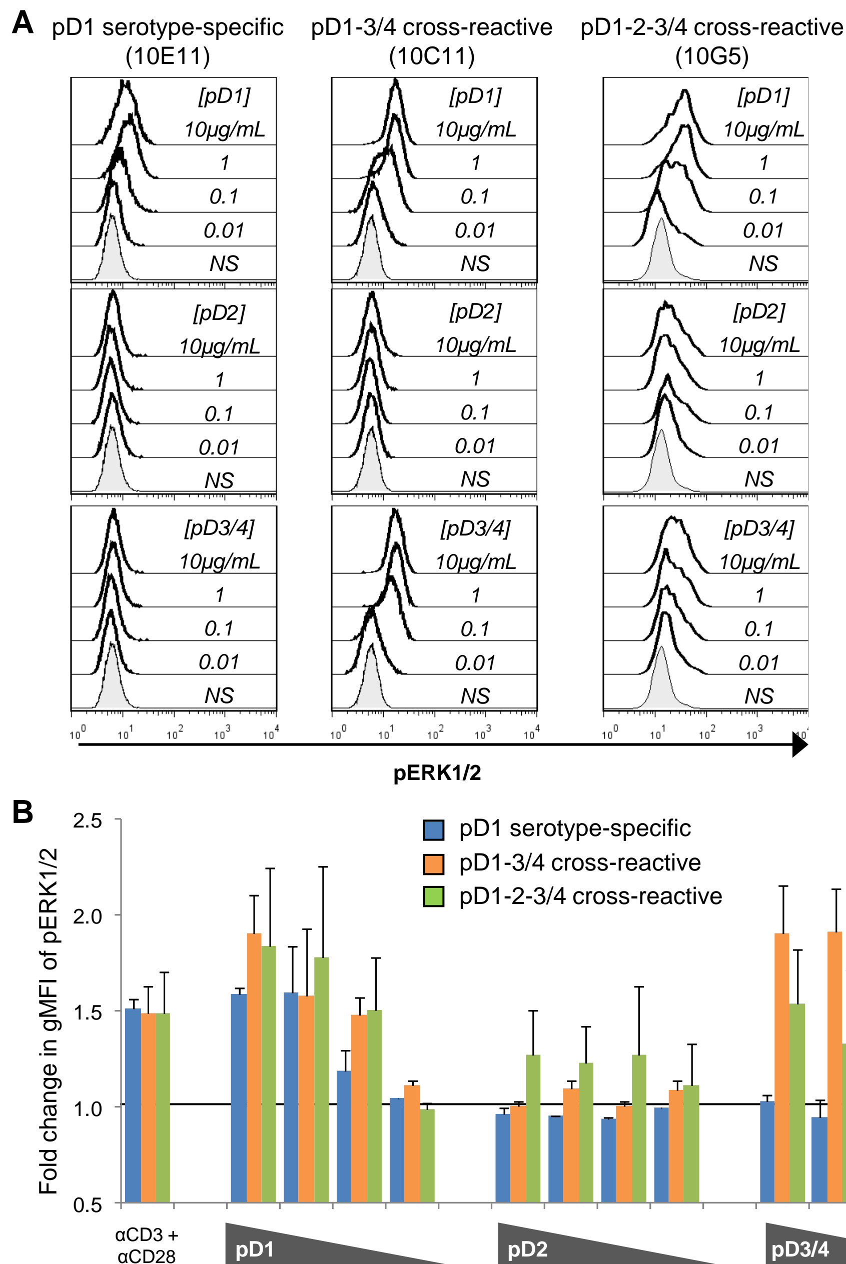

FIGURE 4.9. Phosphorylation of ERK1/2 does not correlate with MIP-1β production.

(A) Peptide dose-dependent phosphorylation of ERK1/2 after stimulation with epitope variants. (B) Average fold change in geometric mean fluorescence intensity (gMFI) of pERK1/2 in the cell lines 10E11 (n = 3), 10C11 (n = 4), and 10G5 (n = 2) after stimulation with decreasing concentrations (10, 1, 0.1, 0.01μg/mL) of the three peptide variants. Fold change is relative to ‘no stimulation’ control.

However, pD2 stimulation of 10C11, which up-regulated MIP-1β in ICS assays, failed to phosphorylate ERK1/2.

H. The DENV-2 A11-NS3$_{133}$ epitope variant acts as a partial agonist

Our data so far have indicated that the pD1 and pD3/4 epitope variants are strong agonists able to induce robust T cell responses using both the calcium and MAP kinase signaling pathways. As pD2 also signals through the calcium pathway, we speculated that pD2 stimulation may induce a delayed pERK1/2 signal which prevented us from detecting it in our previous assays. Therefore we assessed pERK1/2 at 5, 10, 20, and 30 minutes following stimulation of the pD1-3/4 cross-reactive cell line 10C11 with pD2. We were unable to detect up-regulation of pERK1/2, however, at any time point after pD2 stimulation (Figure 4.10A). We next assessed phosphorylation of CD3ζ, a more proximal signal, to confirm that pD2 was specifically engaging the TCR. After three minutes of stimulation, we detected phosphorylated CD3ζ (pCD3ζ) in response to each of the three epitope variants (Figure 4.10B). The extent of pCD3ζ was comparable for all three dengue variants and was higher than that for a control (non-dengue) HLA-A*1101-restricted peptide.

We next analyzed expression of the activation marker CD137 as a further indicator of antigen-specific stimulation (Wolfl*, et al.*, 2007) to confirm the specificity of the relatively small pD2 response by 10C11. At 6 hours after stimulation, pD2 activated a low frequency of T cells to up-regulate CD137 (Figure 4.10C), correlating with its induction of MIP-1β measured by ICS. The pD2-induced CD137 expression was not

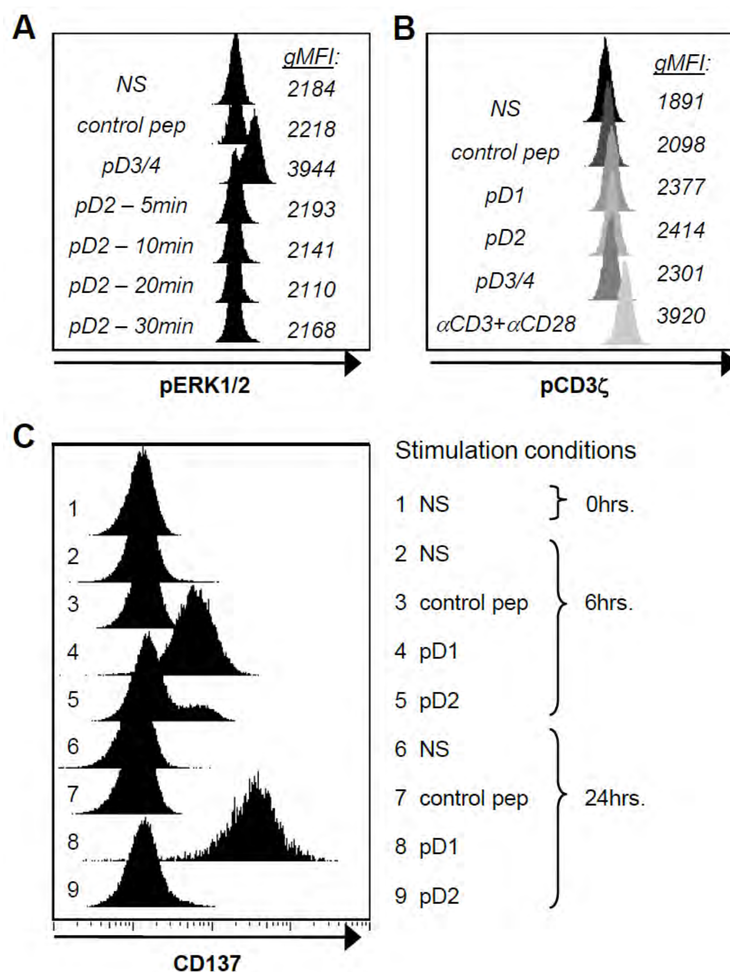

FIGURE 4.10. pD2 induces a short-lived activation signal in a pD1-3/4 cross-reactive cell line.

(A) Time course of pERK1/2 after pD2 stimulation of the cell line 10C11. One of 2 independent experiments is shown. (B) Phosphorylation of CD3ζ after stimulation of 10C11 with each of the three peptide variants. Histograms are shaded to emphasize the change in gMFI. One of 3 independent experiments is shown. (C) Time course of CD137 expression after stimulation of 10C11 with pD1, pD2, and a control non-dengue HLA-A*1101-restricted viral peptide. The peptide concentration used for these experiments was 10μg/mL. NS = no stimulation.

sustained at 24 hours, however, whereas stimulation with a full agonist peptide (pD1) induced robust up-regulation of CD137 at both 6 and 24 hours. These data indicated that pD2 was a partial agonist for 10C11, initiating calcium signaling but failing to up-regulate pERK1/2. This weak stimulus thus induced a short-lived signal that was only able to stimulate MIP-1β production but not degranulation, IFNγ, or TNFα.

I. Chapter summary

To further define the $CD8^+$ T cell response to heterologous DENV serotypes, we assessed functional responses after short-term bulk culture and isolated HLA-A*1101-restricted $NS3_{133}$ epitope-specific $CD8^+$ T cell lines from subjects who had primary DENV infections. These T cell lines exhibited marked cross-reactivity toward epitope variants representing the four DENV serotypes in pMHC tetramer binding and functional assays. Many clones responded similarly to homologous and heterologous serotypes with striking cross-reactivity between the DENV-1 and DENV-3 epitope variants. *In vitro*-stimulated T cell lines consistently revealed a hierarchical induction of MIP-1β > degranulation > TNFα > IFNγ, which depended on the concentration of agonistic peptide. Phosphoflow assays showed peptide dose-dependent phosphorylation of ERK1/2, which correlated with cytolysis, degranulation, and induction of TNFα and IFNγ, but not MIP-1β production. We also show qualitatively different T cell receptor signaling after stimulation with homologous and heterologous peptides; in particular, pD2 can act as a partial agonist. By showing that different effector responses can be elicited from T cells depending on the epitope variant used for stimulation, these data support a model

whereby the order of sequential DENV infections influences the immune response to secondary heterologous DENV infection, contributing to varying disease outcomes.

CHAPTER V

DISCUSSION

Plasma leakage, a hallmark of severe dengue disease, occurs late after infection and is coincident with viral clearance. This, together with evidence that increased disease severity is associated with secondary heterologous DENV infection, suggests the involvement of cross-reactive DENV-specific memory T and B cells in contributing to an immunopathologically-mediated clinical outcome.

We studied the frequency, kinetics, serotype-specificity, phenotype and function of DENV epitope-specific T cells during and/or after acute infection. In comparison with previous studies, our study involved large cohorts of subjects with DF and DHF, uniquely distinguished between those with primary and secondary DENV infections, included infections with all four DENV serotypes and analyzed responses to immunodominant epitopes for two different HLA class I alleles, including detailed analyses of T cell functional responses to heterologous epitope variants. Thus, our data address several gaps in the current literature, test the validity of assumptions underlying the interpretation of previous data and reveal new observations relevant to our understanding of dengue pathology.

A. Antigen-specific T cell frequencies in acute primary versus secondary infection

Mongkolsapaya *et al.* reported low but detectable frequencies of A11-$NS3_{133}$-specific T cells during acute secondary DENV infection in Thai children which peaked in

early convalescence (Mongkolsapaya*, et al.*, 2003). In contrast, Dung *et al.* recently reported that A11-$NS3_{133}$-specific T cells were undetectable during the febrile phase until after the development of DHF among infected Vietnamese children (Dung*, et al.*, 2010). Our data are clearly more consistent with the earlier report, and, in fact, several of our subjects had very high frequencies of epitope-specific T cells during acute infection, prior to the onset of plasma leakage, supporting their potential to contribute to disease pathogenesis. Our data using the B7-$NS3_{222}$ tetramer further confirmed this result, and are consistent with limited data from a previous study from our laboratory using IFNγ ELISPOT assays (Zivna*, et al.*, 2002). The finding of antigen-specific T cell expansion during viremia is also more consistent with observations in other viral diseases (Miller*, et al.*, 2008; Akondy*, et al.*, 2009; Wiesel*, et al.*, 2009) and with other evidence of T cell activation during acute DENV infection (Kurane*, et al.*, 1991; Green*, et al.*, 1999a; Chau*, et al.*, 2008). The divergent findings of Dung *et al.* may reflect differences in experimental technique; a more intriguing possibility is that the expansion of A11-$NS3_{133}$-specific T cells differs between Thai and Vietnamese patients. In this regard, it is of interest that an association with dengue disease severity has been observed for HLA-A11 in our Thai cohort (Stephens*, et al.*, 2002), but was not noted in a study in Vietnam (Nguyen*, et al.*, 2008) despite the similar genetic backgrounds of these two populations.

We found that the frequency of epitope-specific T cells peaked earlier in subjects with primary infection than in those with secondary infection. Prior studies in which DENV-specific T cells were tracked over time had not differentiated between subjects with primary and secondary infections. Given the more rapid proliferation of memory T

cells, and the more rapid clearance of viremia in secondary infection (Vaughn*, et al.*, 2000), this result was not anticipated but is consistent with findings in DENV infection of BALB/c mice (Beaumier*, et al.*, 2008). More rapid activation of memory T cells would not necessarily correspond to an earlier peak T cell frequency, since sustained proliferation signals balanced by apoptosis (Mongkolsapaya*, et al.*, 2003; Myint*, et al.*, 2006) would affect the timing of peak responses to a greater extent. Future studies need to incorporate analysis of these mechanisms as well as other immunomodulatory signals such as regulatory T cells (Luhn*, et al.*, 2007).

B. Lack of correlation between T cell frequency, activation and disease severity

The magnitude of A11-NS3$_{133}$-specific T cells did not correlate with disease severity in our cohort. This held true whether the data were analyzed according to clinical diagnosis (i.e. DF versus DHF) or continuous measures of disease severity such as pleural effusion index, hemoconcentration, or platelet nadir. A similar lack of association was reported in a study in Vietnam (Dung*, et al.*, 2010). Other studies had reported higher frequencies of DENV-specific T cells in patients with DHF, but these associations were found at 2 weeks (Mongkolsapaya*, et al.*, 2003; Mongkolsapaya*, et al.*, 2006) or 6 months (Zivna*, et al.*, 2002) post-infection. Differences in timing or differences in infection history (i.e. serotype of primary and secondary infection) may explain the differences in results between these studies. However, the lack of a correlation with disease severity and the timing of peak tetramer-positive T cell frequencies in early convalescence rather than at the time of plasma leakage (for at least

some subjects) indicate that the frequency of A11-NS3$_{133}$ tetramer-positive T cells is not the principal determinant of disease. T cell responses to other DENV epitopes may be more important contributors to disease, as is suggested by our limited data on B7-NS3$_{222}$-specific T cells in HLA-A11^{-}B7^{+} subjects. Alternatively, characteristics of the DENV-specific T cell response other than quantity, for example effector responses (Bashyam*, et al.*, 2006), may determine its immunopathological contribution.

Our data on expression of phenotypic markers by tetramer-positive T cells demonstrated selective activation of DENV-specific T cells as well as a transition from an effector to a memory phenotype, consistent with findings in other acute human viral infections (Miller*, et al.*, 2008; Akondy*, et al.*, 2009). There were no significant correlations between expression of activation or phenotypic markers and disease severity in this cohort. This stands in contrast to the results of other studies focused on CD69, an early marker of activation (Green*, et al.*, 1999a; Chau*, et al.*, 2008). We did observe slightly higher CD38 expression and a slightly earlier downregulation of CD45RA on tetramer-positive T cells during acute infection in secondary DENV infections compared to primary infections, which may reflect the expansion of pre-existing memory T cells.

C. The presence of serotype-cross-reactive T cells in primary DENV infection

Mongkolsapaya *et al.* found that A11-NS3$_{133}$-specific T cells in patients with secondary DENV infections preferentially stained with pMHC tetramers corresponding to DENV serotypes heterologous to the infecting serotype and interpreted this result to demonstrate original antigenic sin (Mongkolsapaya*, et al.*, 2003). Our study used a

similar approach, but extended this analysis to primary DENV infections, an important comparison group absent in the earlier study. Our *ex vivo* pMHC tetramer staining results demonstrated similar patterns of serotype-cross-reactive T cells in subjects with primary and secondary DENV infections, including cells with preferential binding of heterotypic tetramers. Functional assays performed both *ex vivo* and on numerous A11-$NS3_{133}$-specific T cell lines isolated from patient PBMC confirmed the presence of extensive serotype-cross-reactivity in primary infection. Thus, heterotypic tetramer binding in these subjects does not reflect a history of prior infection with other DENV serotypes; other possible explanations for this phenomenon are heterologous immunity from prior unrelated infections (Welsh*, et al.*, 2010), private specificity of the T cell repertoire (Welsh*, et al.*, 2006), or inherent immunogenicity of different epitope variants (Bashyam*, et al.*, 2006; Beaumier*, et al.*, 2008). Despite great donor-to-donor variability, we noted some similarities in patterns of tetramer staining among individuals with the same infection status (i.e. DENV serotype, primary versus secondary). This suggests that the sequence of infection is a factor in shaping the DENV-specific memory T cell repertoire, but other elements are more important for this epitope.

Our *in vitro* findings support the notion that the order of DENV serotypes with which a particular individual is infected plays a role in the outcome of the secondary immune response. The data suggest that, for the response to the A11-$NS3_{133}$ epitope, DENV-1 infection followed by DENV-3 infection, or vice versa, would trigger the activation of cross-reactive T cells that undergo a vigorous polyfunctional response involving IFNγ production. On the other hand, a secondary DENV-2 infection may elicit

cross-reactive, poorly responding T cells that are skewed toward MIP-1β production. Increased MIP-1β at the site of infection may augment IFNγ and TNFα production by T cells (Lillard*, et al.*, 2003) activated by other viral epitopes and increase TNFα production by monocytes (Nath*, et al.*, 2006), elevating the circulating levels of these cytokines and ultimately contributing to immunopathology. Secondary DENV-2 infection has been observed as a risk factor for increased disease severity in epidemiological studies in Asia (Guzman*, et al.*, 1990; Thein*, et al.*, 1997).

While most severe cases of dengue are seen in patients experiencing a secondary DENV infection, many occur in infants experiencing a primary DENV infection. This was often attributed solely to antibody-mediated enhancement of infection, but serotype-cross-reactive T cell responses might contribute to severe disease in these cases as well. A pool of DENV-specific memory T cells presumably does not yet exist in the first year of life. However, our data suggest that primary infection can elicit a large proportion of serotype-cross-reactive T cells. Such a mix of responding T cells with a variety of affinities toward multiple DENV serotypes means that the serotype of infection holds great sway over the magnitude and type of T cell response induced. Thus, our data indicate that the anti-DENV T cell response may play a greater role in dengue pathogenesis than previously appreciated.

D. Differences between epitope variants

We found that A11-$NS3_{133}$ epitope-specific $CD8^+$ T cell lines isolated from primary DENV immune donors were highly cross-reactive and demonstrated greater

recognition of pD1 and pD3/4 variant peptides as compared to pD2, consistent with *ex vivo* tetramer staining and limited functional data. Dong *et al.* also detected significant pD1-3/4 cross-reactivity within this A11 epitope-specific T cell population after secondary DENV infection (Dong*, et al.*, 2007). Though a different DENV-2 epitope variant was used in that study, a majority of their 'highly cross-reactive' T cell clones recognized pD1 and pD3/4 with greater potency than the DENV-2 variant.

The predominance of pD1-3/4 cross-reactivity may be explained by the primary structure of these epitope variants. Classic A11-restricted epitopes have a small aliphatic residue at position 2 (P2) and a basic residue at the C terminus (Kubo*, et al.*, 1994; Sidney*, et al.*, 1996; Sidney*, et al.*, 2008). The epitope used in our study follows this motif since it contains a threonine at P2 as well as a C-terminal arginine. Recently, a secondary anchor residue for A11-restricted epitopes was identified at P6 or P7, which creates a double bulge of neighboring amino acids exposing likely sites of TCR contact (Li and Bouvier, 2004). Such bulging of the A11 dengue epitope would expose P9, the residue that differs between pD1 or pD3/4 and pD2. However, P9 of a 10-mer epitope from HIV was determined to be the C-terminal MHC anchor residue (Couillin*, et al.*, 1994). No matter which side of the TCR-MHC interface interacts with the P9 residue, the non-conservative amino acid change (and corresponding charge difference) between pD1 or pD3/4 (asparagine) and pD2 (aspartate) could drastically affect binding avidity. These epitope variants have been crystallized together with HLA-A*1101 (Chotiyarnwong*, et al.*, 2007), which indicates tight binding of peptide and MHC. However, three different algorithms predicted that A11 binding for the three peptide

variants reflects a hierarchy of pD3/4>pD1>>pD2. We speculate that poor binding of pD2, compared to pD1 and pD3/4, to either A11 or the TCR may explain its lower functional avidity apparent in our study, consistent with the 'induced fit' or 'antigen-dependent tuning of peptide-MHC flexibility' mechanisms of cross-reactivity (Yin and Mariuzza, 2009).

E. Discordance in the epitope variant hierarchy between pMHC tetramer binding and functional avidity

The hierarchical preference of pD1>pD3/4>pD2 detected when cells were directly stained *ex vivo* with pMHC tetramers was similarly detected after short-term bulk culture of peptide-stimulated patient PBMC as well as for individual epitope-specific T cell lines. However, functional assays suggested that pD3/4 was a better agonistic ligand than pD1, supporting their predicted MHC binding affinity hierarchy of pD3/4>pD1>pD2. This was evidenced by the different peptide dose response curves in ^{51}Cr release and ICS, though not phosphoflow, assays performed on the isolated T cell lines. Other studies have shown a similar discordance between T cell function and pMHC tetramer binding by antigen-specific T cells (Derby, *et al.*, 2001; Kalergis, *et al.*, 2001; Rubio-Godoy, *et al.*, 2001; Dutoit, *et al.*, 2002) and suggested that pMHC tetramer dissociation assays were a better predictor of functional avidity than direct binding (Rubio-Godoy, *et al.*, 2001; Dutoit, *et al.*, 2002). Kalergis *et al.* used point mutations to show that TCR-pMHC engagement had to be sufficiently long to allow for efficient T cell activation and that too long (or too short) of an interaction could compromise the "agonist potency of pMHC

variants" (Kalergis, *et al.*, 2001). Our data fit this model in that the hierarchy of functional avidity (pD3/4>pD1>pD2) was evident in assays that required a long incubation time (i.e. ^{51}Cr release and ICS) but was obscured (pD1>pD3/4>pD2) in assays with short incubation times (i.e. pMHC tetramer binding and phosphoflow). Thus, the extent of serotype-cross-reactivity detected by pMHC tetramer staining of PBMC *ex vivo* might underestimate the true functional serotype-cross-reactive potential of A11-NS3$_{133}$ epitope-specific T cells.

F. Functional similarities of epitope-specific T cells

Regardless of their serotype-cross-reactivity, the functional response of all the A11-NS3$_{133}$-specific CD8$^+$ T cell lines we tested revealed a hierarchical induction of MIP-1β > degranulation > TNFα > IFNγ. This is in line with a recent report from our laboratory characterizing PBMC from DENV vaccinees in response to *in vitro* stimulation of homologous and heterologous HLA-A*0201-restricted epitope variants (Bashyam, *et al.*, 2006). Imrie *et al.*, however, found that a larger proportion of CD8$^+$ T cell clones specific for a B*5502-restricted epitope on the DENV NS5 protein produced IFNγ compared to TNFα (Imrie, *et al.*, 2007). Studies of HIV-specific T cells demonstrated a hierarchical induction of MIP-1β > degranulation > IFNγ > TNFα > IL-2 (Betts, *et al.*, 2006; Ferre, *et al.*, 2009). Patients who were better able to control their HIV viral loads had higher numbers of T cells exhibiting multiple effector functions in both PBMC (Betts, *et al.*, 2006) as well as rectal mucosa (Ferre, *et al.*, 2009), suggesting a protective role for polyfunctional CD8$^+$ T cells. While the hierarchical induction of

MIP-1β > degranulation > cytokines in virus-specific $CD8^{+}$ T cells was observed in all of these studies, it is unclear what factors affect the particular order of cytokine production. The fact that A11-$NS3_{133}$-specific T cells appear to initiate TNFα production before IFNγ production may suggest their predisposition toward secreting immunopathogenic (TNFα), as opposed to protective (IFNγ), cytokines. In this regard, it is important to note that HLA-A11 is reported to have a modest association with severe disease (Stephens*, et al.*, 2002).

Previous studies have shown that varying degrees of TCR-ligand interaction elicit a hierarchical order of response thresholds for different effector functions (Valitutti*, et al.*, 1995; Valitutti*, et al.*, 1996; Itoh and Germain, 1997; Hemmer*, et al.*, 1998; Betts*, et al.*, 2004; La Gruta*, et al.*, 2004). It was determined that, regardless of ligand potency, the extent of TCR engagement as measured by TCR down-regulation dictated the functional response elicited from the T cell. Specifically, cytotoxicity was elicited after very low levels of TCR occupancy by peptide-MHC whereas cytokine responses required higher occupancy for induction. Our data support these findings in that cytotoxicity (measured by target cell lysis and degranulation) was induced at the lowest concentrations of full agonist peptides used as well as high concentrations of partial agonist peptides. TNFα and IFNγ production was only seen after stimulation with full agonist peptides, and the proportion of cells producing these cytokines increased with increasing concentrations of those peptides, supporting the idea that greater TCR occupancy triggers cytokine production. Additionally, phosphoflow assays performed in our study confirmed that low TCR occupancy qualitatively altered peptide-induced

signaling pathways (i.e. pD1 and pD3/4 induced pERK1/2 whereas pD2 did not), which helps explain differential effector responses. This is the first time phosphoflow has been utilized to demonstrate variations in peptide-induced signaling in T cells, and it has proven to be an effective tool for measuring such differences. Altogether our data argue that production of the chemokine MIP-1β has an even lower signal threshold than cytotoxicity, supporting other studies which suggest MIP-1β is a more sensitive measure of antigen-specific cells than IFNγ (De Rosa*, et al.*, 2004; Betts*, et al.*, 2006).

The polyfunctional nature of the effector responses we detected from epitope-specific $CD8^+$ T cell lines was not necessarily reflected in ICS responses detected *ex vivo*. Many responses from subjects with primary infection were dominated by a single effector function, though this was less evident in responses from subjects with secondary infection. Other studies have noted the prevalence of mono- or oligo-functional responses detected *ex vivo* (Bashyam*, et al.*, 2006; Duangchinda*, et al.*, 2010). The samples we used for *ex vivo* stimulation were taken within one week after defervescence, indicating that T cells detected in subjects with primary infection have not yet differentiated into memory T cells. This may explain the lower frequency of polyfunctional T cells in these subjects versus those with secondary infection, as memory T cells more rapidly induce multiple effector functions simultaneously compared to naïve T cells (Zimmermann*, et al.*, 1999; Veiga-Fernandes*, et al.*, 2000).

G. Study limitations

The interpretation of our data is subject to several limitations. Although the patient cohort used for *ex vivo* analysis was relatively large, the small number of HLA-A11$^+$ subjects with the same DENV serotype, serologic response (primary/secondary infection), and clinical outcome (DF/DHF) limits the statistical power for important subgroup analyses, and data from HLA association studies suggest that the influence of T cell responses may not be the same for all four serotypes (Stephens*, et al.*, 2002). Additionally, although subjects were followed daily during acute illness, adequate specimens were not available for all of these flow cytometry studies from all time points for all subjects. Given that rapid changes were observed in tetramer-positive T cell frequencies during acute infection, it is likely that peak frequencies were missed in some subjects. Furthermore, some PBMC had very high frequencies of epitope-specific T cells, but we were not able to reconfirm the data due to limited sample volume, leading us to interpret the results with caution. However, repeat staining of selected samples where we had multiple vials gave consistent results. Few of the DHF patients in our study experienced shock (i.e. DHF grades III or IV). As Mongkolsapaya *et al.* found the highest frequencies of tetramer-positive T cells in patients with shock (Mongkolsapaya*, et al.*, 2003; Mongkolsapaya*, et al.*, 2006), it is possible that the milder disease in our study cohort concealed a relationship to the most severe disease. Moreover, while our study is the first to assess T cell responses to more than one DENV epitope within individual subjects, we cannot exclude the possibility that these epitopes are not representative of the global DENV-specific CD8$^+$ T cell response. Duangchinda *et al.*

recently showed higher cytokine responses in children with DHF compared to DF when overlapping peptides covering the entire NS3 protein were used (Duangchinda*, et al.*, 2010). However, that study only analyzed PBMC collected several weeks post-infection and the same association was not detected by Simmons *et al.* (Simmons*, et al.*, 2005a). Finally, human studies are limited to analysis of blood samples, and we may have missed pathogenic T cells that were bound to the endothelium or located in other tissues.

Our study was also limited to characterizing $CD8^+$ T cell lines specific for a single epitope, whereas the overall T cell response would reflect responses to multiple epitopes with varying patterns of serotype-cross-reactivity. Our limited *ex vivo* analysis of B7-$NS3_{222}$-specific T cells in HLA-$A11^+B7^+$ versus $A11^-B7^+$ subjects suggested a possible influence of particular HLA haplotypes on the expansion of epitope-specific T cells. Future analyses should investigate whether the immunodominance hierarchy of epitope-specific T cells is maintained in subjects with varying combinations of HLA alleles. Our cell line study was ultimately limited to four patients, but the subjects did vary with regard to the infecting serotype, age, illness grade as well as genetic background. We isolated many T cell lines from each patient that showed similar patterns of serotype-cross-reactivity to the A11-$NS3_{133}$ epitope. Further functional studies on individuals infected with other DENV serotypes, experiencing different illnesses and who have different genetic backgrounds than our donors will add to our understanding of the contribution of this and other cross-reactive epitope-specific T cell populations to disease.

In addition to lifelong immunity to the currently-infecting serotype, primary DENV infection induces transient but protective immunity to heterologous DENV

serotypes in patients for at least two months after illness (Sabin, 1952). The patient samples from which we isolated epitope-specific T cell lines (and detected *ex vivo* functional responses) were collected eight months or less after primary infection. It is possible that the cross-reactive T cell responses we observed might not represent stable, long term T cell memory. Analysis of memory T cell responses after longer intervals would be complicated by the substantial potential for re-exposure to DENV in individuals living in DENV-endemic areas. However, our group has detected serotype-cross-reactive T cell responses in vaccine recipients several years after immunization (Zivny, *et al.*, 1999; Mangada and Rothman, 2005; Bashyam, *et al.*, 2006) suggesting that primary exposure to the virus generates a long-lived cross-reactive memory T cell response.

H. Summary, implications, and revisions to the T cell immunopathogenesis model

In summary, our data points to a complex picture of T cell involvement in DENV infection. Specifically, this thesis showed:

- a diverse pattern of serotype-cross-reactivity in both primary and secondary infections
- selective activation and expansion of DENV-specific T cells during acute infection
- kinetic differences in peak frequency and phenotypic evolution (i.e. CD45RA expression) of T cells in primary versus secondary DENV infection
- no correlation between the magnitude of DENV tetramer-positive T cells and disease

- variable agonistic properties of different A11-$NS3_{133}$ epitope variants

These results alter the way we think about T cell involvement in dengue pathology.

Prior to DENV infection, the naïve T cell repertoire theoretically includes T cells with a variety of specificities for the four DENV serotypes. Our group and others hypothesized that the pool of memory T cells generated during primary DENV infection largely comprises serotype-specific T cells with a smaller proportion of cells with low affinity to heterologous serotypes (Figure 5.1A and B). Upon secondary heterologous DENV infection, for example DENV-3 infection following a prior DENV-1 infection, the memory T cells with low affinity to DENV-3 are preferentially re-activated (due to their lower threshold for activation compared to higher affinity, DENV-3 specific naïve T cells). The low affinity interaction with heterotypic antigen (i.e. antigen from the currently infecting, heterologous serotype) elicits a sub-optimal T cell response (e.g. high TNFα production) that ultimately contributes to disease pathology.

However, the data generated in this thesis suggest that serotype-cross-reactivity in the response to primary infection is much more extensive than previously appreciated (Figure 5.1C). This changes our view of how T cells may be activated upon secondary infection. T cells with high affinity for DENV-1 do not necessarily have low affinity for DENV-3, effectively increasing the pool of DENV-specific memory T cells available to respond in secondary heterologous infection (Figure 5.1D). The serotype of the infecting virus in secondary infection then plays a greater part in determining the final T cell effector response, as the extent and position of amino acid changes within the epitope variant's sequence will affect its ability to activate T cells. Based on our data with A11-

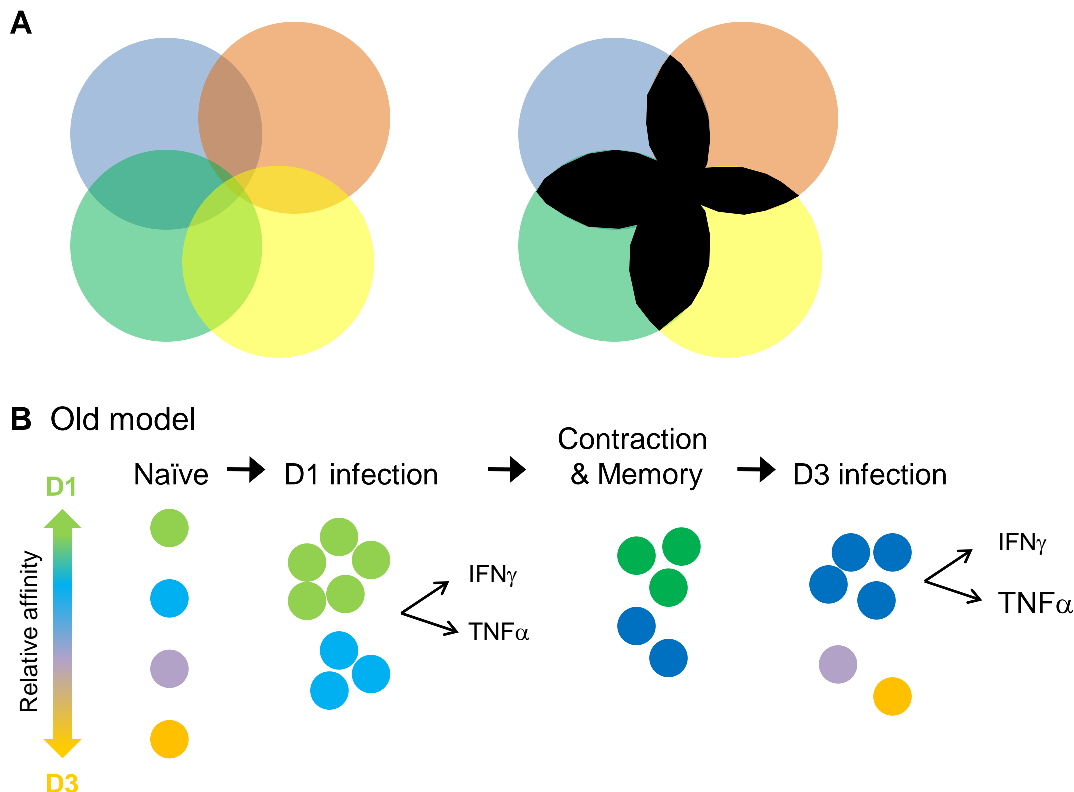

FIGURE 5.1. Revisions to the model of T cell involvement in dengue pathogenesis.

(A) Following a primary infection with any serotype of DENV, the majority of memory T cells generated are specific to the infecting serotype while a relatively small proportion of T cells are cross-reactive to other serotypes. Each colored circle represents the T cell response for one of the four DENV serotypes. The extent of serotype-cross-reactivity is shaded in black. (B) The old model for T cell-mediated dengue pathogenesis indicates that T cells differ in their relative affinity for DENV serotypes (shown here for DENV-1 and -3). The highest affinity T cells are activated in primary infection and transition to memory after viral clearance. Upon secondary heterologous infection, low affinity, serotype-cross-reactive memory T cells have an advantage (in frequency and threshold for activation) over high affinity naïve T cells and mediate a skewed cytokine response which ultimately contributes to dengue pathology. Each colored circle represents a single T cell clonotype. Light colors indicate naïve/effector T cells; darker colors indicate memory T cells.

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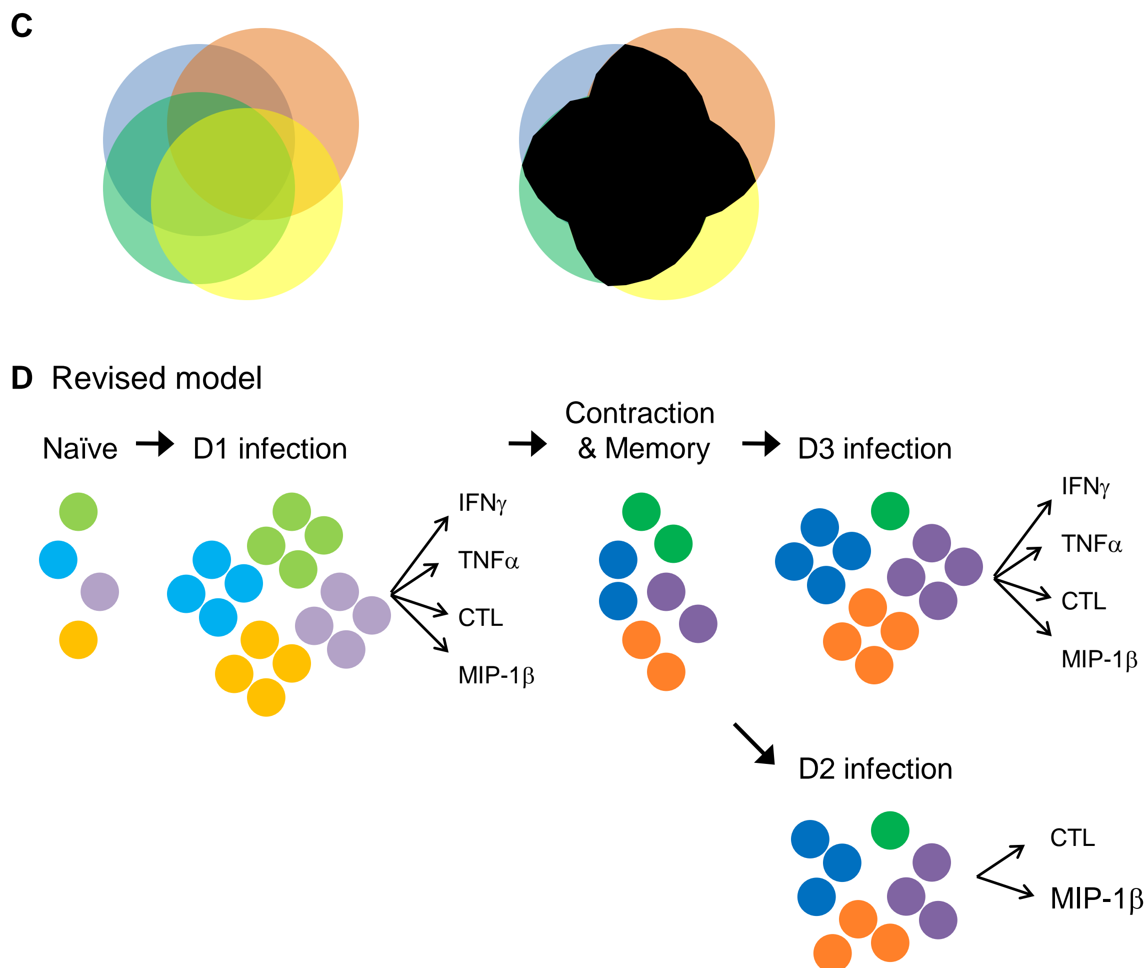

FIGURE 5.1. Revisions to the model of T cell involvement in dengue pathogenesis. (*cont'd*)
...(C) In our revised model, primary infection generates a greater proportion of memory T cells that are serotype-cross-reactive than previously appreciated. Each colored circle represents the T cell response specific for one of the four DENV serotypes. The extent of serotype-cross-reactivity is shaded in black. (D) The revised model recognizes the presence of naïve T cells with a variety of serotype-specific affinities, which do not necessarily have low affinity for heterologous serotypes. The pool of T cells available for (re-)activation is thus larger than previously expected in both primary and secondary infection. Based on our data regarding A11-$NS3_{133}$-specific T cells, DENV-1 followed by DENV-3 or -4 infection might re-activate a response akin to that seen for DENV-1 infection, which includes IFNγ production, likely leading to protection from severe disease. However, DENV-1 followed by DENV-2 infection might re-activate a smaller response (due to its partial agonist effect) which would be dominated by MIP-1β production, possibly leading to enhanced cytokine (e.g. TNFα) production by other nearby T cells or monocytes. Each colored circle represents a single T cell clonotype. Light colors indicate naïve/effector T cells; darker colors indicate memory T cells.

$NS3_{133}$ epitope-specific T cells, DENV-1 followed by DENV-3 infection might activate a comparable response to primary DENV-1 infection whereas secondary DENV-2 infection might activate a weaker response that exhibits an altered functional profile (i.e. MIP-1β without IFNγ production).

We believe that severe disease results when a combination of host genetics and viral factors add to an altered T and B cell profile in the host (Figure 5.2). This thesis sought to better understand features of the T cell response that may contribute to dengue disease and found extensive serotype-cross-reactivity; responses are complex and can vary even for a single epitope. Even if we consider proliferative and functional responses to two epitopes out of a given individual's global anti-DENV T cell response, there are several possible outcomes. Therefore, the role that T cells play in influencing dengue disease may be bigger than previously anticipated. Ultimately, altered T cell responses elicited by heterologous variants of DENV together with the viral load, antibody response, host genetics and other factors determine disease outcome.

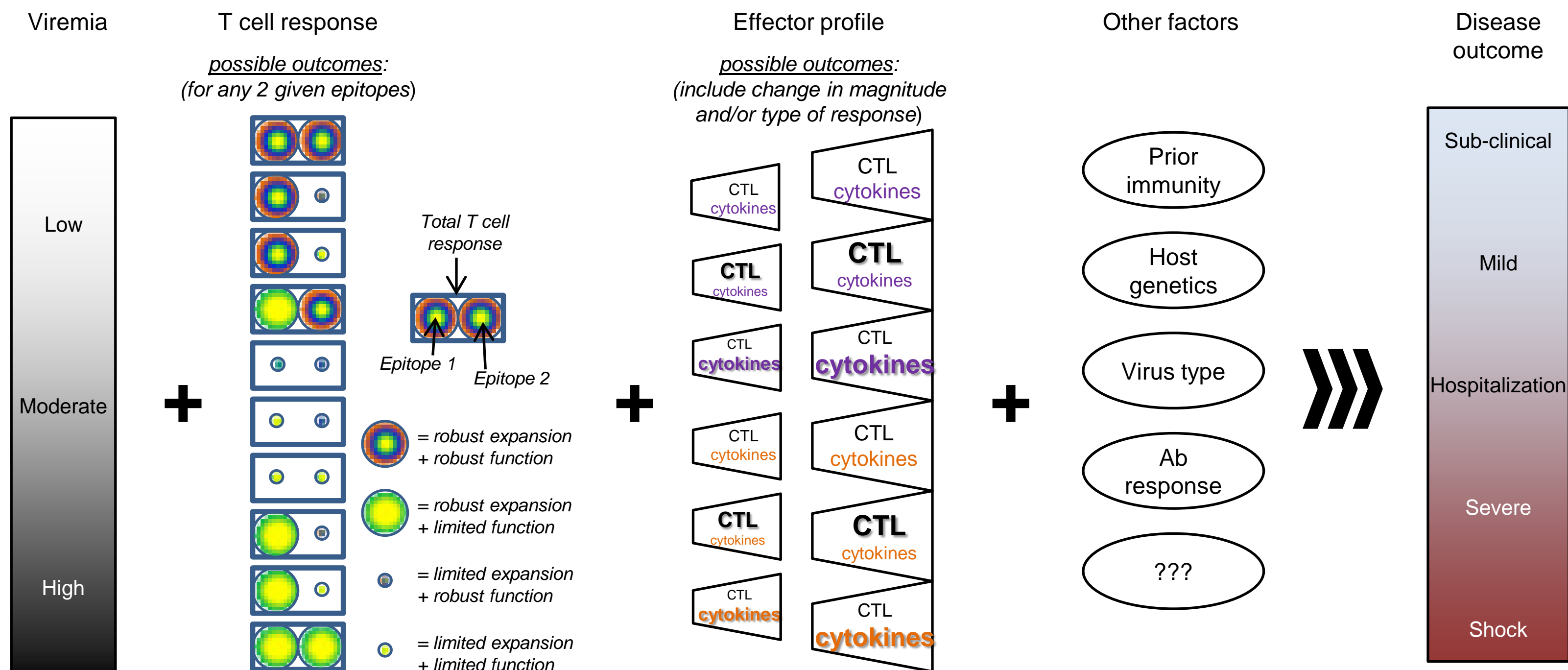

FIGURE 5.2. Disease outcome is governed by a variety of factors.

DENV infection leads to one of many possible disease outcomes, which is governed by a variety of factors, including T cell responses. Viremia affects the size and type of T cell response and vice versa. The global T cell response (blue boxes) is a culmination of responses by T cells specific for multiple epitopes (shown here for any 2 epitopes; circles within each box). Shown are possible responses by 2 epitope-specific T cell populations which can proliferate robustly or not (represented by the size of the circle) and exhibit a robust or limited effector response (represented by the number of different colors within each circle). The effector profile of the T cells can also vary by magnitude of the response (represented by big or small trapezoid shapes) as well as type of response (the relative effect of cytolysis [CTL] and cytokine production is shown by size of lettering; a change in type of cytokine response [e.g. IFNγ>TNFα changing to TNFα>IFNγ] is represented by different colors). The antibody response, host genetic factors, and other variables also influence a patient's final outcome, which ranges in severity from asymptomatic infection to shock and possible death.

CHAPTER VI

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