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Cross-Reactive Memory CD4$^+$ and CD8$^+$ T Cells Alter the Immune Response to Heterologous Secondary Dengue Virus Infections in Mice: A Dissertation

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CROSS-REACTIVE MEMORY $CD4^+$ AND $CD8^+$ T CELLS ALTER THE IMMUNE RESPONSE TO HETEROLOGOUS SECONDARY DENGUE VIRUS INFECTIONS IN MICE

A Dissertation Presented

By

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ABSTRACT

Dengue virus (DENV) infects 50-100 million people worldwide every year and is the causative agent of dengue fever (DF) and the more severe dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). There are four genetically and immunologically distinct DENV serotypes (DENV-1, DENV-2, DENV-3, and DENV-4). Evidence suggests that an increased risk for DHF/DSS during secondary infection with a heterologous DENV serotype is due to an immunopathological response mediated by serotype-cross-reactive memory T cells from the primary infection. Furthermore, epidemiological studies have shown that the sequence of infection with different DENV serotypes affects disease severity. Though much has been learned from human studies, there exist uncontrollable variables that are intrinsic in this system such as genetic factors and unknown infection histories. These factors can skew experimental results, making interpretations difficult. Therefore, a murine model to study the immunologic aspects of sequential dengue infections would be an asset to the field of dengue research.

To examine the effect of sequential infection with different DENV serotypes on the CD8+ T cell response, we immunized Balb/c mice with a primary DENV infection on day 0 and subsequently challenged with a heterologous secondary DENV infection on day 28. We tested all possible sequences of infection with the four serotypes. We analyzed the T cell response to two previously defined epitopes on the DENV E (L^d-restricted) and NS3 (K^d-restricted) proteins. Using ELISPOT and intracellular cytokine staining, we measured the frequency of T cells secreting IFNγ and TNFα in response to stimulation with these epitopes during three phases: acute primary, acute secondary, and

the memory phase after primary infection. We found that the T cell response in heterologous secondary infections was higher in magnitude than the response in acute primary infection or during the memory phase. We also found that the hierarchy of epitope specific responses, as measured by IFNγ secretion, was influenced by the sequence of infections. The adoptive transfer of immune serum or immune splenocytes suggested that memory T cells from the primary infection responded to antigens from the secondary infection. *In vitro* experiments with T cell lines generated from mice with primary and secondary DENV infections suggested the preferential expansion of cross-reactive memory T cells.

In testing all of the different possible sequences of infection, we observed that two different sequences of infection (e.g., DENV-2 followed by DENV-1 versus DENV-2 followed by DENV-3) resulted in differential $CD8^+$ T cell responses to the NS3 peptide even though both secondary infection serotypes contain the identical peptide sequence. To investigate this phenomenon, we examined the role of $CD4^+$ T cell help on the memory $CD8^+$ T cell response. We found that $CD4^+$ T cell cytokine responses differ depending on the sequence of infection. In addition, it was also shown that cross-reactivities of the $CD4^+$ T cell response are also sequence-dependent. Moreover, dengue-specific memory $CD4^+$ T cells can augment the secondary $CD8^+$ T cell response. Taken together, we demonstrated that this serotype sequence-dependent phenomenon is the result of differential help provided by cross-reactive memory $CD4^+$ T cells.

The findings in this novel mouse model support the hypothesis that both $CD4^+$ and $CD8^+$ serotype-cross-reactive memory T cells from a primary dengue virus infection alter the immune response during a heterologous secondary dengue virus infection. These

data further elucidate potential mechanisms whereby the specific sequence of infection with different dengue virus serotypes influences disease outcomes in humans.

CHAPTER I

INTRODUCTION

Epidemiology of dengue

Dengue virus (DENV) is the causative agent of dengue and a significant source of global morbidity. The first known epidemic given the name dengue occurred in Spain in 1801 (Soler, Pascual et al. 1949). However, there is evidence of epidemics of dengue-like illness throughout the world long before the nineteenth century. The first such report was during the Chin Dynasty of China between 265 and 420 AD (Nobuchi 1979). Outbreaks consistent with dengue occurred in the West Indies and Panama in 1635 and 1699, respectively (Howe 1977; McSherry 1982). The first reports of major epidemics widely recognized as likely to be dengue were in Asia, Africa, and North America in 1779 and 1780 (Rush 1789; Hirsch 1883; Pepper 1941; Howe 1977). Today DENV infects 50 – 100 million people world wide every year. Most of these infections are asymptomatic or cause an uncomplicated illness, dengue fever (DF), but 500,000 develop into severe dengue disease, dengue hemorrhagic fever (DHF), which has a 2.5% fatality rate. Most of these cases of DHF are in children (Pinheiro and Corber 1997).

DENV is a member of the *Flaviviridae* family and genus *Flavivirus*. Other members of this genus include yellow fever virus, West Nile virus, and Japanese encephalitis virus. DENV is an arthropod-borne virus, transmitted predominantly by the mosquito vector *Aedes aegypti* (Gubler 1989). This particular species of mosquito is well adapted to urban areas and feeds during the daytime – an advantageous adaptation for

feeding on humans since the density of humans outside is higher during the day. The virus is passed to the mosquito by feeding on an infected person. After eight to ten days of incubation of the virus in the mosquito, the mosquito can then pass on the virus by feeding on another human, thus continuing the transmission cycle. Though *A. aegypti* is the principal vector for dengue, the virus may also be spread by the mosquito *Aedes albopictus* (Gubler 1989).

Dengue virus genome, structure, and proteins

There are four immunologically distinct serotypes of DENV: dengue-1 virus (DENV-1), dengue-2 virus (DENV-2), dengue-3 virus (DENV-3) and dengue-4 virus (DENV-4). These viruses share approximately 70% sequence identity. The genome of DENV is a positive-sense single-stranded RNA, which has a length of ~11kb (Henchal and Putnak 1990). Ten proteins are encoded by the RNA. Three proteins are structural components of the virion: nucleocapsid (C), envelope (E), and membrane (M). The remaining seven are nonstructural (NS) proteins: NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5. The RNA is packaged by the nucleocapsid protein, which is then further enclosed in a lipid bilayer containing the envelope and membrane proteins (Henchal and Putnak 1990).

Each protein DENV encodes has an indispensable function, some proteins having several functions. The C protein forms the structural component of the nucleocapsid (Henchal and Putnak 1990). The prM (precursor M) protein is cleaved into the mature M protein that is involved in the morphogenesis of the virion and affects viral infectivity (Randolph, Winkler et al. 1990). The E protein is a receptor binding and membrane

fusion protein (Allison, Schalich et al. 2001; Lorenz, Allison et al. 2002). It is also a target for neutralizing antibodies (Monath, Arroyo et al. 2002). The functions of the nonstructural proteins are less well understood. NS1 is known to be a complement fixing protein and can exist in either cell-associated or secreted forms (Lee, Crooks et al. 1989). NS3 has many functions. It has helicase activity and is involved is the hydrolysis of the anhydride bond of the nucleotide triphsophates (Bazan and Fletterick 1989; Benarroch, Selisko et al. 2004). In addition, it is a serine protease and functions with the NS2B cofactor (Wengler and Wengler 1991; Zhang, Mohan et al. 1992). NS4a and NS4b are both hydrophobic and function in inhibiting interferon (IFN) signaling (Munoz-Jordan, Laurent-Rolle et al. 2005). NS5 is the viral RNA dependent RNA polymerase and plays a role in capping through its GTP-binding site and methyltransferase function (Egloff, Benarroch et al. 2002).

Dengue life cycle

The DENV life cycle begins through receptor mediated endocytosis. Though the major cellular receptor for DENV is not definitely known, some potential receptors are DC-SIGN, GRP78, and CD14-associated molecules (Chen, Wang et al. 1999; Tassaneetrithep, Burgess et al. 2003; Jindadamrongwech, Thepparit et al. 2004; Lozach, Burleigh et al. 2005). A second molecule, possibly a coreceptor, also appears to be required for viral entry (Martinez-Barragan and del Angel 2001). Potential co-receptors are glycosaminoglycans such as heparan sulfate (Chen, Maguire et al. 1997). Acidification of the endosomal vesicle causes conformational changes in the virion, leading to fusion of the viral and endosomal membranes and the release of the

nucleocapsid into the cytoplasm. Dissociation of the RNA from the capsid follows. The positive sense RNA is translated into one polyprotein that is then post-translationally cleaved and processed. Replication takes place on intracellular membranes. Virus assembly occurs in the endoplasmic reticulum (ER). Particles form by the budding of the RNA-capsid complex into the ER where E and M are already present. These immature viral particles coated with prM-E heterodimers enter and travel through the trans-Golgi network. Cleavage of prM by a furin protease results in the formation of E homodimers and the mature virion. Mature virions are released through exocytosis (Mukhopadhyay, Kuhn et al. 2005).

Clinical manifestations of dengue virus infections

DENV infection of humans results in a continuum of disease manifestations ranging from no symptoms to severe disease. The majority of infected persons remain asymptomatic. Dengue fever (DF) is the less severe outcome of dengue disease. It is a self-limited disease resulting in fever, flu-like symptoms, myalgia, headache, nausea, vomiting, arthralgia, rash, and retrorbital pain (Kalayanarooj, Vaughn et al. 1997). Dengue hemorrhagic fever (DHF) is the most severe form of dengue disease. In addition to the above-mentioned symptoms of DF, DHF is characterized by an increase in the permeability of the vascular endothelium leading to plasma leakage. DHF has the potential to result in shock (dengue shock syndrome, DSS) and, left untreated, may be fatal (Guzman and Kouri 2002). DHF generally occurs between days 3-5 of illness, corresponding to the time of defervesence and resolution of viremia. The timing of the

onset of illness, after viral clearance, suggests that DHF is caused by the immune response as opposed to the virus itself (Halstead 1982).

Dengue is a systemic disease. Hematologic findings include leukopenia and thrombocytopenia; these manifestations may be caused by a combination of bone marrow suppression and/or platelet destruction (Guzman and Kouri 2002). In addition, elevated serum aminotransferases are also seen in DENV-infected patients, indicating liver involvement (Kalayanarooj, Vaughn et al. 1997). Necrosis of the liver cells has been reported in fatal cases (Guzman and Kouri 2002). CNS involvement (encephalitis) in dengue rarely occurs (Guzman and Kouri 2002).

Risk factors for severe dengue disease

The risk for developing DHF is influenced by individual, epidemiological, and viral factors.

Individual factors

Risk factors for DHF for the individual include age, race, nutritional status, human leukocyte antigen (HLA)-type, and sequential infections. DHF is more prevalent in young children, usually under the age of eleven (Kliks, Nimmanitya et al. 1988). Some authors have suggested that this is due to the fact that the baseline vascular permeability of children is higher than in adults (Gamble, Bethell et al. 2000). As for race, epidemiological studies in Cuba and Haiti suggest that Caucasian populations are more susceptible to DHF than those of African descent (Guzman, Alvarez et al. 1999). In addition, unlike many other diseases, DHF appears to be less common in malnourished

individuals (Kalayanarooj and Nimmannitya 2005). This phenomenon may be due to immune suppression.

Correlations have been drawn between increased probability for severe dengue disease and HLA type. Stephens *et al*, in a study of 263 Thai patients, reported that there was an association of HLA class I alleles HLA-A*0203 and HLA-B*52 with the less severe DF (Stephens, Klaythong et al. 2002). They also showed that there were correlations between HLA-A*0207 and HLA-B*51 and an increased risk for DHF. However, a study in a Caucasian Brazilian population did not find an increase in frequency of an particular HLA class I antigens in patients exhibiting DF as compared to the control group of healthy individuals (Polizel, Bueno et al. 2004). A relationship was found between HLA-A2 and HLA-B blank and a greater possibility for manifesting DHF. HLA-B13 had a negative correlation with the risk of DHF. Paradoa Perez *et al* confirmed that HLA-A1 and HLA-B blank were linked with severe disease along with HLA-Cw1 and HLA-A29 (Paradoa Perez, Trujillo et al. 1987). In a different Cuban cohort, HLA-B*15 and HLA-DRB1*04 were found at increased frequency among patients that had either DF or DHF compared to healthy control subjects (Sierra, Alegre et al. 2007). Thus, these studies suggest a genetic risk factor for dengue hemorrhagic fever and also provide evidence for an immunopathogenic mechanism for severe disease.

Sequential infections with DENV have been noted as one of the most important risk factors for severe dengue disease (Halstead 1982; Sangkawibha, Rojanasuphot et al. 1984). After infection with one serotype of DENV, one develops lifelong homotypic immunity to that serotype. Since a primary infection with one serotype offers only transient (months) protection against a secondary infection with another serotype,

secondary infections with other DENV serotypes can occur. DHF uncommonly occurs during a primary infection (Halstead 1982; Sangkawibha, Rojanasuphot et al. 1984). A Thai study in 1970 showed that 85% of patients with DHF had high titers of cross-reactive antibodies, suggesting a previous DENV infection (Halstead, Nimmannitya et al. 1970). Following DENV epidemics in Thailand in the early 1980's, two reports emerged stating that patients contracting a secondary infection were 15 to 80 times more likely to have severe disease than those experiencing a primary infection. A 1981 Cuban outbreak of DENV-2 followed a DENV-1 outbreak in 1977: 98% of DHF patients had a secondary infection (Guzman, Kouri et al. 1987; Burke, Nisalak et al. 1988). More recently, another outbreak occurred in Cuba in 2001-2002 and also linked the occurrence of DHF/DSS with secondary infection (Alvarez, Rodriguez-Roche et al. 2006). These epidemiological data suggest that there is an increased risk of DHF in secondary infection. This risk factor of secondary infections suggests an immunopathological mechanism.

Epidemiological factors

A rise in the density of the dengue vector *Aedes aegypti* increases the global risk for dengue (Gubler 1989). Uncontrolled urbanization due to population growth has contributed to substandard housing and sanitation and lack of mosquito control. These scenarios promote the larval habitats of *Aedes*, such as the accumulation of used tires and non-biodegradable plastics, and thereby enhance DENV transmission (Gubler 1989).

Globalization also increases the circulation of DENV. There has been an increase in the amount of airplane travel, leading to travelers being infected in endemic areas and then bringing the virus back when they return. This can also lead to the creation of hyperendemic (co-circulation of multiple serotypes) regions. Hyperendemic areas lead to

a situation allowing heterologous secondary infections, a risk factor for DHF/DSS (Gubler and Trent 1993; Gubler and Clark 1995; Gubler 2002).

Viral factors

Viral characteristics, such as serotype and genotype, affect the risk for DHF. Certain serotypes of DENV, especially DENV-2 and DENV-3, have been associated with severe disease more frequently than the other serotypes (Guzman and Kouri 2002). Different genotypes of DENV within a particular serotype have also been shown to have differential outcomes in disease severity. Though studies in Asia had shown that secondary infection with DENV-2 caused an increased risk for DHF, no DHF/DSS was seen in DENV-2 epidemics in the Americas until Asian genotype DENV-2 strains were introduced. Watts *et al* examined a DENV-2 outbreak in Peru in 1995 where only mild illness occurred (Watts, Porter et al. 1999). The virus isolated from serum was sequenced and determined to be an American genotype DENV-2 strain (Leitmeyer, Vaughn et al. 1999). Epidemiological data from Asian outbreaks would have predicted that DENV-2 infections should cause up to 10,000 cases of DHF; however, no cases of DHF were detected. These observations suggest that Asian genotype strains cause more severe disease in children as well as adults than American genotype strains.

Several molecular determinants in the virus have been positively associated with disease severity. *In vitro* and *in vivo* studies describe mutations in the E protein of dengue that affects its virulence. Mutations that inhibit antibody recognition of the protein have been hypothesized to potentially cause more severe disease, whereas mutations that affect the function of the protein, such as those in the fusion loop, are believed to attenuate the virus (Roehrig, Bolin et al. 1998). Changes in the 3' and 5' non-

coding regions (NCR) of dengue are predicted to change the secondary structure of the RNA and therefore interfere with viral translation (Leitmeyer, Vaughn et al. 1999).

The specific sequence of DENV serotypes in secondary infections also has an important effect on the risk for DHF. An epidemiologic study by Sangkawibha *et al* examined a 1980 outbreak in Thailand and found the highest risk for severe disease in secondary DENV-2 infections that followed a primary DENV-1 infection. Primary DENV-3 infection followed by secondary DENV-2 appeared to cause the second highest incidence of DHF, followed by a primary DENV-4 infection preceding an exposure to DENV-2. DENV-1 infection followed by a secondary DENV-2 infection was also linked with several DHF epidemics (Sangkawibha, Rojanasuphot et al. 1984). Endy *et al*, in a prospective study of Thai school children from 1998-2000, showed that DHF occurred in the following DENV serotype sequences: DENV-4 followed by DENV-2, DENV-1 followed by DENV-3, DENV-2 followed by DENV-3, and DENV2-followed by DENV-1 (Endy, Nisalak et al. 2002). The Cuban epidemic of 2001-2002 identified an association between the occurrence of severe disease and the sequence of primary DENV-1 infection followed by a secondary infection with DENV-3 (Alvarez, Rodriguez-Roche et al. 2006).

Immunological response to dengue virus infections

Upon infection with DENV, the human immune system mounts a potent innate, humoral, and T cell response in an effort to clear the virus.

Innate Immune Response

Dendritic cells have been shown to be a potential target cell for DENV. However, dendritic cells were also proven to elicit a potent anti-viral response. The dendritic cell membrane protein CD209/DC-SIGN is able to bind dengue virus E protein, and therefore may act as a receptor for viral entry into dendritic cells, promoting viral propagation (Lozach, Burleigh et al. 2005). IFNα/β production by DENV-infected dendritic cells was abrogated by the down regulation of Tyk2-STAT, STAT1, and STAT2 by the nonstructural DENV proteins, suggesting possible immune evasion activity by DENV (Ho, Hung et al. 2005).

On the other hand, dendritic cells appear to participate in the immune response against DENV. *In vitro* infection of $CD14^{+}$ dendritic cells resulted in the production of TNFα, IFNγ, and IL-10 (Palmer, Sun et al. 2005; Deauvieau, Sanchez et al. 2007). An increase in IL-12p70 has also been demonstrated *in vitro* upon dendritic cell infection (Libraty, Pichyangkul et al. 2001). Human RIG-I, in dendritic cells, has been found to recognize double-stranded-RNA and is required for the production of type I interferons in response to flavivirus infections (Chang, Liao et al. 2006).

Other cells are also likely key players in the innate immune response against dengue. Natural killer (NK) cells have been found to be involved in antibody-dependent cell-mediated cytotoxicity (ADCC) of DENV-infected cells (Kurane, Hebblewaite et al. 1986; Laoprasopwattana, Libraty et al. 2007). Many other cell types, including epithelial and endothelial cells, mast cells and myelomonocytic cell lines, primary human monocytes, and monocyte-derived macrophages, have been shown to be susceptible to infection with DENV in vitro and respond to infection by producing the chemokines IL-

6, IL-8, RANTES, MIP-1α, and MIP-1β (Spain-Santana, Marglin et al. 2001; Lee, Su et al. 2007).

Antibody Responses

Primary infection with DENV induces the generation of antibodies against the structural proteins E and prM and also against the non-structural protein NS1. *In vitro* studies by Roehrig *et al* have shown that anti-E antibodies are able to neutralize the virus and also prevent viral binding and fusion (Roehrig, Bolin et al. 1998). *In vivo* studies have shown that passive immunization of mice with antibodies directed against E, prM, or NS1 can protect animals against lethal DENV infection. Anti-NS1 antibodies are able to fix complement and mediate viral lysis. Passive immunization of mice with anti-PrM, NS1 and E antibodies have been shown to protect against lethal dengue infection (Kaufman, Summers et al. 1987; Schlesinger, Brandriss et al. 1987; Kaufman, Summers et al. 1989).

T-Cell Responses

A vast amount of research has been conducted on the importance of T cells functions during the immune response to DENV. DENV-specific proliferation was found in both $CD8^+$ and $CD4^+$ T cell populations in peripheral blood mononuclear cells (PBMC) from DENV-immune donors using both tritiated thymidine incorporation and also by flow cytometry analysis of dilution of CFSE in cells stained pre-stimulation (Kurane, Meager et al. 1989). In bulk PBMC and clones, a predominantly T_H1 response was found, demonstrated by the production of IFNγ, TNFα, IL-2, and TNFβ (Hober, Poli et al. 1993; Green, Vaughn et al. 1999; Mustafa, Elbishbishi et al. 2001). Small amounts of the T_H2 cytokine, IL-4, have been found to be produced (Mori, Kurane et al. 1997).

Though most of the cytolytic responses measured in short-term T cell lines reflect $CD8^+$ T cell responses, lysis of DENV-infected cells by $CD4^+$ T cells has also been demonstrated. These $CD4^+$ cells lysed DENV peptide-pulsed target cells by a perforin-mediated mechanism and also lysed bystander cells through Fas-FasL interactions (Gagnon, Ennis et al. 1999). These latter interactions may contribute to the hepatocyte damage seen in dengue (Burke 1968; Ishak, D.H. et al. 1982).

Immune responses to secondary dengue virus infection

As noted above, evidence has suggested that secondary DENV infections are a risk factor for developing DHF/DSS. Memory immune cells generated from the primary DENV infection have been shown to modulate the immune response upon heterologous secondary DENV infection, suggesting that the pathology seen in secondary infection is immunologically mediated. Both antibody and T cell responses have been postulated to contribute to this phenomenon.

Antibody Dependent Enhancement (ADE)

Several studies have shown that viral burden was correlated with disease severity (Murgue, Roche et al. 2000; Libraty, Endy et al. 2002). Earlier, it was observed that DENV can replicate to a higher titer in PBMC from DENV-immune patients compared to PBMC from DENV-naïve individuals (Halstead, Nimmannitya et al. 1967). DENV-specific antibodies in the sera of the immune patients caused this increased viral replication. This phenomenon was designated antibody dependent enhancement (ADE) (Halstead, Nimmannitya et al. 1967). ADE is caused by anti-envelope antibodies resulting from a primary infection that are cross-reactive but non-neutralizing to the

secondary infecting virus. These antibodies bind the secondary virus and enhance its uptake into cells through the FcR receptor, potentially leading to increased viral load. ADE has been proposed as an immunopathogenic mechanism for DHF (Halstead, Nimmannitya et al. 1967).

Several *in vitro* studies have demonstrated a link between ADE and dengue disease. Kliks *et al,* demonstrated that serum from mothers of infants with DHF contained anti-DENV antibodies that enhanced infection of monocytes *in vitro* (Kliks, Nimmanitya et al. 1988). Also, the age when the infant fell sick with DHF correlated with the amount of antibody acquired from the mother. When DHF occurs during a primary DENV infection, it is primarily in infants six to twelve months of age whose mothers have been previously infected by DENV. In contrast, between the years of one and three, DHF is very rare during primary infection. This is a time window in which any antibody passed from the mother would no longer be present (Kliks, Nimmanitya et al. 1988). Seropositive infants infected with DENV under the age of three months were asymptomatic and were protected from disease. Another report by Kliks *et al* showed that pre-infection sera from immune Thai children who later had DHF enhanced DENV infection of monocytes *in vitro*, whereas sera from children that had less severe dengue disease were unable to enhance infection (Kliks, Nisalak et al. 1989).

In contrast, Laoprasopwattana *et al* also found that pre-illness plasma from Thai children enhanced DENV infection *in vitro* in K562 cells; however, no correlations were found between the ability of patient pre-secondary infection plasma samples to enhance viral infection and disease severity (Laoprasopwattana, Libraty et al. 2005). Laopraspwattana *et al* also measured antibody-dependent cellular cytotoxicity (ADCC)

mediated by these pre-illness plasma samples (Laoprasopwattana, Libraty et al. 2007). They demonstrated that ADCC activity of the plasma inversely correlated with the viremia levels in the patients undergoing DENV-3 infection, but not in those undergoing DENV-2 infection. Taken together, these studies suggest that pre-existing antibodies modify secondary DENV infections; however, controversy remains in whether these effects are pathogenic or protective.

Cross-reactive T Cell Immunity

The principle of original antigenic sin describes a situation in which cross-reactive memory immune cells generated from a primary infection alter the immune response during secondary infection (Francis 1953; Klenerman and Zinkernagel 1998). Specifically, it is hypothesized that lower avidity memory T cells can dominate the immune response over naïve T cells with higher avidity for the virus causing the secondary infection. The presence of cross-reactive T cells after primary DENV infections is, therefore, an implicit assumption in this model. DENV-specific $CD4^+$ and $CD8^+$ T cells have been detected in PBMC of patients after primary infection (Bukowski, Kurane et al. 1989). *In vitro* experiments showed that, though the predominant responses of the PBMC were to the homologous infecting serotypes, cross-reactive responses were found in all samples (Kurane, Innis et al. 1989). Kurane *et al* and Mathew *et al* also demonstrated the existence of DENV serotype-crossreactive T cells, the majority of which recognized nonstructural DENV proteins, especially NS3 (Kurane, Meager et al. 1989; Mathew, Kurane et al. 1996). Mathew *et al* also reported that, after secondary DENV infections, the majority of the DENV-specific memory T cells were serotype-cross-reactive and recognized nonstructural proteins (Mathew, Kurane et al. 1998).

Many reports have provided *in vitro* evidence for modulated immune responses during a secondary DENV infection. Mongkolsapaya *et al* demonstrated original antigenic sin *in vivo* for dengue by staining PBMC obtained during acute DENV infection with tetramers of HLA class I molecules loaded with variant peptides for each DENV serotype (Mongkolsapaya, Dejnirattisai et al. 2003). More $CD8^+$ T cells stained with tetramers matching a heterologous serotype than stained with the tetramer corresponding to the currently infecting virus. This evidence suggests that the majority of cells responding were not specific to the secondary infecting virus but were crossreactive memory T cells generated during the earlier primary infection. In addition, it has been demonstrated that cross-reactive $CD8^+$ T cells could mount a cytolytic response *in vitro* to variant peptides from other serotypes (Kurane, Innis et al. 1989). Other studies have shown that $CD4^+$ cells from DENV-immune donors produced more IFNγ in response to peptides from the homologous serotype, but that the ratio of TNFα- to IFNγ-producing cells was greater in response to heterologous serotypes (Mangada and Rothman 2005). In addition, HLA-A2-restricted $CD8^+$ T cells from vaccinees who received primary immunizations with different serotypes showed different IFNγ, TNFα, and MIP-1β cytokine profiles depending on which variant peptide was used for stimulation in vitro (Bashyam, Green et al. 2006). This *in vitro* evidence demonstrated that priming with one serotype can alter the response to another serotype. A particular peptide in this study, specifically a DENV-3 variant, proved to be immunodominant regardless of the serotype that the donor was infected with. These data provide evidence for a scenario where antigen from the secondary infecting virus could be immunogenic to memory cells generated from a primary infection regardless of serotype. These studies

also offer substantiation for the concept that a particular sequence of heterologous infection may influence the resulting immune response, supporting the epidemiologic observations that the serotype sequence of infection can influence dengue disease (Sangkawibha, Rojanasuphot et al. 1984).

Mechanisms of immunopathology in dengue hemorrhagic fever

Although potentially life-threatening, DHF is a self-limiting disease and generally causes no long-term damage. Therefore, vascular leakage in DHF is probably caused by functional rather than destructive effects on the endothelial cells. Several studies have thus investigated the effect of the dengue-induced immune response on endothelial cells. Though direct infection of endothelial cells *in vivo* is still debated, it has been shown that endothelial cells may be infected with DENV in vitro and as a result produce the pro-inflammatory cytokines IL-8, RANTES, and IL-6 (Avirutnan, Malasit et al. 1998; Spain-Santana, Marglin et al. 2001; King, Anderson et al. 2002; Lee, Su et al. 2007). Changes in the cytoskeletal structure and expression of adhesion molecules were observed in endothelial cells upon infection (Talavera, Castillo et al. 2004). These cells were also more permeable to small molecules. Addition of a blocking anti-IL-8 antibody was able to partially reverse the IL-8 induced effects on the cells. Infection of human umbilical vein endothelial cells (HUVEC) with dengue virus in the presence of dengue immune sera resulted in the activation of complement (Avirutnan, Malasit et al. 1998). Cardier *et al,* reported that *in vitro* the presence of serum from dengue infected patients caused activation and apoptosis of endothelial cells (Cardier, Marino et al. 2005). This phenomenon could be reversed with the addition of anti-TNFα antibody. Anti-DENV

NS1 antibodies can also bind HUVEC and induce the production of IL-6, IL-8, and MCP-1 (Lin, Chiu et al. 2005). These data suggest that inflammatory molecules induced as a result of DENV infection may cause an increase in permeability of bystander endothelial cells seen in DHF.

T cells have also been implicated in the inflammation seen in secondary DENV infections. Many markers of T cell activation and inflammation have been shown to be elevated in patients with increased disease severity. Studies by Green *et al*, Hober *et al*, and Kurane *et al* found elevated serum levels of TNFα, soluble IL-2 receptor, soluble CD8, soluble CD4 and soluble TNFRII in patients with DHF as compared to those with less severe DF. In addition, the highest levels of soluble TNFRII occurred two days before the appearance of plasma leakage, supporting the notion of a role for T cell activation in the genesis of DHF (Kurane, Brinton et al. 1991; Hober, Poli et al. 1993; Green, Vaughn et al. 1999). Bethell *et al* strengthened these results by showing that there were even higher levels of sTNFRII in patients with DSS than in patients with less severe DHF without shock (Bethell, Flobbe et al. 1998). Mangada *et al* demonstrated that there was an association between *in vitro* TNFα production of pre-illness PBMC to DENV antigens and more severe disease in subsequent infection (Mangada, Endy et al. 2002). These studies provide strong evidence that the increased risk of severe dengue disease upon secondary DENV infection is due to an immunopathological mechanism involving increased T cell activation and production of inflammatory cytokines.

Status of vaccine development against dengue

Secondary infection as a risk factor for DHF, plus the strong evidence for an immunopathological mechanism for DHF, present a significant hurdle for generating a vaccine against dengue. To help circumvent this complication, tetravalent vaccines directed against all four serotypes have been the focus of several groups (Halstead 2002). Clinical trials on two of these vaccines, resulted in 80 - 90% seroconversion to all four DENV serotypes, but the levels of immunity induced may not be enough to provide long-lasting protection (Halstead 2002).

Chimeric flaviviruses have also been under development for dengue vaccines. One such tetravalent vaccine uses the Yellow Fever virus strain 17D as a backbone and replaces the preM and E genes with those from DENV. The results from the chimeric vaccines have been encouraging, and neurovirulence tests in mice were negative. However, in primates, some serotypes induced a lower neutralizing antibody response than others. In addition, there was animal to animal variation with respect to their responses to the individual serotypes (Guirakhoo, Pugachev et al. 2004). Other chimeric vaccines have employed a DENV-2 or DENV-4 backbone and replaced the preM and E genes with those of other serotypes (Blaney, Sathe et al. 2007). Several of these vaccines have shown promise in animals by showing an attenuated phenotype compared to the wild-type viruses and also appear to confer protection in several murine models (Blaney, Sathe et al. 2007).

Plasmid vaccines have also been studied for DENV as well as for other flaviviruses such as Japanese encephalitis, Murray Valley Encephalitis, Tick Borne Encephalitis and louping ill viruses. These plasmids encode preM and E. Coexpression

of preM and E is required for correct folding of E, and also leads to secretion of virus-like particles, which may increase the immunogenicity of the vaccine (Timofeev, Butenko et al. 2004).

The nonstructural protein NS1 is another interesting choice for vaccine design. It has been shown to elicit an antibody response. In addition, antibodies directed against NS1 also has been demonstrated to induce a potent complement-mediated virus inactivation activity (Schlesinger, Brandriss et al. 1987). Vaccine studies using NS1 from Tick Borne Encephalitis virus in a murine model have induced a high-titer protective antibody response (Jacobs, Stephenson et al. 1994). However, anti-DENV NS1 antibodies have been shown to be cross-reactive to cell surface proteins in the walls of blood vessels (Lin, Lei et al. 2002). Therefore, a vaccine targeting NS1 could induce anti-NS1 antibodies that may be more harmful than protective.

A successful vaccine for DENV will most likely be tetravalent. That is, eliciting an immune response to all four serotypes simultaneously. This concurrent response to all four viruses is thought to prevent any immunopathology from occurring. Efforts to design such vaccines have included chimeric and plasmid vaccines, since such vaccines have the potential to tailor the immune response towards specific immunogenic molecules. They also are able to include these viral molecules from several serotypes in one reagent, potentially resulting in the desired tetravalent response. Results from vaccine studies have appeared promising. However, challenges remain for developing a vaccine that both induces equal immunity to all four serotypes and confers life-long immunity.

Murine models of dengue infection

The clinical studies described above have shown the existence of DENV serotype-cross-reactive memory T cells and also increased T cell activation and cytokine production in patients with acute dengue disease. However, there are limitations intrinsic to these human studies. In most patients with secondary DENV infections, the serotype of the previous infection is unknown; this could complicate interpretation of the immune response to the current DENV infection. Prior exposure to other flaviviruses is also not usually known. As a result, the influence of a specific serotype as the primary infection on the immune response directed to the secondary infection cannot be determined. Finally, different HLA genotypes influence the susceptibility of the patients to disease and these differences may complicate the analysis of data compiled from patients with different HLA types. Therefore, it is important to examine sequential infections in a system that minimizes these confounding factors. An animal model would be useful to examine the immune response to secondary DENV infections, since the history of viral infection could be controlled. A murine system would also be beneficial since the history of viral infection could be controlled. In addition, the use of inbred mouse strains would control for the influence of genetic factors on study outcomes, since HLA-type in humans appear to affect disease outcome.

Many mouse models have been utilized to study DENV infection, involving both immunodeficient and immunocompetent mice. Table 1 summarizes the major features of each of these models, which are discussed below.

Table I-1 Mouse Models of Dengue Infection

Authors	Mouse Strain	Virus	Viral Strain	Dose	Route	Viral Replication	Disease	Mortality (%)	Day of Death
Huang *et al.*	Balb/c, C57Bl/6, A/J	DENV-2	PL046	10^8 pfu	i.v.	yes	transient thrombocytopenia and paraplegia	0	NA
Atrasheuskaya *et al.*	Balb/c	DENV-2	P23085	$5LD_{50}$	i.p.	yes	anemia, thrombocytopenia, pre-terinal paralysis, shock, death	100	7
Shresta *et al.*	A/J	DENV-2	PL046	$10^{7.5}$ pfu	i.v.	yes	paralysis, elevated hematocrit, elevated WBC count	55	8-14
Chen *et al.*	C57Bl/6	DENV-2	16681	10^8 pfu	i.v.	yes	elevated serum liver enzyme levels, lymphocyte infiltration into the liver	0	NA
Paes *et al.*	Balb/c	DENV-2	Rio de Janeiro	10^4TCID_{50}	i.p.	yes	apoptotic and necrotic hepatocytes, steatosis	0	NA
Barth *et al.*		DENV-2	Rio de Janeiro	$TCID_{50}$	i.p.	yes	transient liver inflammation, multifocal endothelial cell injury	0	NA
Barreto *et al.*	Balb/c	DENV-2	Rio de Janeiro	10^4TCID_{50}	i.p. and i.v.	yes	transient lung inflammation	0	NA
Wu *et al.*	SCID + human PBL	DENV-1	not reported	10^6 pfu	i.p.	yes	none	0	NA
Lin *et al.*	SCID + K562	DENV-2	PL046	10^7 pfu	i.p.	yes	paralysis	100	17
An *et al.*	SCID + HepG2	DENV-2	Tr1751	10^6 pfu	i.p.	yes	thrombocytopenia, hematocrit, blood urea nitrogen levels	80	12.8
Bente *et al.*	NOD-SCID + human CD34+ cells	DENV-2	K0049	$10^{4.7}$ pfu	s.c	yes	fever, rash, thrombocytopenia	0	NA
Johnson and Roehrig	AG129	DENV-2	16681	10^6 pfu	i.p.	yes	hind-leg paralysis, blindness, and death	100	10-12
Shresta *et al.*	AG129	DENV-2	PL046	$10^{4.4}$ pfu	i.v.	yes	elevated hematocrit, paralysis, death	100	15
Shresta *et al.*	STAT-1 -/-	DENV-2	PL046	10^7-10^8 pfu	i.v.	yes	paralysis	0	NA
Shresta *et al.*	AG129	DENV-2	D2S10	10^7 pfu	i.v.	yes	death	100	5
Rothman *et al.*	Balb/c and B10.RDD	DENV-1	Hawaii	$10^{6.5}$ pfu	i.p.	no	None	0	NA
Rothman *et al.*	Balb/c and B10.RDD	DENV-2	NGC	$10^{6.5}$ pfu	i.p.	no	None	0	NA
Rothman *et al.*	Balb/c and B10.RDD	DENV-3	PR6	$10^{6.5}$ pfu	i.p.	no	None	0	NA
van der Most *et al.*	Balb/c	DENV-2 (secondary to YF/DENV-2 chimeric virus)	NGC	$10^{4.5}$ pfu	i.c.	not reported	death	75	10

Immunocompetent Mouse Models

Several immunocompetent murine models have been developed to study dengue. Huang *et al* infected immunocompetent Balb/c, C57Bl/6, and A/J mice with a high titer of DENV-2 strain PL046 intravenously, which caused transient thrombocytopenia in the animals (Huang, Li et al. 2000). Atrasheuskaya *et al* infected Balb/c with $5LD_{50}$ ip. of mouse-adapted DENV-2 strain P23085. These animals exhibited anemia, thrombocytopenia, pre-terminal paralysis, shock, and death (Atrasheuskaya, Petzelbauer et al. 2003). A one-hundred percent mortality rate was reported. There were increased serum levels of TNFα, IL-1β, IL-6, IL-10, IL-1R antagonist and soluble TNFRI. The addition of an anti-TNFα antibody decreased the mortality by 60%. Shresta *et al* intravenously infected A/J mice, also with a high titer ($10^{7.5}$ pfu) of DENV-2 strain PL046 (Shresta, Kyle et al. 2004). However, the normal phenotype of A/J mice includes a lower number of NK cells and lower NK cell activity than other strains, as well as a deficiency in complement component C5. The authors reported that 55% of infected mice developed paralysis, the presence of DENV in the central nervous system, and an elevated hematocrit and white blood cell count. They also noticed increased numbers of $CD69^+$ NK cells and B cells, an IgG and IgM response, and also an elevation in splenocyte production of IFNγ. Chen *et al* infected C57Bl/6 mice with a high titer (10^8 pfu) of DENV-2 strain 16681 intravenously (Chen, Lai et al. 2004). They observed elevated serum liver enzyme levels and lymphocyte infiltration into the liver. A homologous secondary DENV infection was given seven days after the initial infection and an increase in the clinical and biochemical signs of liver injury followed. The kinetics of liver injury correlated with T cell activation; serum liver enzyme elevation and

the appearance of apoptotic hepatocytes occurred on days 3, 5, and 7, the same days in which the numbers of activated T cells were high. Paes *et al* conducted histological studies showing that some liver damage occurred after infection of Balb/c mice with a DENV-2 strain from a Rio de Janeiro outbreak (Paes, Pinhao et al. 2005). This damage included apoptotic cells, necrotic hepatocytes and steatosis. They also showed an increase in liver enzymes. Barth *et al* reported that intraperitoneal infection with 10^4 $TCID_{50}$ of the Rio de Janeiro DENV-2 strain led to transient liver inflammation and multifocal endothelial injury (Barth, Barreto et al. 2006). Most recently, Barreto *et al* infected Balb/c mice intraperitoneally or intravenously with the Rio de Janeiro strain of DENV-2 (Barreto, Takiya et al. 2007). The mice demonstrated transient inflammation of the lungs by histological study. Using electron microscopy, they found that the endothelial cells of the animals' capillaries exhibited phyllopodia, suggesting their activation. The results from these immunocompetent mouse models of dengue infection correlated pathology with immune activation.

Immunodeficient Mouse Models

A significant number of murine models employed immunodeficient mice. Several models utilized mice with severe combined immunodeficiency (SCID). SCID mice are from the C.B-17 inbred strain (Balb/c C57Bl/K*a-Igh-1*^{b}/Icr N17F34) and lack functional T and B cells due to a mutation of the protein kinase, DNA activated, catalytic polypeptide gene (Prkdc) located on chromosome 16. Wu *et al* showed that some SCID mice engrafted with human peripheral blood lymphocytes became viremia after they were injected with DENV-1 (Wu, Hayes et al. 1995). Lin *et al* used K562 cells, a human myeloid cell line, as a graft in SCID mice; upon infection with 10^7 pfu DENV-2 strain

PL046 i.p., paralysis was seen and correlated with viremia (Lin, Liao et al. 1998). An *et al* found that, after engraftment with HepG2 cells, a human liver cell line, and i.p. infection with DENV-2 strain Tr1751, mice at first showed the highest viral titers in the serum and liver (An, Kimura-Kuroda et al. 1999). Later, on day 12 post infection when paralysis was seen, the highest viral titers were in the serum and brain. The animals also exhibited thrombocytopenia, and increases in hematocrit and blood urea nitrogen levels. Elevated TNFα was also found in the paralyzed mice. A more recent report by Bente *et al* used non-obese diabetic (NOD)-SCID mouse strain and reconstituted the animals with human $CD34^+$ cells from human cord blood (Bente, Melkus et al. 2005). After infection with $10^{7.7}$ pfu DENV-2 strain K0049, mice developed fever, rash and thrombocytopenia. These humanized mouse models demonstrated the pathological effect of dengue in relation to human tissues.

Immunodeficient mice other than SCID have also been used. Johnson and Roehrig infected AG129 mice, an IFNα/β/γ receptor knockout strain on the 129Sv(ev) mouse strain background, with 1×10^6 pfu DENV-2 strain 16681 i.p. (Johnson and Roehrig 1999). The infection caused hind-leg paralysis and blindness and was eventually lethal in these mice, thereby providing evidence of the necessity of IFN for viral clearance in mice. Shresta *et al* performed extensive work in dengue mouse models. Two of these reports also utilized AG129 mice. The first demonstrated that these mice were susceptible to DENV-induced elevation of hematocrit, paralysis, and death. Since AG129 lack the interferon receptors, both studies solidified the importance of IFNα/β and IFNγ for controlling and clearing the infection. Mice deficient in $CD4^+$ and $CD8^+$ T or B cells were not susceptible to DENV (Shresta, Kyle et al. 2004). Shresta *et al* also

performed experiments analyzing the importance of STAT-1 in DENV infection (Shresta, Sharar et al. 2005). Using mice deficient in STAT-1, they found that a STAT-1-dependent mechanism was required for clearing the initial viral load after infection with a high titer of DENV. However, they also provided evidence that a STAT-1-independent mechanism was essential for controlling viremia and preventing disease, seen in this study as paralysis. Another study involving AG129 mice used a new mouse adapted DENV-2 strain, D2S10, isolated by alternately passing the virus between mosquito cells and AG129 mice (Shresta, Sharar et al. 2006). Infection with this virus was lethal at an earlier time point and resulted in the production of large amounts of TNFα. Death appeared to be TNFα mediated since blocking this cytokine reduced mortality. These murine studies reported a mechanism by which the immune system controls DENV and DENV-induced disease, specifically the importance of both type 1 and type 2 interferons.

Murine T Cell Responses to Dengue

Several studies in mouse models specifically examined the T cell response to DENV. Rothman *et al* defined a K^d-restricted NS3 epitope and an L^d-restricted E epitope recognized by $CD8^+$ T cell clones generated from DENV-2-infected mice (Rothman, Kurane et al. 1996). Spaulding *et al* isolated a T cell clone from a DENV-3-infected mouse that recognized the DENV-3 variant of the NS3 epitope (Spaulding, Kurane et al. 1999). Using cytotoxicity assays, they demonstrated that DENV-2 NS3-specific T cell clones were not cross-reactive to the DENV-3 variant; however, the DENV-3 specific clone was cross-reactive and recognized the DENV-2 variant. These variant NS3 peptides differed by only one amino acid at a non-anchor residue. Van der Most *et al* immunized Balb/c mice subcutaneously with a chimeric yellow fever/dengue virus and

then challenged the mice 14 days later with $100LD_{50}$ DENV-2 strain New Guinea C (NGC) intracranially (van Der Most, Murali-Krishna et al. 2000). They observed a high frequency of DENV-specific activated $CD8^+$ T cells in the central nervous system using intracellular cytokine staining for IFNγ. The chimeric virus vaccination protected the mice against lethal dengue encephalitis. In a study by An *et al* using Hep-G2 cell engrafted SCID mice, mice were infected with DENV-2 with or without adoptive transfer of DENV-specific $CD8^+$ T cell clones (An, Kimura-Kuroda et al. 1999). Mice that received $CD8^+$ T cells had decreased mortality when compared to mice receiving DENV-2 and naïve thymocytes (80% vs. 100%). However, deaths occurred five days earlier in mice that received DENV-specific T cells. These findings provided evidence for both pathogenic and protective roles for DENV-specific T cells. These T cell studies demonstrated the induction of serotype-crossreactive DENV-specific T cells by primary DENV infection in mice and also potential protective and pathogenic roles of these cells upon secondary DENV infection.

The murine models described above provided valuable observations regarding the immune response to DENV and the potential role of immune activation in dengue disease. It was determined that interferons are essential for viral clearance. In addition evidence was presented suggesting that TNFα causes disease manifestations in DENV infection. It was also shown that vaccination with DENV structural proteins protected animals from disease upon DENV challenge. However, these systems have limitations. The majority of the studies using immunocompetent mice utilized high titers of virus for immunization. In comparison, the amount of DENV thought to be inoculated by *Aedes aegypti* mosquito in natural infections is on the order of 10^4 pfu (Gubler 1989). In

addition, the majority of disease signs seen in these animals are neurologic, which has little clinical relevance to human infections. Similarly the use of mice with immunodeficiencies results in a system that is somewhat artificial. Since one postulated mechanism for severe dengue disease involves an immunopathological response, a murine model with an intact immune system is necessary to study this phenomenon. Lastly, none of the mouse models used heterologous secondary DENV infections to examine the effect of DENV-specific memory T cells on the immune response to secondary infection; as noted earlier, this is a proposed immunopathological mechanism for dengue disease.

Thesis objectives

Human studies have shown the existence of serotype-cross-reactive memory T cells and also increased T cell activation and cytokine production in patients with acute dengue disease. However, no direct evidence has been reported that serotype-crossreactive memory T cells are activated to augment the T cell response. Although many mouse models had been established to examine the immune response in DENV virus infections, the response to sequential DENV infections had not previously been described. We therefore developed an immunocompetent mouse model to examine the immune responses to heterologous secondary DENV infections and demonstrate the role of cross-reactive memory T cells in this response. **We hypothesized that the T cell responses in heterologous secondary DENV infections are enhanced compared to the acute and memory response to primary DENV infection. Further, we hypothesize that the augmentation of the immune response in secondary DENV infections is due to**

serotype-cross-reactive memory T cells and also results in a change in the hierarchy of epitope immunodominance. This work is presented in two parts:

Chapter III: Cross-reactive Memory CD8$^+$ T cells Alter the Immune Response to Heterologous Secondary Dengue Virus Infections in Mice in a Sequence-Specific Manner

a. Kinetics of IFNγ responses to primary and secondary dengue virus infections in mice

b. The magnitude and specificity of the T cell response to secondary dengue virus infections and sequence dependency

c. Memory T cells are preferentially activated during heterologous secondary dengue virus infections

d. Enhanced TNFα production following heterologous secondary dengue virus infections

e. Altered specificity of cytokine and cytotoxic responses after secondary challenge

Chapter IV: Sequence-Specific Augmentation of the Dengue-Specific Memory CD8$^+$ T Cell Response by Memory CD4$^+$ T Cells

a. Difference in magnitude of the CD4$^+$ T cell responses in secondary DENV-1 versus secondary DENV-3 infections

b. Influence of memory CD4$^+$ T cells on the memory CD8$^+$ T cell response

c. Activation of cross-reactive memory CD4$^+$ T cells by heterologous antigen

CHAPTER II

MATERIALS AND METHODS

A. Viruses

DENV-1 strain Hawaii, DENV-2 strain New Guinea C (NGC), DENV-3 strain CH53489, and DENV-4 strain 814669 were used in our experiments. All viruses were propagated in C6/36 mosquito cells and their titers were determined by plaque assay in Vero cells.

B. Peptides

H-2K^d-restricted NS3 and H-2L^d-restricted E epitopes were previously characterized by our laboratory (Rothman, Kurane et al. 1996). Peptides with amino acid sequences corresponding to each epitope for each serotype were synthesized at the University of Massachusetts Peptide Core Facility (Table II-1). The amino acid sequences for the NS3 epitope in DENV-1 and DENV-3 are identical as are DENV-2 and DENV-4, and the respective peptides are referred to as D1/3 NS3 and D2/4 NS3. The peptides corresponding to the E epitopes for each serotype are referred to as D1E, D2E, D3E, and D4E.

C. Preparation of dengue antigens

Lysates of DENV-infected Vero cells were prepared as previously described (Mangada and Rothman 2005). Vero cell monolayers were infected with either DENV-1, DENV-2, DENV-3, or DENV-4. The cells were incubated until >50% of the cells

Table II-1. K^d- and L^d- restricted dengue epitopes used in this study[a]

Peptide	MHC	Serotype	Sequence
NS3 (298-306)	K^d	D1/3	G**Y**ISTRV*G***M**
		D2/4	G**Y**ISTRV*E***M**
E (331-339)	L^d	D1	A**P**CKIPF*SS*
		D2	S**P**CKIPF*EI*
		D3	A**P**CKIPF*ST*
		D4	A**P**CKVP*IEI*

[a]Anchor residues are shown in bold; differences in amino acid sequence between serotypes for each epitope is shown in italics.

showed cytopathic effect. The cells were harvested and fixed by treatment with 0.025% gluteraldehyde for 15 minutes at 4^{o} C. The cells were then sonicated on ice followed by centrifugation. The supernatants were harvested and stored at -70°C. Control antigen was made from uninfected Vero cells.

D. Immunization

Balb/c mice 4-6 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME) and immunized with 2 $x10^5$ pfu ip. of DENV or an equivalent volume of C6/36 culture supernatant. For secondary infection, mice were immunized 28-56 days after the primary infection with 2 x 10^5 pfu ip. of heterologous or homologous DENV serotypes. At the indicated time points, mice were sacrificed and splenectomized. Single cell suspensions were made. All mice were maintained in the Animal Facility at the University of Massachusetts Medical School, which is regulated by AWA-1995, PHS-1986, and MA140-1985, following the American Association for the Accreditation of Laboratory Animal Care 1965 guidelines.

E. Cell line generation

Splenocytes were placed in a T25 flask at a concentration of $5x10^6$ cells per milliliter in RPMI 1640 medium with 10% fetal bovine serum and 14.3 mM β-mercaptoethanol. Recombinant murine IL-2 (50 U/ml) and peptide (10μg/mL) or DENV antigen (1:100 final dilution) were also added. Every 14 days, peptide-stimulated cells were restimulated with 10μg/mL of peptide and γ-irradiated (3500 rads) P815 cells.

F. IFN-γ ELISPOT

Peptide-specific IFNγ-secreting T cells were quantified by ELISPOT. Briefly, wells of 0.45 μm filter plates (Millipore) were pre-coated with purified anti-mouse IFN-γ mAb (AN18, Mabtech) in PBS overnight. 2.5 x 10^5 splenocytes per well were incubated overnight with 4 μg/mL peptide, 5μg/mL concanavalin A (Sigma-Aldrich), or medium alone. Wells were washed between each step with phosphate buffered saline (PBS). Biotinylated anti-mouse IFN-γ mAb (R4-6A2, Mabtech) was added and incubated at room temperature for 2 hours. After washing, streptavidin-horseradish peroxidase (Mabtech) was added and incubated at room temperature for 1 hour. After further washes, Nova Red substrate solution (Vector Laboratories) was added and incubated for 15 minutes at room temperature. The plate was then washed with tap water. Plates were allowed to dry and spots were counted either manually or by CTL Immunospot ELISPOT plate reader. All data shown represent values in which the medium control backgrounds were subtracted. The median background value was 7 spot forming cells (SFC) per million splenocytes.

G. Intracellular cytokine staining

Peptide-specific IFNγ and TNFα-secreting T cells were quantified by intracellular cytokine staining (ICS) assay as described (Mangada and Rothman 2005). Briefly, 5 x 10^5 splenocytes were incubated with 10 μg/mL peptide, PMA/Ionomycin, or medium alone with 5 μL/mL Brefeldin A (GolgiPlug, BD Bioscience) for 5 hours at 37^o C. Cells were washed twice with FACS buffer (2% FBS and 0.1% sodium azide in PBS). Anti-CD16/CD32 (2.4G2, BD Bioscience) was added and the samples were incubated at 4^o C

for 15 minutes. Anti-mouse CD3ε - PerCP (145-2C11, BD Bioscience) and anti-mouse CD8α-FITC (5.3-C711, eBioscience) were added and the samples were incubated at 4°C for 30 minutes. Cells were washed. Cytofix/Cytoperm buffer (BD Bioscience) was added to each sample and incubated at 4°C for 20 minutes. Cells were washed twice with Perm/Wash buffer (BD Bioscience). PE-conjugated anti-mouse TNF-α (MP6-XT22, eBioscience) and/or APC-conjugated IFN-γ (XMG1.2, eBioscience) was added and the samples were incubated at 4°C for 30 minutes. Cells were washed with Perm/Wash and were resuspended in FACS Buffer. Data were acquired by the Flow Cytometry Core Laboratory at the University of Massachusetts using a FACSCalibur and analyzed using FlowJo software (Tree Star). A small lymphocyte gate was drawn on forward and side-scatter low populations and further gated on $CD3^+$ $CD8^+$ cells. The frequencies of cytokine-positive cells from samples cultured with medium alone were subtracted from those of samples stimulated with peptide.

Intracellular cytokine staining of cells stimulated with antigen was performed as above with a few modifications. Cells were stimulated overnight with a 1:20 dilution of viral antigen or control vero antigen in the presence of 5 μL/mL each of Brefeldin A and monensin. The frequencies of cytokine-positive cells from samples cultured with control vero cell antigen were subtracted from those of samples stimulated with viral antigen.

H. ^{51}Cr Release Assays

P815 Target cells were labeled with 10μL (250 μCi) of ^{51}Cr for one hour at 37°C. After labeling, cells were washed three times, followed by incubation with 10mg/mL of peptide for 30 minutes in a 96-well round bottom plate. Effector cells were added to 2 x

10^3 target cells per well at an effector to target ration of 50:1. The cells were incubated for 4 hours at 37^{o}C, and then supernatants were harvested using the Skatron supernatant collection system. The amount of ^{51}Cr released was measured in a gamma counter. Percent specific lysis was measured by the following equation: (cpm experimental release – cpm spontaneous release)/(cpm maximum release – cpm spontaneous release) x 100. Spontaneous release values were obtained from supernatants from target cells incubated in medium without effectors. Maximum release values were acquired from supernatants from target cells incubated with Renex detergent (1:20 dilution) without effectors. All assays were performed in triplicate.

I. Isolation of $CD4^+$ and $CD8^+$ T cells

$CD4^+$ and $CD8^+$ T cells were collected by negative selection through magnetic separation. Splenocytes were isolated from DENV-2-immune mice and incubated with biotin-antibody cocktail (Miltenyi Biotec) followed by incubation with anti-biotin microbeads. After washing with buffer (0.5% fetal bovine serum and 2mM EDTA in PBS pH 7.2), the cells were collected as the effluent when passed through an LS column (Miltenyi Biotec) in a MidiMACS separator (Milteni Biotec).

J. Adoptive Transfer

For whole splenocyte transfer, 2 x 10^7 Thy 1.2^+ splenocytes from DENV-3-immune Balb/c mice that were immunized at least 28 days prior or naïve Balb/c mice were transferred in 100 μL i.v. into congenic Thy 1.1^+ Balb/c mice. The next day, mice

were infected with 2 x 10^5 pfu DENV-2 i.p. Day 9 post infection, mice were sacrificed and splenectomized.

For transfer of $CD4^+$ and $CD8^+$ T cells, 1 x 10^6 $CD8^+$ T cells and/or 3 x 10^6 $CD4^+$ T cells were transferred i.v. in a volume of 100μL into naïve Balb/c mice. The following day, the mice were immunized with 2 x 10^5 pfu ip. of either DENV-1 or DENV-3. Nine days later, the mice were sacrificed and intracellular cytokine staining was performed on their splenocytes.

K. Thy 1.2^+ cell depletion

Cells were incubated with Thy 1.2 microbeads (Miltenyi Biotec) at 4^oC for 15 minutes. Cells were washed. The cell suspension was applied to an LD depletion column (Miltenyi Biotec) in a MidiMACS separator (Miltenyi Biotec).

L. Serum transfer

On day -1, naïve male Balb/c mice were passively immunized ip. with 200 μL of undiluted sera from DENV-3-immunized or control mice. On day 0, mice were immunized with 2 x 10^5 pfu ip. DENV-2. On day 9 post-infection, mice were sacrificed.

M. Statistics

Medians, absolute deviations, confidence intervals, and standard errors were computed using SPSS and Microsoft Excel. ELISPOT and ICS data were compared between groups using the Mann-Whitney test, ANOVA, and Tukeys pot hoc analysis; p values <0.05 were considered significant.

CHAPTER III

CROSS-REACTIVE MEMORY CD8$^+$ T CELLS ALTER THE IMMUNE RESPONSE TO HETEROLOGOUS SECONDARY DENGUE VIRUS INFECTIONS IN MICE IN A SEQUENCE-SPECIFIC MANNER

Secondary DENV infection is a risk factor for DHF. Extensive evidence points to immunopathology as a hypothesized mechanism for this phenomenon. Due to limitations of human studies, an animal model would be useful to examine the immune response to secondary DENV infections. Several mouse models have been utilized to study DENV infection. In these cases either immunodeficient mice were used or immunocompetent mice were infected with a high DENV inoculum or infected intracranially (Lin, Liao et al. 1998; An, Kimura-Kuroda et al. 1999; Johnson and Roehrig 1999; Huang, Li et al. 2000; van Der Most, Murali-Krishna et al. 2000). In contrast, immunocompetent mice infected with lower doses of DENV by the intraperitoneal route remain disease-free but develop antibody and T cell responses that parallel human immune responses to primary infection. However, the response to sequential DENV infections has not previously been described. We infected immunocompetent Balb/c mice sequentially with low doses of heterologous serotypes of DENV. We then examined IFNγ and TNFα induction by CD8$^+$ T cells after infection. The effect of memory T cells and antibodies on the immune response was determined.

A. Kinetics of IFN-γ responses to primary and secondary dengue virus infections in mice

To understand the differences in the T cell response to DENV after primary and secondary infection, we first compared the kinetics of the immune response following primary or heterologous secondary dengue virus infection. IFNγ ELISPOT assays were used to quantify the T cell responses in DENV-infected mice. Mice were immunized with a primary DENV infection. On days 4 – 14 post infection, the splenocytes were assayed for IFNγ production in response to the previously identified NS3 and E epitopes corresponding to each serotype (Table II-1) (Rothman, Kurane et al. 1996). The peak IFNγ ELISPOT response was seen between days 8 – 10 (Table III-1). The highest overall response was seen after primary DENV-4 infection. DENV-2 gave the next highest response, whereas DENV-1 and DENV-3 gave low responses. The NS3 epitope was immunodominant compared to the E epitope in all immunization groups. In addition, the D2/4 NS3 peptide elicited a larger response than the D1/3 NS3 peptide regardless of the infecting serotype (Figs. III-1 and III-2) .

Twenty-eight days after the primary infection, mice were boosted with a secondary infection of a heterologous DENV serotype. Days 4 – 14 post infection, cytokine production of the splenocytes was measured. Though the magnitude of the response was greater after secondary infection as compared to after primary infection, the timing of the peak response was similar, at 8 – 10 days post infection (Table III-1). The NS3 epitope was immunodominant compared to the E epitope after secondary infection and the D2/4 NS3 peptide elicited a higher response than the D1/3 NS3 peptide.

Table III-1. Kinetics of IFN-γ response to dengue epitopes following primary and secondary DENV-2 infection*

			Median SFC / Million Splenocytes (Absolute Deviation)					
					Peptide			
		N	D1/3 NS3	D2/4 NS3	D1 E	D2 E	D3 E	D4 E
Primary DENV-2	**Days Post Infection**							
	4	4	19 (11)	36 (15)	<7	<7	<7	11 (11)
	6	4	<7	67 (28)	81 (19)	16 (7)	<7	57 (28)
	8	3	10 (7)	177 (82)	<7	71 (53)	<7	83 (83)
	9	4	<7	68 (24)	<7	65 (74)	<7	67 (42)
	10	2	<7	265 (77)	23 (0)	65 (39)	12 (12)	59 (59)
	14	3	<7	33 (74)	<7	<7	<7	<7
Heterologous Secondary DENV-2								
	4	4	<7	27 (45)	81 (27)	27 (13)	<7	43 (34)
	6	3	24 (21)	68 (39)	13 (14)	41 (32)	11 (19)	65 (34)
	8	4	<7	<7	17 (27)	<7	<7	17 (57)
	9	3	291 (146)	584 (187)	79 (119)	99 (171)	104 (172)	233 (199)
	10	4	<7	92 (68)	15 (12)	17 (17)	<7	<7
	14	4	<7	29 (18)	9 (8)	<7	10 (11)	8 (10)

* Data shown were compiled from two separate experiments

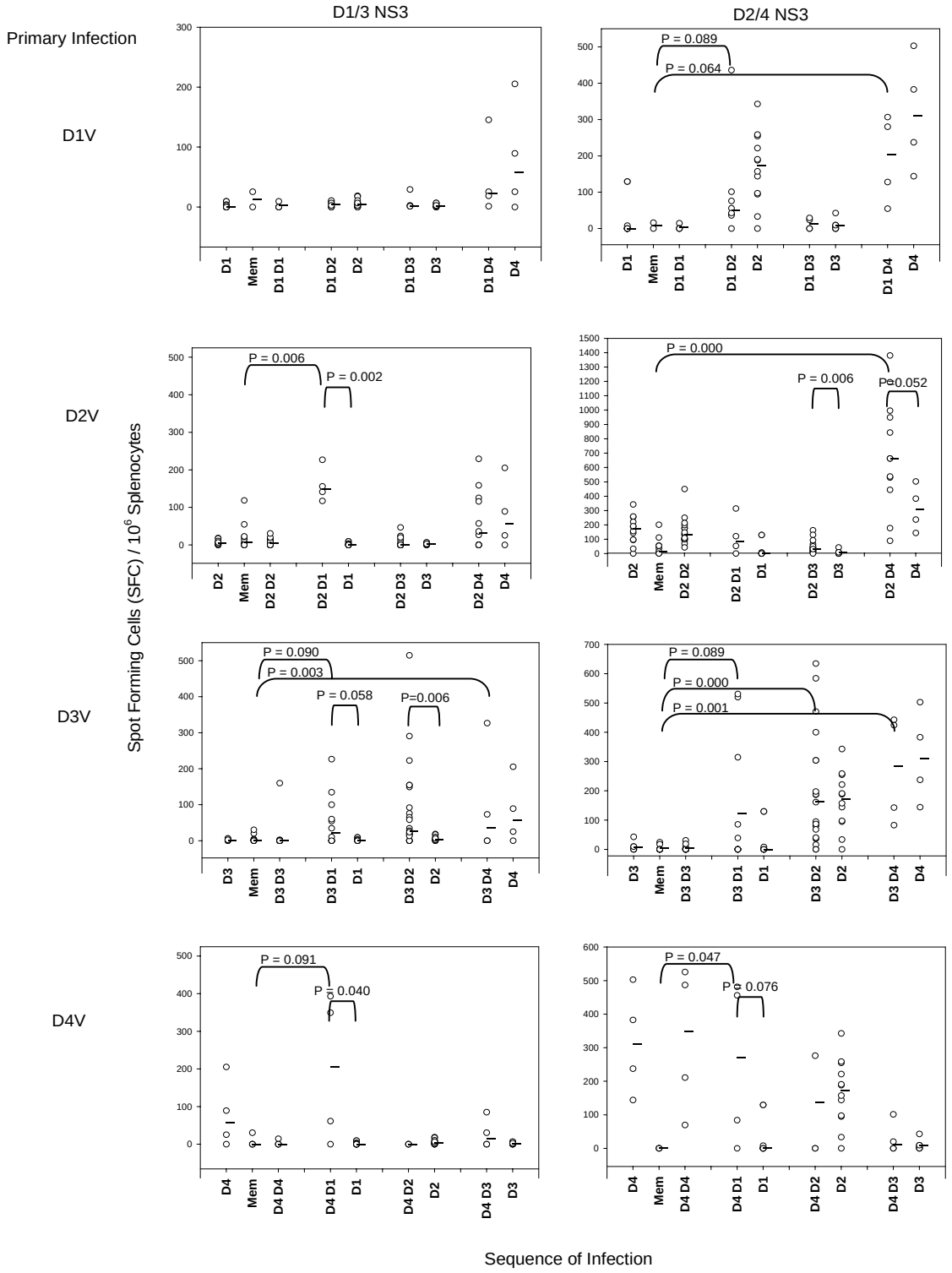


FIGURE III-1. IFNγ ELISPOT responses to the dengue virus NS3 epitope. Mice were administered sequential heterologous or homologous dengue virus infections as noted. The IFNγ responses to the D1/3 NS3 (left column) and D2/4 NS3 (right column) peptides were measured 8-10 days post infection by ELISPOT. The X axis represents the sequence of infection that the animals received. For example, D1 D2 refers to mice that received a primary DENV-1 infection followed by a secondary DENV-2 challenge. 'Mem' indicates responses at 36-38 days after primary infection. All 16 potential sequences were tested; data for secondary infections are grouped by the primary serotype (listed on the left). Data for primary infections with each serotype are repeated in each graph for ease of comparison. The Y axis represents the ELISPOT responses in spot forming cells per million splenocytes. Each point represents an individual animal; median values are noted by horizontal lines. P values for the comparisons between different groups were calculated using the Mann-Whitney test. P values < 0.10 are shown.

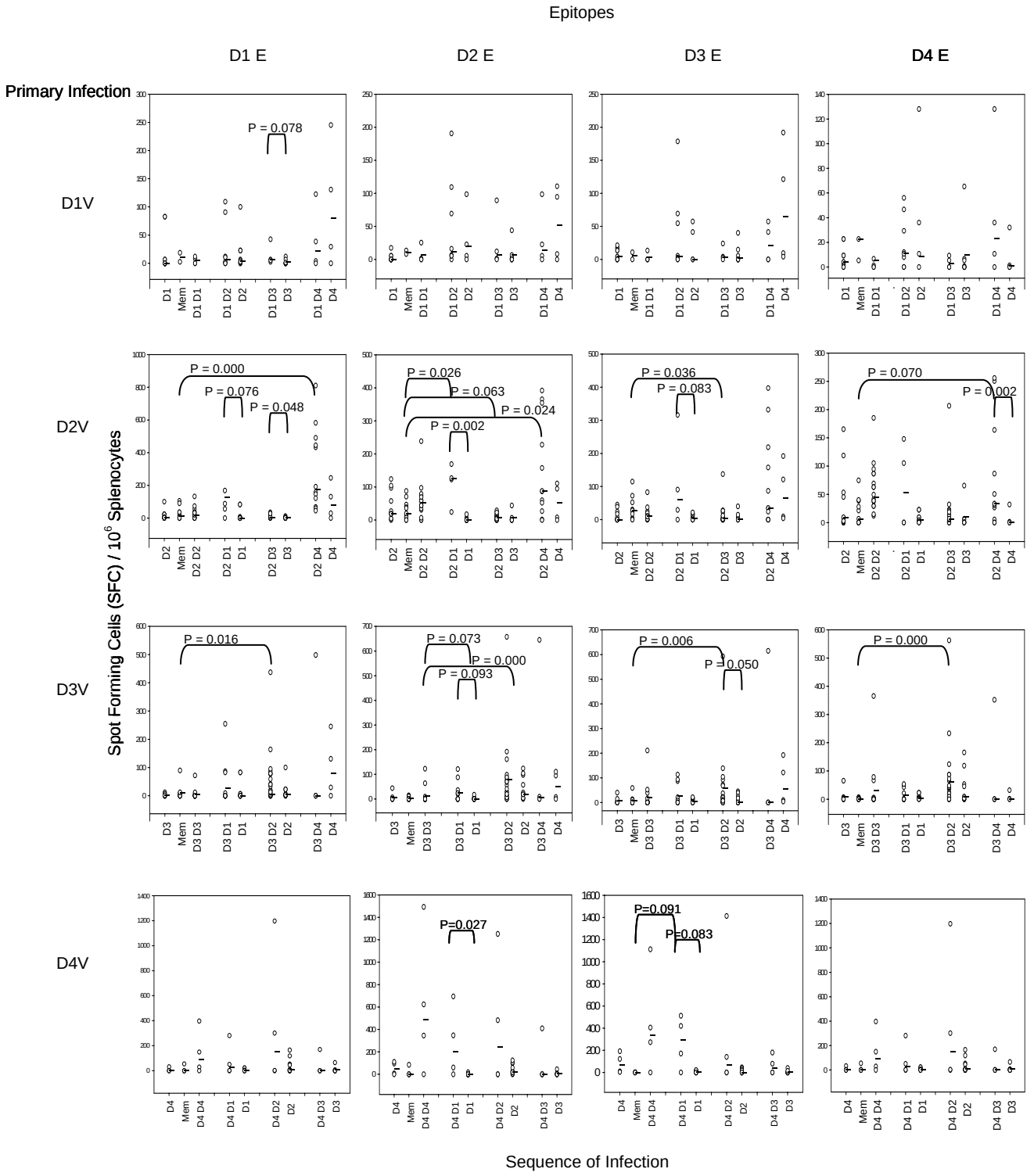


FIGURE III-2. IFNγ ELISPOT responses to the dengue virus E epitope. Mice were administered sequential heterologous or homologous dengue virus infections as described. IFNγ responses to the D1E (leftmost column), D2E (second column), D3E (third column), and D4E (rightmost column) peptides were measured 8-10 days post infection by ELISPOT. Data are presented as described in the Figure III-1 legend. N = 4 – 22 mice per infection group.

B. The magnitude and specificity of the T cell response to secondary dengue virus infections and sequence dependency

The model of original antigenic sin and data in humans suggest that prior infection with DENV generates memory cells that are cross-reactive to antigens from heterologous DENV serotypes (Halstead, Rojanasuphot et al. 1983; Mongkolsapaya, Dejnirattisai et al. 2003). These memory cells would have a lower threshold of activation than naïve cells that are specific to the secondary infecting DENV and therefore would be expected to dominate the immune response to the secondary infection. To determine whether sequential infections have similar effects on the immune response to DENV in this murine model, animals were sequentially infected with all combinations of two DENV serotypes. On days 8-10 post primary infection, the splenocytes from subgroups of mice were analyzed in IFNγ ELISPOT assays. The remaining mice were given a secondary immunization on day 28 post primary infection. Days 8-10 post secondary infection, ELISPOT assays were performed to measure the immune response to the secondary infection.

Overall, heterologous secondary infection caused an increase in the frequencies of epitope-specific IFNγ-secreting cells compared to the response after primary infection (Fig. III-1). However, the effect was sequence dependent. For example, a boost in the immune response to all peptides tested was seen in the group of mice that received a primary infection with DENV-3 followed by a secondary infection with DENV-2. These observations were not universal in all sequences and the epitope to which the boosted immune response was directed varied (Fig. III-1 and III-2).

A change in the epitope hierarchy was also observed after heterologous secondary DENV infections. The most striking example was the immune response to the D1/3 NS3 epitope. When mice were challenged with DENV-2 after a primary DENV-3 infection, the immune response to the D1/3 NS3 epitope was significantly increased when compared to the response during the memory phase after a primary DENV-3 immunization (Fig. III-1). Another such scenario occurred after a primary DENV-2 infection followed by a DENV-1 challenge, where the immune response to the D2E epitope was boosted over that after a primary DENV-1 infection (Fig. III-2). In both of these cases, the epitope to which the enhanced response was directed corresponded to the primary infecting serotype.

C. Memory T cells are preferentially activated during heterologous secondary dengue virus infections

Since antibody dependent enhancement is a proposed mechanism of dengue immunopathology, we considered the possibility that pre-existing antibodies from the primary infection enhanced the secondary infection resulting in the boost in T cell response observed. Therefore, we tested whether passive immunization of mice with DENV-immune sera would enhance the response to immunization with a heterologous serotype. Mice were given sera from mice immunized with DENV-3 28 days prior and were challenged with DENV-2. The IFNγ ELISPOT response was measured on day 8. The T cell response from mice that received the immune sera was lower than that from the mice that received the control sera (Fig. III-3). The decrease in response after adding the sera could be the result of inhibition of infection by neutralizing antibodies in the

sera. These data suggest that the boosted T cell response we observed after heterologous secondary infection was not due to enhanced antigen presentation by antibody-complexed virus.

These findings suggested that serotype-crossreactive epitope-specific memory T cells from the primary infection were being preferentially recalled during the heterologous secondary infection resulting in the boost of the immune response. To determine if this were the case, DENV-3 immune or naïve Thy 1.2^+ splenocytes were adoptively transferred intravenously into congenic Thy 1.1^+ mice and the mice were infected with DENV-2. Nine days later, the IFNγ response was measured by IFNγ ELISPOT and intracellular cytokine staining. To distinguish between the donor and recipient cells' response by ELISPOT, the splenocytes were depleted of the Thy 1.2^+ donor cells prior to the assay. The response of the depleted splenocytes was compared to the response of the undepleted splenocytes (Fig. III-4a). Depleting the memory cells significantly decreased the response, showing that the memory cells were responsible for the majority of the immune response during secondary infection.

To directly compare the peptide-specific responses of donor and recipient T cells, intracellular cytokine staining (ICS) assays were done after the adoptive transfer using the Thy1 marker to distinguish the donor and recipient cells. A significantly higher frequency of the donor CD8+ T cells responded to peptide stimulation than the endogenous CD8+ T cells. Figure III-4b shows data from one animal that received DENV-3-immune Thy 1.2^+ splenocytes prior to DENV-2 challenge. In this figure, 0.6% and 1.3% of donor CD8+ T cells responded to D1/3 NS3 and D2/4 NS3, respectively, as compared to 0.11% and 0.17% of host CD8+ T cells. Three adoptive transfer

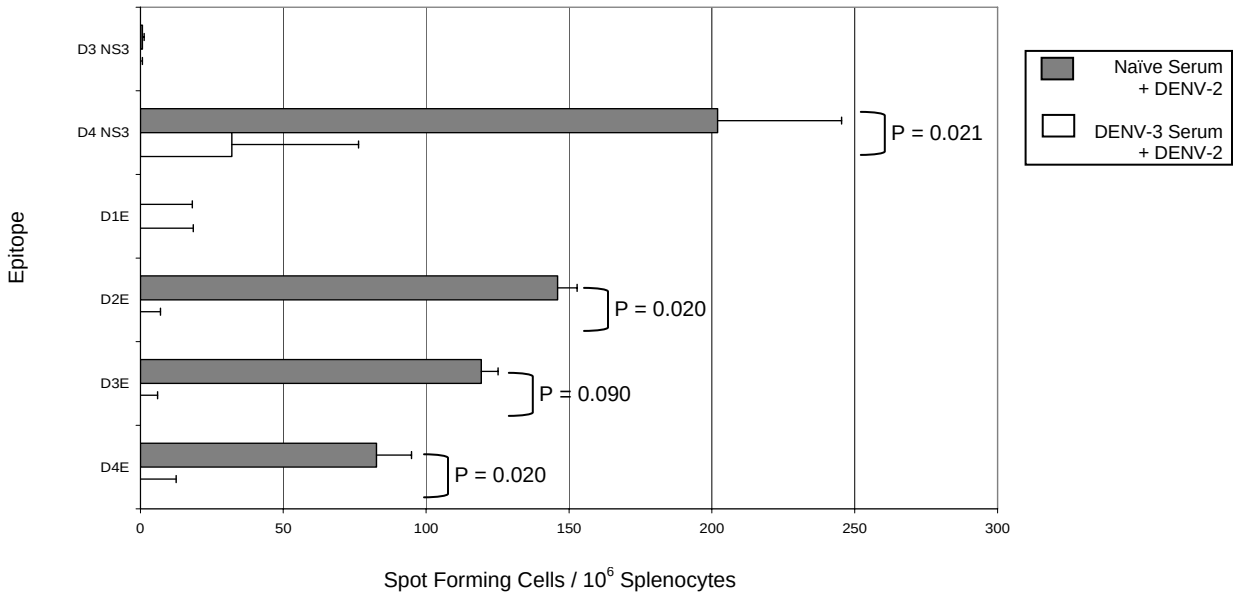
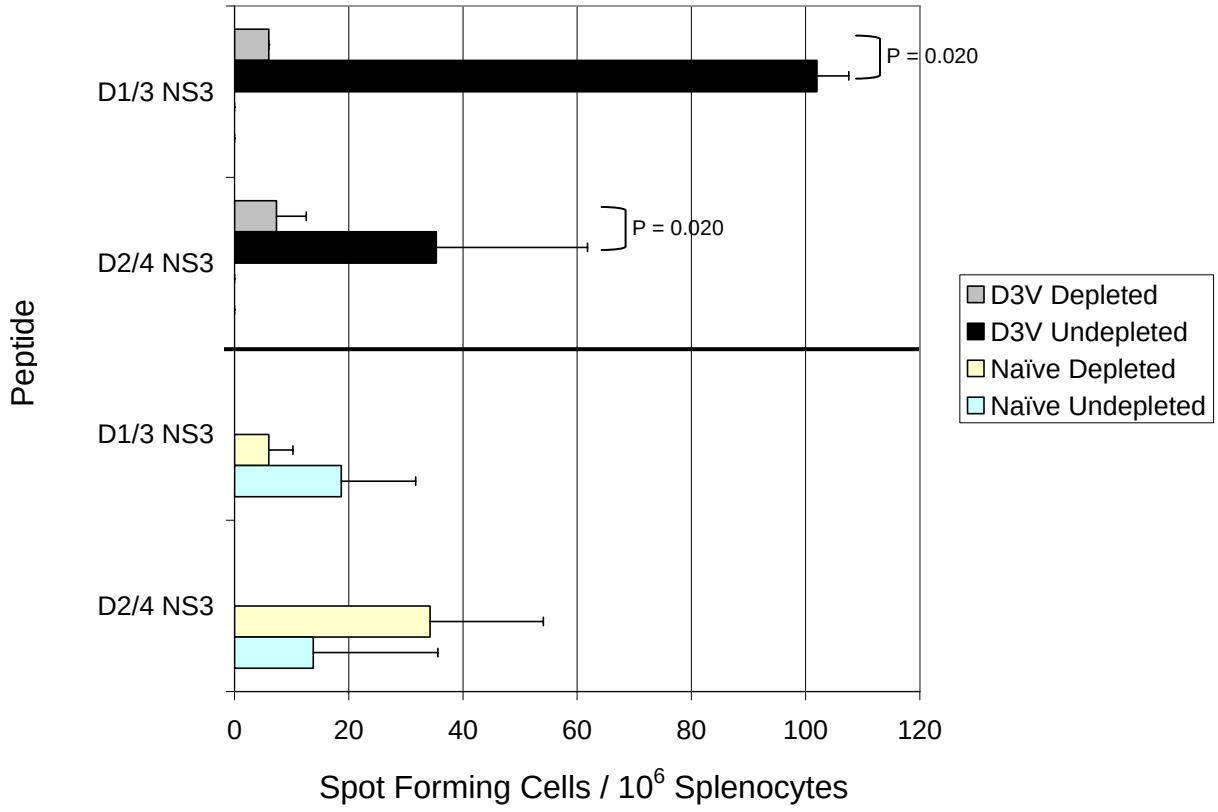
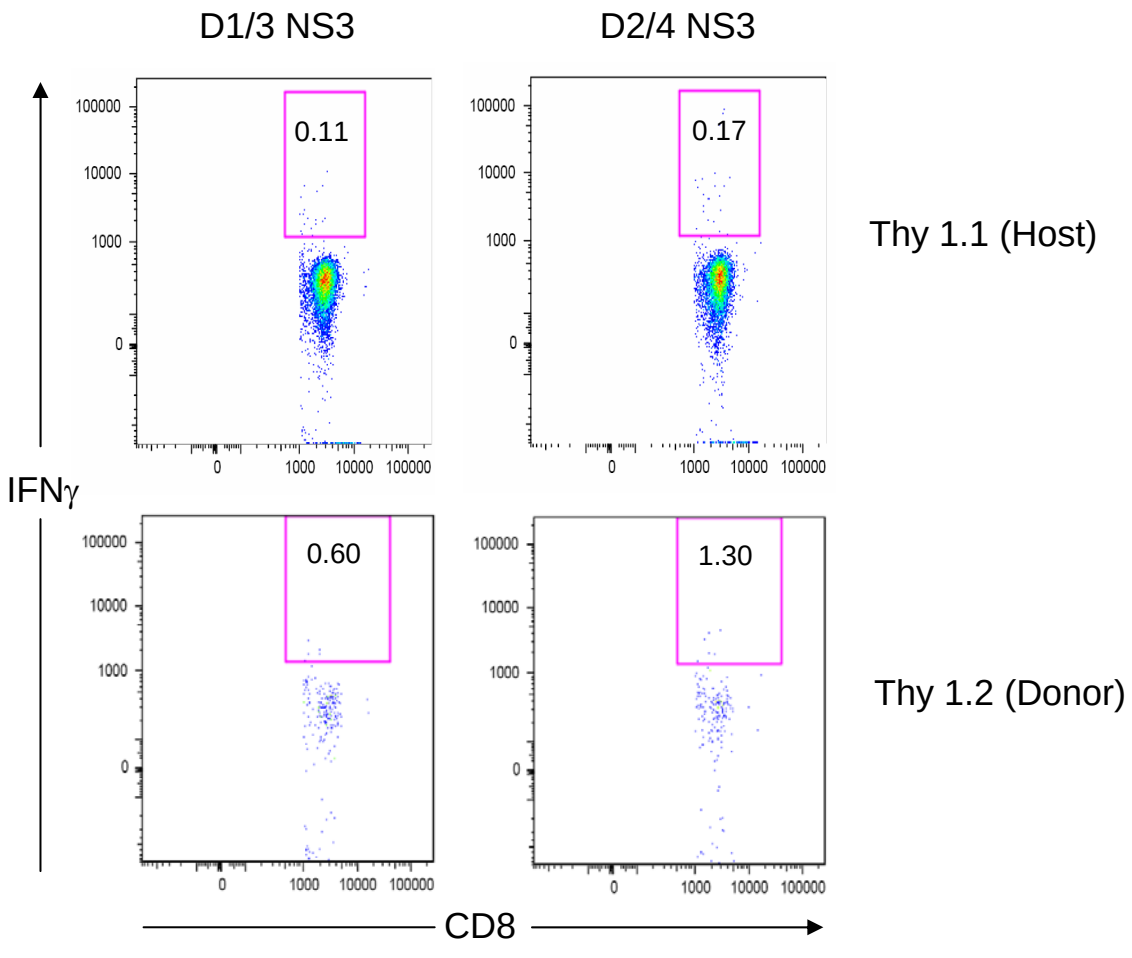


FIGURE III-3. T cell responses to dengue virus infection after passive immunization of mice with dengue virus-immune serum. Four- to six-week old male Balb/c mice were given 200 μL DENV-3-immune or naïve serum i.p. The following day, mice were infected with $2x10^5$ pfu DENV-2 i.p. On day 8 post infection, the IFNγ response to the 6 peptides indicated was measured by ELISPOT. Median values (N = 4 per group) and 95% confidence intervals are shown. P values were calculated by the Mann-Whitney test. P values <0.05 are considered significant and those <0.10 are shown. N = 4 mice per group

A.


D1/3 NS3
D2/4 NS3
D1/3 NS3
D2/4 NS3
P = 0.020
P = 0.020
Peptide
D3V Depleted
D3V Undepleted
Naïve Depleted
Naïve Undepleted
0
20
40
60
80
100
120
Spot Forming Cells / 10^6 Splenocytes

B.


D1/3 NS3
D2/4 NS3
0.11
0.17
0.60
1.30
IFNγ
CD8
Thy 1.1 (Host)
Thy 1.2 (Donor)

C.

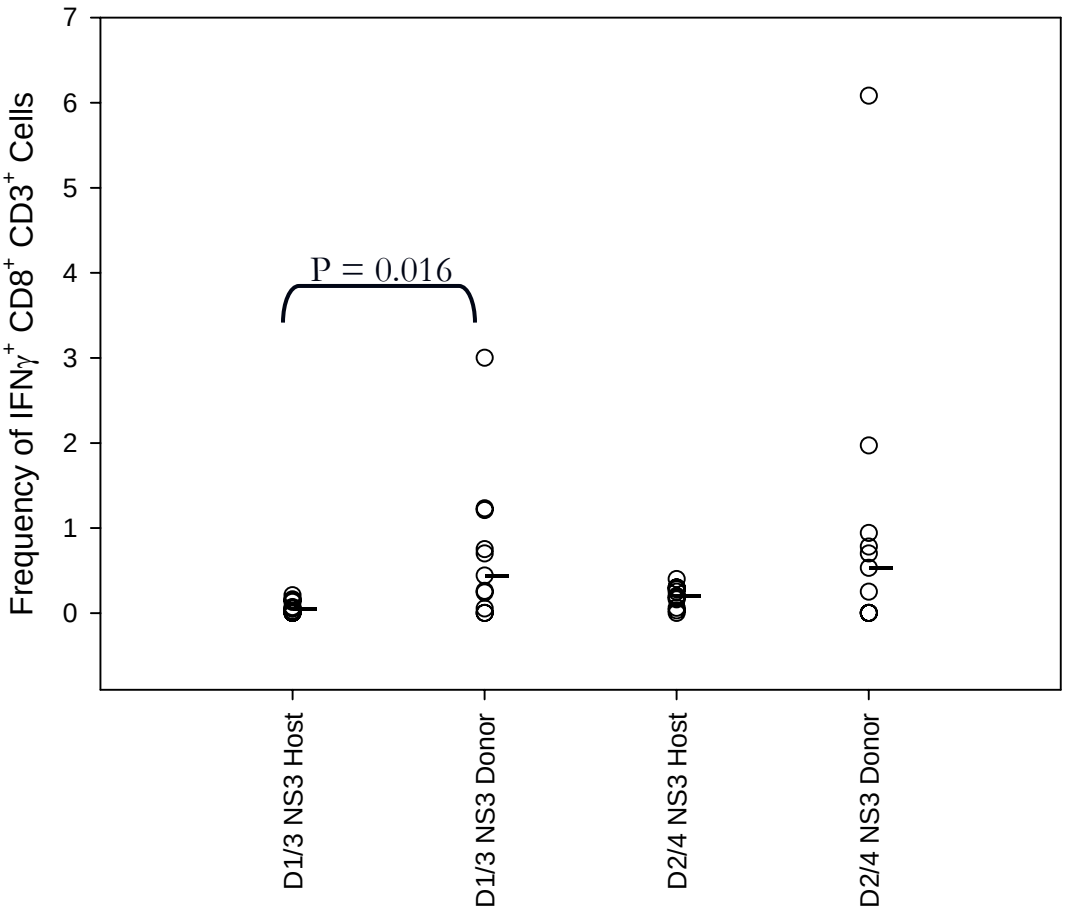


FIGURE III-4. Preferential activation of donor dengue virus-immune splenocytes in dengue infection following adoptive transfer of splenocytes from DENV-3-immune mice. Thy 1.2^+ Balb/c mice were infected with $2x10^5$ pfu DENV-3 i.p. Splenocytes were isolated from mice 28 days post infection, and $2x10^7$ cells were transferred i.v. to naïve Thy1.1^+ congenic mice. The following day mice were infected with $2x10^5$ pfu DENV-2 ip. 9 days post infection mice were sacrificed and their splenocytes were used in ELISPOT and intracellular cytokine staining (ICS) assays to examine peptide-specific IFNγ responses. *a*. IFNγ ELISPOT responses. To determine the effect of memory cells on the response, Thy 1.2^+ donor cells were depleted from splenocytes by magnetic cell separation prior to the ELISPOT assay. The responses of depleted and undepleted cells were compared. Median values (N=4 per group) and 95% confidence intervals are shown. *b.* Intracellular cytokine staining for IFNγ of splenocytes following adoptive transfer. Data were analyzed by gating on Thy 1.1^+ (recipient) or Thy 1.2^+ (donor) $CD3^+$ $CD8^+$ lymphocytes. The frequency of $CD8^+$ $IFN\gamma^+$ cells was measured in the gated population. Data shown are representative of 3 experiments performed. *c.* Summary data for intracellular cytokine staining assays for all experiments performed. Horizontal lines represent median values. P values were calculated using the Mann-Whitney test. P values <0.05 were considered significant and those <0.10 are shown. N = 11 mice per group from a total of 3 experiments.

experiments (N=11 mice) yielded comparable results; the median frequencies of host CD8+ T cells responding to D1/3 NS3 and D2/4 NS3 were 0.05% and 0.2% respectively, and the median frequencies of donor CD8+ T cells responding to D1/3 NS3 and D2/4 NS3 were 0.44% and 0.53%, respectively. The difference in the frequency of $CD8^+$ donor versus host cells secreting IFNγ in response to D1/3 NS3 was statistically significant ($p = 0.016$). These results directly show that cross-reactive memory T cells were preferentially recruited during the secondary DENV infection.

D. Enhanced TNF-α production following heterologous secondary dengue virus infections.

In human studies, TNF-α has been implicated in dengue immunopathology. Both DENV-infected cells and DENV-specific T cells have been shown to be capable of TNFα secretion (Mangada, Endy et al. 2002; Mangada and Rothman 2005). We therefore determined whether there was an enhanced TNF-α response in mice after secondary infection when compared to primary infection using ICS (Fig. III-5). Though not statistically significant, the frequency of $TNF\alpha^+$ $CD8^+$ T cells was increased after heterologous secondary infection when compared to both the TNFα response during acute primary infection and also during the memory phase after primary infection. This augmented response was seen after stimulation with all the DENV peptides tested. These observations are similar to our findings on the IFNγ response, and indicate that serotype-cross-reactive TNFα-producing memory T cells were expanded in secondary infection.

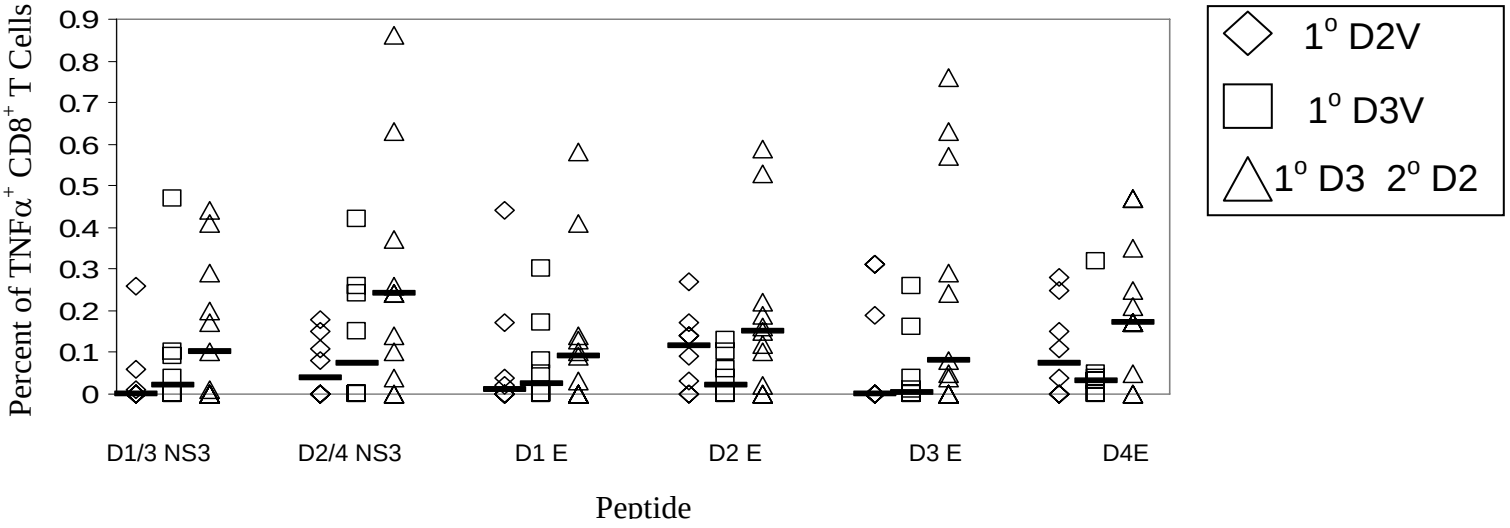


FIGURE III- 5. Enhanced TNFα responses to heterologous secondary dengue virus infection. Mice were infected with a primary or secondary dengue virus infection, as indicated. TNFα responses to the six peptides corresponding to the NS3 and E epitopes were measured by intracellular cytokine staining 8-10 days post infection. Data are presented as the percent of $CD8^+$ $CD3^+$ cells that were TNFα+. Each data point represents an individual mouse. Diamonds, squares, and triangles represent mice that received primary DENV-2 infection, primary DENV-3 infection, or heterologous secondary DENV-2 infection, respectively. Median values for each group are shown. P values were calculated by Mann-Whitney test. All p values were greater than 0.05 and therefore not considered significant and not shown. N = 7 – 11 mice per group from a total of 3 experiments.

E. Altered specificity of cytokine and cytotoxic responses of T cell lines generated after primary vs. secondary dengue virus infections.

Previous studies in dengue have described cross-reactive proliferative and cytotoxic responses. Thus, we hypothesized that the cytokine response was not the only T cell effector function affected in the cross-reactive response to secondary infection. Therefore, we examined the serotype-cross-reactivity of the responding T cell populations in greater detail. To generate a greater frequency of DENV-specific T cells, peptide-specific cells were expanded *in vitro* from mice with primary DENV-3 or DENV-2 infections or from DENV-3-immune mice that were challenged with DENV-2 (secondary DENV-2 infection). Splenocytes from each mouse were stimulated with peptide D1/3 NS3 or D2/4 NS3. Five days post stimulation, chromium release assays were done to determine the killing activity and specificity of these cell lines. Cell lines which were generated from splenocytes from mice with primary DENV-3 infection after stimulation with either D1/3 NS3 or D2/3 NS3 peptides showed very low specific lysis (Fig. III-6). The low cytotoxic activity seen was comparable against target cells pulsed with either the D1/3 NS3 or D2/4 NS3 peptides. This result correlated with ELISPOT results that indicated that primary DENV-3 immunization resulted in low IFNγ responses. Cell lines generated from mice that received primary DENV-2 after stimulation with D2/4 NS3 peptide preferentially killed targets pulsed with the D2/4 NS3 peptide over those pulsed with D1/3 NS3 (Fig. III-6), showing high specificity for DENV-2. In contrast, cell lines that were generated from mice that received primary DENV-2 after stimulation with D1/3 NS3 showed equivalent lysis of targets pulsed with D1/3 NS3 peptide or D2/4 NS3. These data indicate that the majority of responding T cells in mice

with primary DENV-2 infection were specific for D2/4 NS3 but that a sub-population of DENV-2-specific T cells activated by primary DENV-2 infection was cross-reactive and able to be expanded *in vitro* with the heterologous peptide D1/3 NS3. However, cell lines derived from mice that received primary DENV-3 followed by secondary DENV-2 infection after stimulation with either peptide responded with lower but similar killing of targets pulsed with either D1/3 NS3 or D2/4 NS3 (Fig. III-6). These results indicate that the populations of cells that were expanded in both of these cultures were cross-reactive. The magnitude and cross-reactivity of killing by cell lines generated from mice with secondary DENV-2 infection were similar to those of cell lines established from mice with primary DENV-3 infection, suggesting that DENV-3-specific T cells were cross-reactively expanded by the heterologous infection *in vivo*. These data suggest that the specificity of cytotoxic activity of DENV-specific T cells was altered in secondary DENV-2 infection as compared to primary DENV-2 infection.

To further evaluate the serotype-crossreactivity of these T cell lines, the bulk cultures were also stained with H-2K^{d}-D1/3 NS3 and H-2K^{d}-D2/4 NS3 tetramers. Patterns of tetramer staining mirrored those of cytotoxic activity. Bulk cultures established from mice that were administered primary DENV-3 had low but equal staining for both the D1/3 NS3 and D2/4 NS3 tetramers, suggesting cross-reactivity. Cell lines generated from primary DENV-2-infected mice and stimulated with D1/3 NS3 stained equally with the D1/3 NS3 and D2/4 NS3 tetramers. The majority of the tetramer-positive cells were double stained with both tetramers, suggesting that these cells were cross-reactive. (Fig. III-6 and III-7b). Cultures from the same primary DENV-2-infected mice but stimulated with D2/4 NS3 had greater tetramer staining with

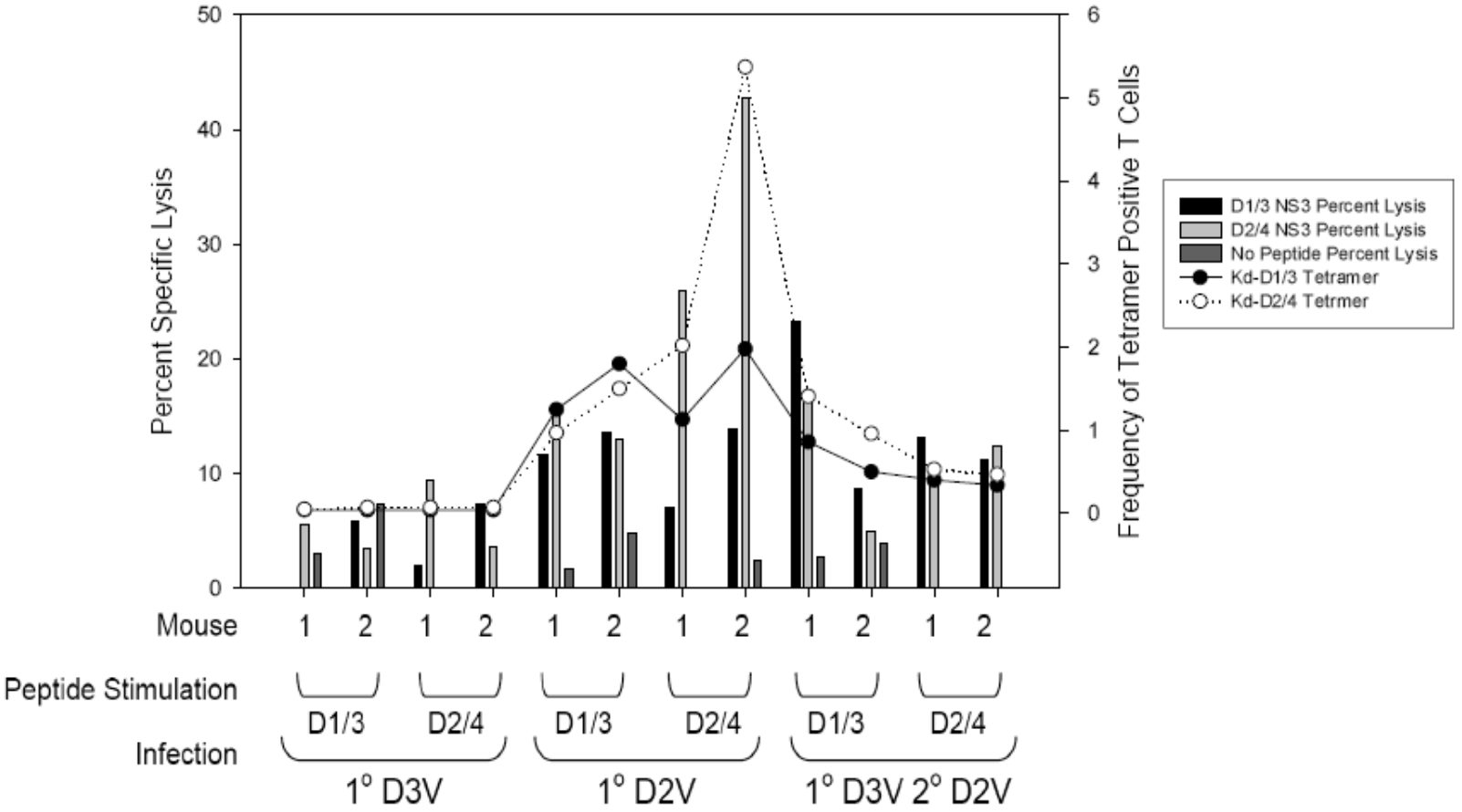
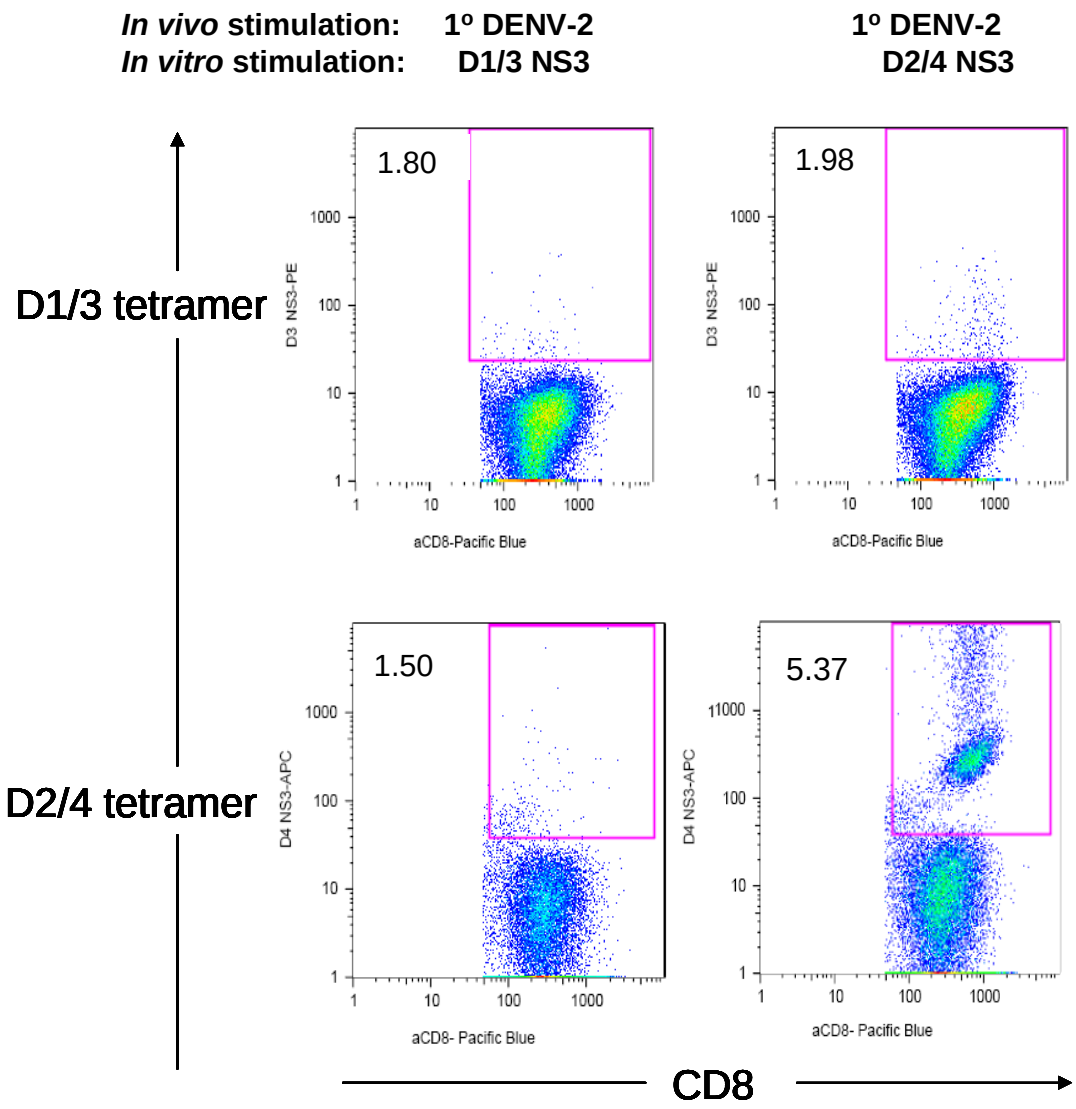


FIGURE III-6. CTL activity and tetramer staining in bulk culture T cell lines. Bulk culture T cell lines were generated from mice that received either primary DENV-2 infection, primary DENV-3 infection, or primary DENV-3 followed by secondary DENV-2 infection. Each cell line was stimulated with either D1/3 NS3 or D2/4 NS3 peptide. Five days post stimulation, cell lines were stained with K^d-D1/3 NS3 and K^d-D2/4 NS3 tetramers. Chromium release assays were also performed using these lines with P815 target cells pulsed with either peptide.

A.


In vivo stimulation: 1º DENV-2
In vitro stimulation: D1/3 NS3
1º DENV-2
D2/4 NS3
D1/3 tetramer
D2/4 tetramer
CD8
1.80
1.98
1.50
5.37
D3 NS3-PE
D4 NS3-APC
aCD8-Pacific Blue
aCD8- Pacific Blue
1
10
100
1000
11000

B.

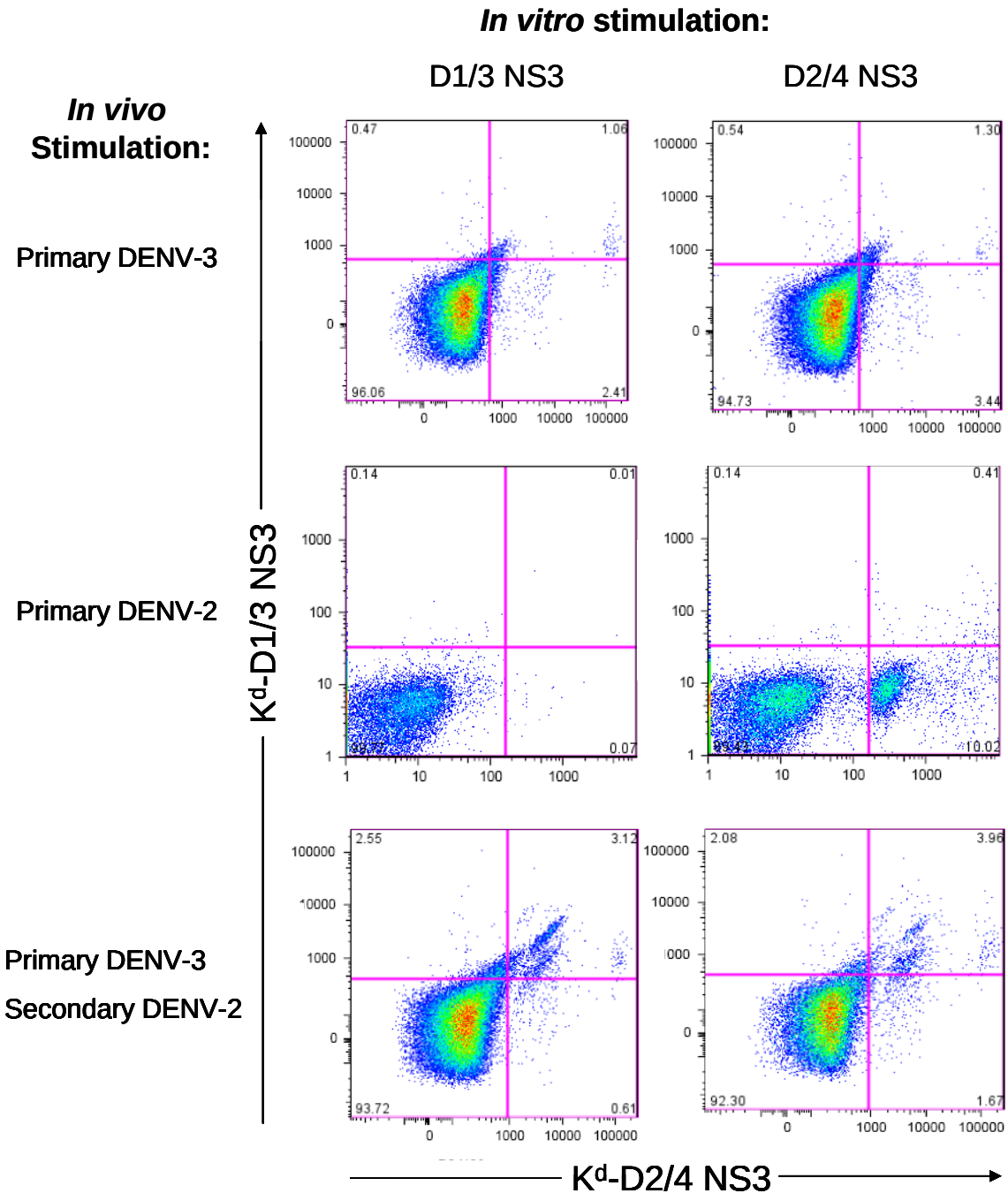


FIGURE III-7. Representative tetramer staining of day 5 bulk culture T cell lines. Bulk culture T cell lines were generated from mice that received either primary DENV-2 infection, primary DENV-3 infection, or primary DENV-3 followed by secondary DENV-2 infection. Each cell line was stimulated with either D1/3 NS3 or D2/4 NS3 peptide. Five days post stimulation, lines were stained with Kd-D1/3 NS3 and Kd-D2/4 NS3 tetramers. Cells were gated on CD3+ CD8+ CD19- F4/80^{-} small lymphocytes. *(a).* Representative single tetramer stain. *(b).* Representative double tetramer stain.

the K^d-D2/4 NS3 tetramer than the K^d-D1/3 NS3 tetramer, indicating the expansion of a DENV-2-specific population. These tetramer staining data correlate with the specific lysis results and indicate that cells generated by D1/3 NS3 stimulation were more cross-reactive than those stimulated with D2/4 NS3. Finally, cultures from mice secondarily infected with DENV-2 had a low frequency of tetramer-positive cells, but the staining was comparable with both the K^d-D2/4 NS3 tetramer and the K^d-D1/3 NS3 tetramers. As with the lines from the primary DENV-3-infected mice, the majority of the tetramer-positive cells were stained with both tetramers (Fig. III-7b). Akin to the cytotoxicity data, the tetramer staining patterns of the secondary DENV-2 T cell lines were similar to those of the primary DENV-3 cultures, suggesting expansion of DENV-3-specific memory cells to heterologous stimulation. These results are in agreement with our sequential infection data in that we had observed that memory cells generated during a primary DENV-3 infection were preferentially recruited to respond to antigen from a secondary DENV-2 challenge.

To generate a broader picture of the functional profile of these cells, the cell lines were also assayed for cytokine secretion, namely IFNγ and TNFα. Additional rounds of stimulation were required for expansion of enough cells for analysis. After three additional rounds of stimulation, several lines had failed to expand. The majority of these lines were from mice with primary DENV-3 infection. This lack of cell expansion correlates with the low T cell responses that we saw in ELISPOT assays and day 5 bulk cultures. The remaining lines were stained with the K^d-D1/3 NS3 and K^d-D2/4 NS3 tetramers and tested for peptide-specific IFNγ- and TNFα-producing $CD8^+$ T cells by intracellular cytokine staining. The one culture remaining from the mice with primary

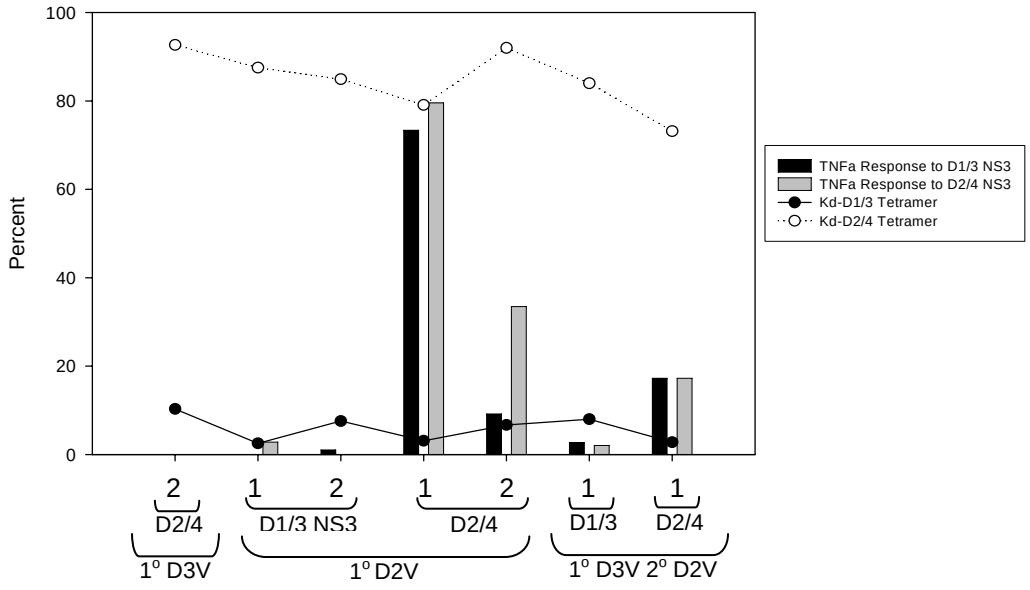


FIGURE III-8: Comparison of tetramer staining and the frequency of TNFα$^+$ CD8 T cells. Bulk culture T cell lines were generated from mice that received either primary DENV-2 infection, primary DENV-3 infection, or primary DENV-3 followed by secondary DENV-2 infection. Each cell line was stimulated with either D1/3 NS3 or D2/4 NS3 peptide. Cells were restimulated at 14-day intervals with peptide and irradiated P815 cells. After three rounds of stimulation, cells were stained with the K^d-D1/3 NS3 and K^d-D2/4 NS3 tetramers and assayed for TNFα response to peptide by intracellular cytokine staining.

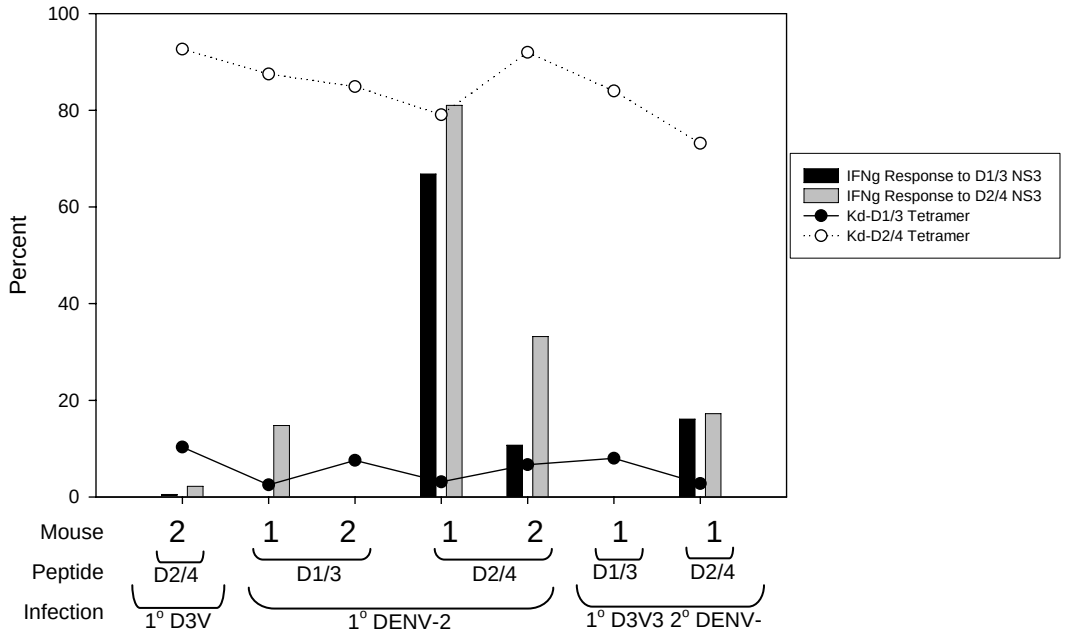


FIGURE III-9: Comparison of tetramer staining and the frequency of IFNγ$^+$ CD8$^+$ T cells. Cell lines were generated as described in Figure III-8. After three rounds of stimulation, cells were stained with the K^d-D1/3 NS3 and K^d-D2/4 NS3 tetramers and assayed for IFNγ response to peptide by intracellular cytokine staining.

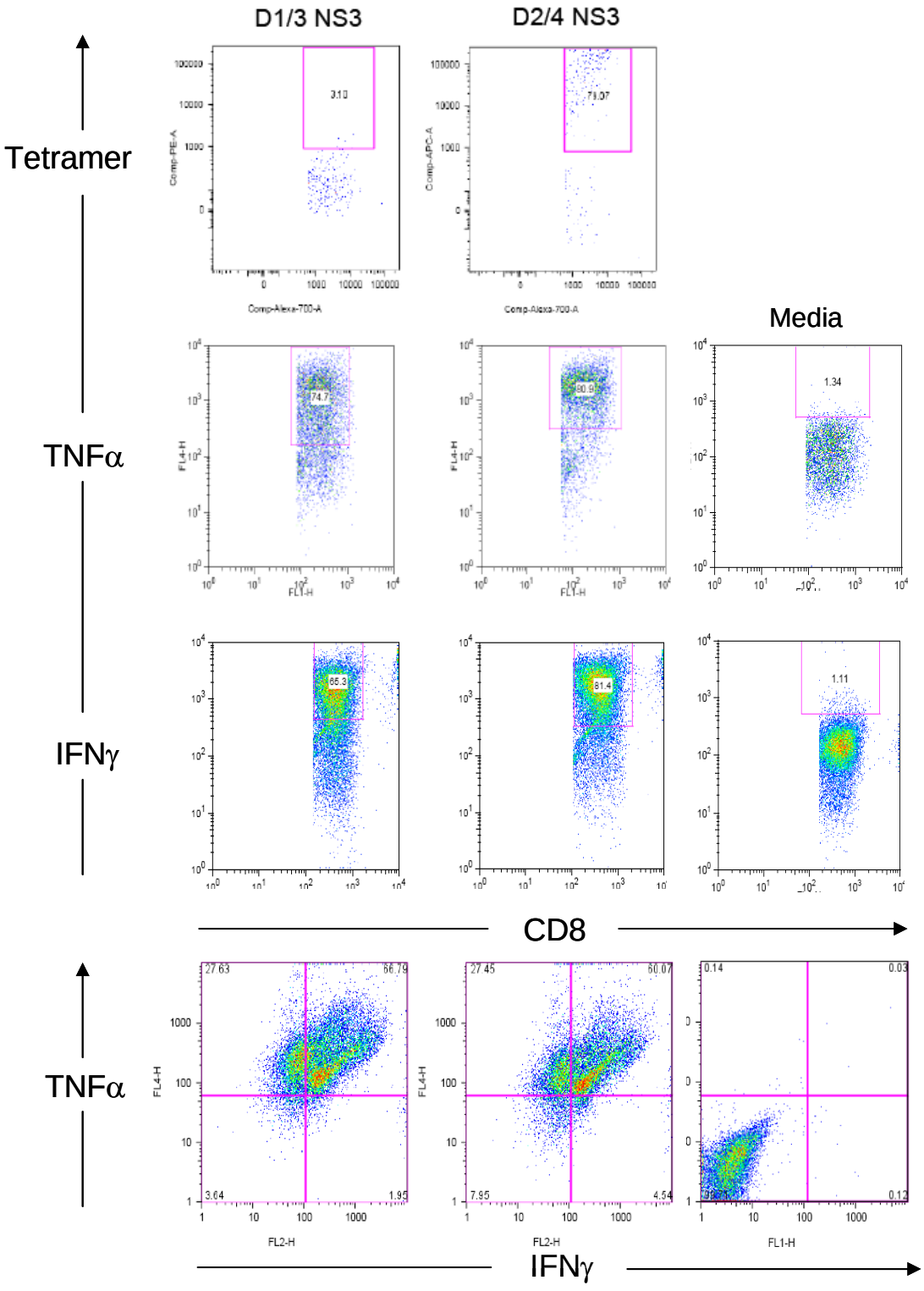


FIGURE III-10: Representative tetramer and intracellular TNFα staining of bulk culture T cell lines. Cell lines were established as described in Figure III-8. After 3 rounds of stimulation, cells were stained with K^d-D1/3 and K^d-D2/4 NS3 tetramers and assayed for TNFα production by intracellular cytokine staining.

DENV-3 infection, stimulated *in vitro* with the D2/4 NS3 peptide, showed minimal cytokine response to either NS3 peptide. Tetramer staining revealed that the cells were mostly specific to the K^d-D2/4 NS3 tetramer, with much lower staining by the K^d-D1/3 NS3 tetramer. (Figs. III-8 – III-10) These results are in contrast to those from tetramer staining performed five days post stimulation. In the majority of the cell lines, five days post stimulation, the frequencies of both K^d-D1/3 NS3 and K^d-D2/4 NS3 positive cells were similar. However, the fact that the vast majority of the cells six weeks post stimulation are now K^d-D2/4 NS3 positive indicates expansion of cells that may have been originally D1/3 NS3 specific, but were cross-reactive and had a higher affinity for D2/4 NS3. These data correlate with the results from sequential infection experiments, since the D2/4-NS3 peptide was the most immunogenic in the sequential infection experiments, regardless of the serotype of challenge.

Cell lines generated from mice with primary DENV-2 infection stimulated with either D1/3 NS3 or D2/4 NS3 peptide had a greater frequency of K^d-D2/4 NS3 tetramer positive cells than K^d-D1/3 NS3 tetramer positive cells (Figs. III-8 - III-10). Both the IFNγ and TNFα responses of the cell lines that were stimulated *in vitro* with the D1/3 NS3 peptide were barely detectable, whereas the cell lines that were stimulated with the D2/4 NS3 peptide showed a very high frequency of IFNγ and TNFα positive cells in reaction to both peptides (Fig. III-8 – III-10). These results suggest that cells induced by primary DENV-2 infection *in vivo* were less cross-reactive to DENV-3-driven *in vitro* expansion.

T cell lines established from mice with heterologous secondary DENV-2 infection stimulated with either peptide had a much percentage of K^d-D2/4 NS3 positive cells than

K^d-D1/3 NS3 positive cells. Nevertheless, cytokine production was the same in response to stimulation with either D1/3 NS3 or D2/4 NS3 peptides (Figs. III-8 – III-10). In addition, the cytokine responses in these cell lines were decreased compared to that from cell lines generated from animals with a primary DENV-2 infection. These differences indicated different cell populations expanding and responding between the two infection groups. Since the cytokine responses in lines established from secondary DENV-2 challenged mice is less robust than those from the lines generated from the DENV-2 primary infected mice, the responding cell population may be D1/3-NS3-specific but cross-reactive for D2/4 NS3. These data demonstrate cross-reactivity of D1/3 NS3-specific cells for D2/4 NS3.

CHAPTER IV

MEMORY CD4$^+$ T CELLS AUGMENT THE MEMORY CD8$^+$ T CELL RESPONSE DURING SECONDARY DENGUE VIRUS INFECTIONS

Heterologous secondary infections are a significant risk factor for developing DHF/ DSS. However, the sequences of serotypes do not all have equal likelihood of inducing severe dengue disease. Epidemiological data point to specific serotype sequences of infection leading to an increased frequency of DHF/DSS. In particular, the following infection sequences have been linked to DHF: DENV-1 followed by DENV-2, DENV-2 followed by DENV-1, and DENV-4 followed by DENV-2 (Sangkawibha, Rojanasuphot et al. 1984; Endy, Nisalak et al. 2002; Alvarez, Rodriguez-Roche et al. 2006). The mechanism(s) by which one sequence causes more severe illness than another is/are not known.

In our studies of the CD8 T cell response to sequential infections described in Chapter III, we noted that the effects of secondary infection could not necessarily be predicted by the amino acid sequences of the CD8 T cell epitopes. For example, we observed that, after primary DENV-2 infection followed by secondary DENV-1 boost, there was an increase in the immune response to the D1/3 NS3 peptide. However, there was no increase in response to this peptide in mice that received the same primary infection but were rechallenged with DENV-3. This phenomenon was of interest since the amino acid sequence for the NS3 peptide was the same for both DENV-1 and DENV-3. We found this scenario to be an opportunity to study the differences in sequences of infections. Since both DENV-1 and DENV-3 induced low CD8$^+$ T cell responses in the

mice as a primary infection, we hypothesized that the differential response in secondary infections could be a result of differences in the $CD4^+$ T cell response. We hypothesized that DENV-1 stimulated a more robust $CD4^+$ T cell response in secondary infection than DENV-3 and that these $CD4^+$ T cells may provide help to the memory $CD8^+$ T cells, resulting in an increased frequency of IFNγ producing $CD8^+$ T cells.

A. Difference in magnitude of the $CD4^+$ T cell responses in secondary DENV-1 versus secondary DENV-3 infections

To determine if there was a difference in the magnitude of the $CD4^+$ T cell response, we performed ICS on splenocytes from mice that were administered primary DENV-2 followed by DENV-1 or DENV-3 and also on splenocytes from mice that were immunized with a primary DENV-1, DENV-2, or DENV-3 infection. Since the murine $CD4^+$ T cell response to dengue is not well characterized, we tested the $CD4^+$ T cell response to whole virus antigens using infected Vero cell lysates (Figure IV-1), as has been done to measure DENV-specific human CD4 T cell responses (Mangada and Rothman 2005). The IFNγ responses to DENV antigens were low after primary infection regardless of viral serotype (Figure IV-2). Responses in mice that received DENV-3 after DENV-2 were slightly higher and the highest responses were observed in mice that received secondary DENV-1 infection after primary DENV-2 infection. The frequency of $IFN\gamma^+$ T cells in cells from mice with secondary DENV-1 infection in response to in vitro stimulation with DENV-2 antigen was significantly higher than in cells from mice that were administered secondary DENV-3 (Fig. IV-2; representative data from one experiment are shown in Fig. IV-1). This finding is particularly interesting since DENV-2

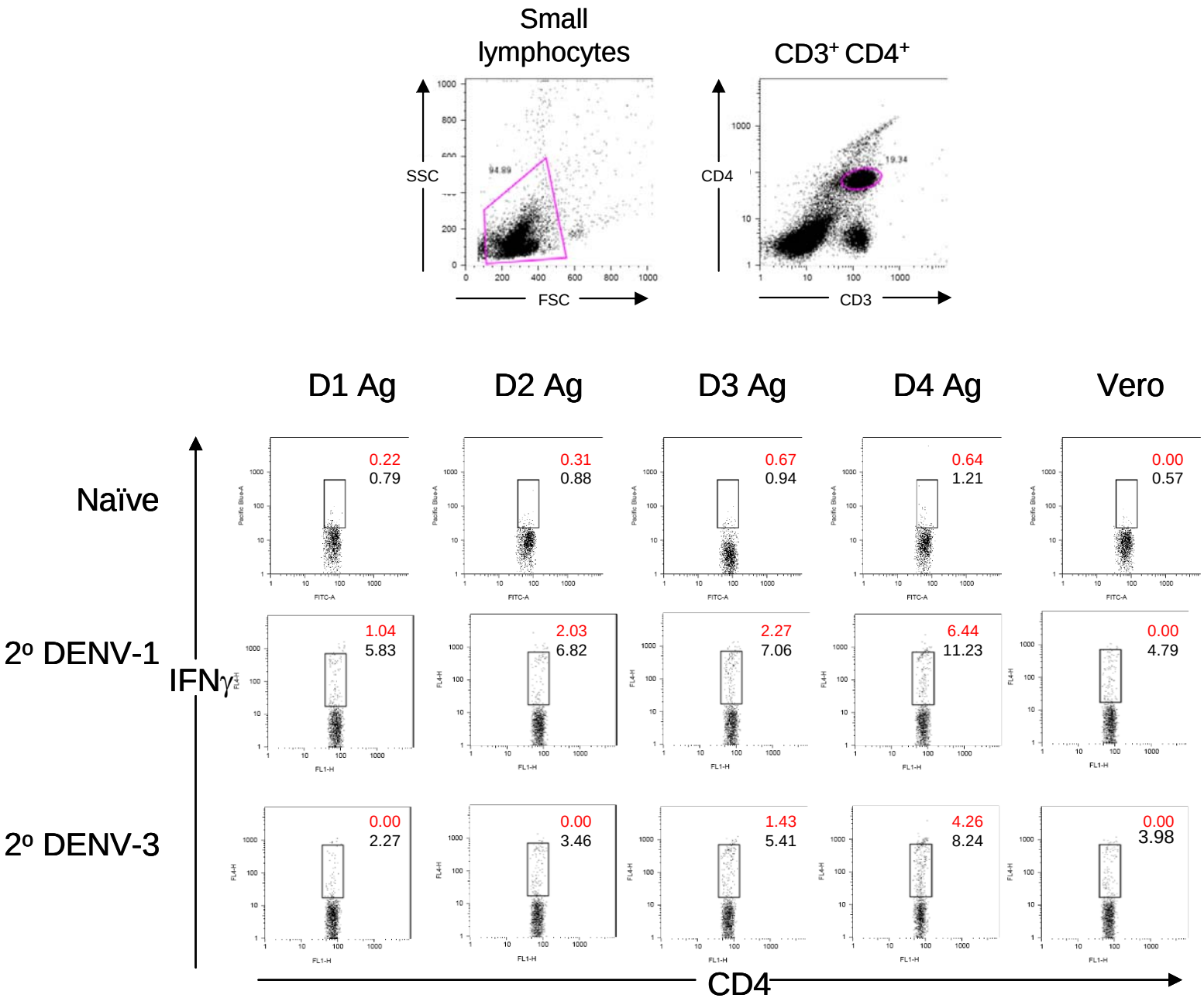


Figure IV-1: Representative IFNγ staining in response to inactivated DENV antigens. Mice were infected with primary DENV-2 infection and rechallenged 28 days later with either DENV-1 or DENV-3. Eight days post secondary infection, mice were sacrificed and their splenocytes were assayed for IFNγ production to DENV-infected Vero cell lysates by intracellular cytokine staining. The frequencies of DENV-specific $IFN\gamma^+$ $CD4^+$ T cells were calculated by gating on the $IFN\gamma^+$ $CD4^+$ $CD3^+$ small lymphocytes and then subtracting the frequency of $IFN\gamma^+$ $CD4^+$ T cells in samples incubated with a control (uninfected) Vero cell lysate from the frequency in samples stimulated with the viral antigens. Frequencies in black type are before subtraction of the control values. Frequencies in red type are after the subtraction of the control values.

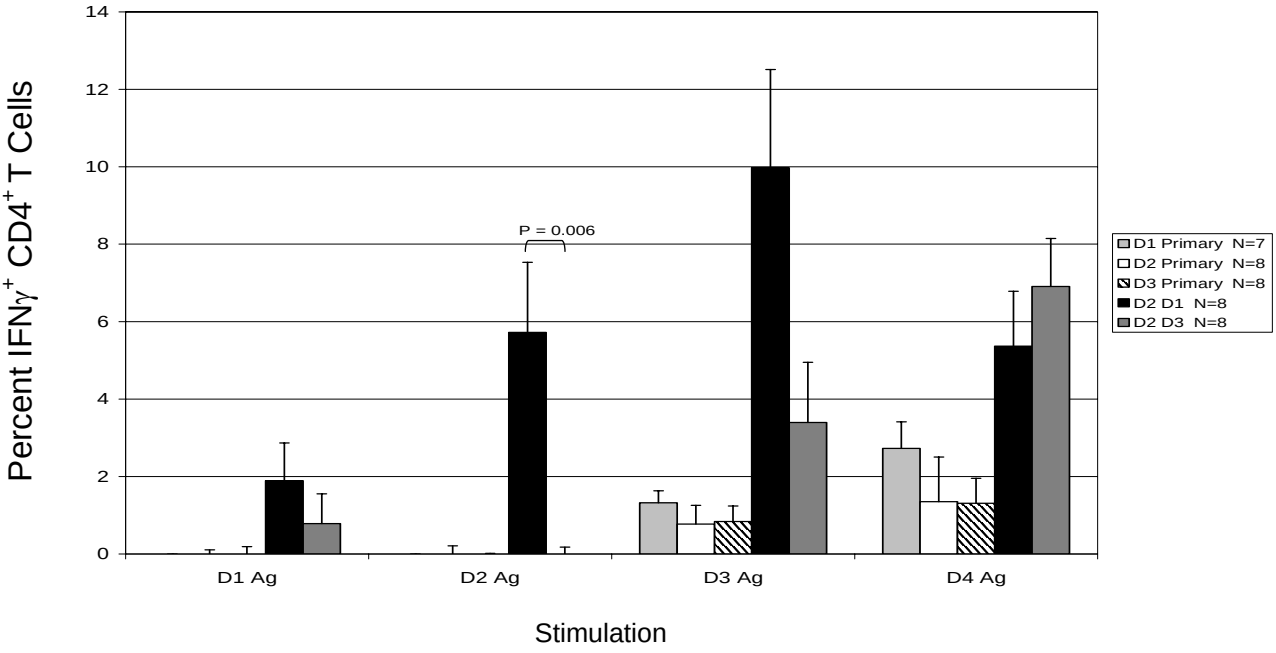
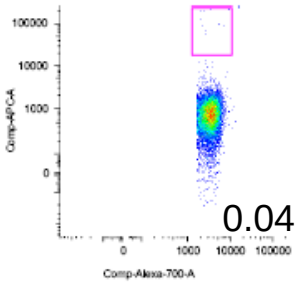
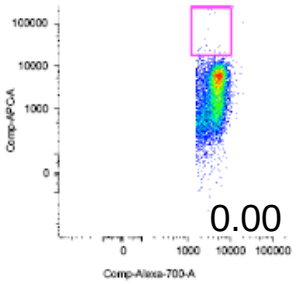
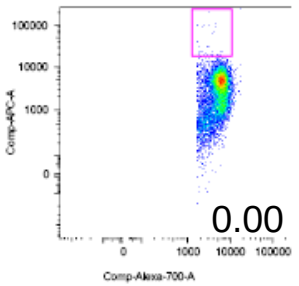
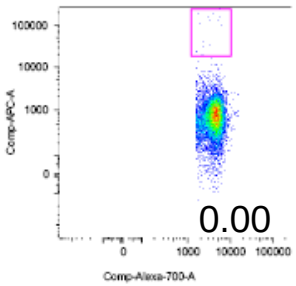
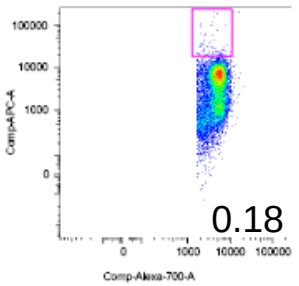
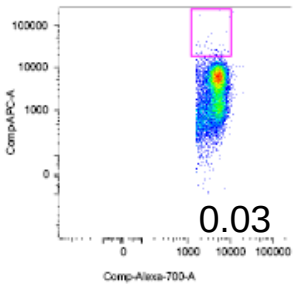
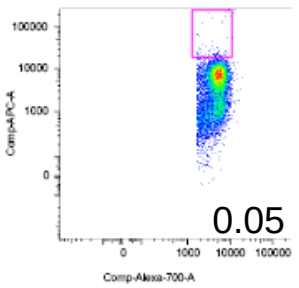
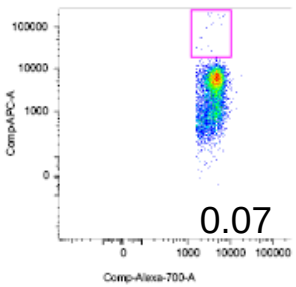


FIGURE IV-2: Comparison of $CD4^+$ T cell responses in primary versus secondary dengue virus infections. Intracellular IFNγ staining was performed on splenocytes from mice that received either primary DENV-1 infection, primary DENV-2 infection, primary DENV-3 infection, or primary DENV-2 followed by secondary DENV-1 or DENV-3 infection. $CD4^+$ T cell responses to inactivated DENV antigens were measured. Medians, 95% confidence intervals, and p values were calculated by the Mann-Whitney test. P values <0.05 were considered significant and < 0.10 are shown. N = 7 – 8 mice per infection group from a total of two experiments.

A.

Infection	$CD8^+$	$CD4^+$	D1/3 NS3	D2/4 NS3
DENV-1	+	-		
DENV-3	+	-		
DENV-1	+	+		
DENV-3	+	+		

B.

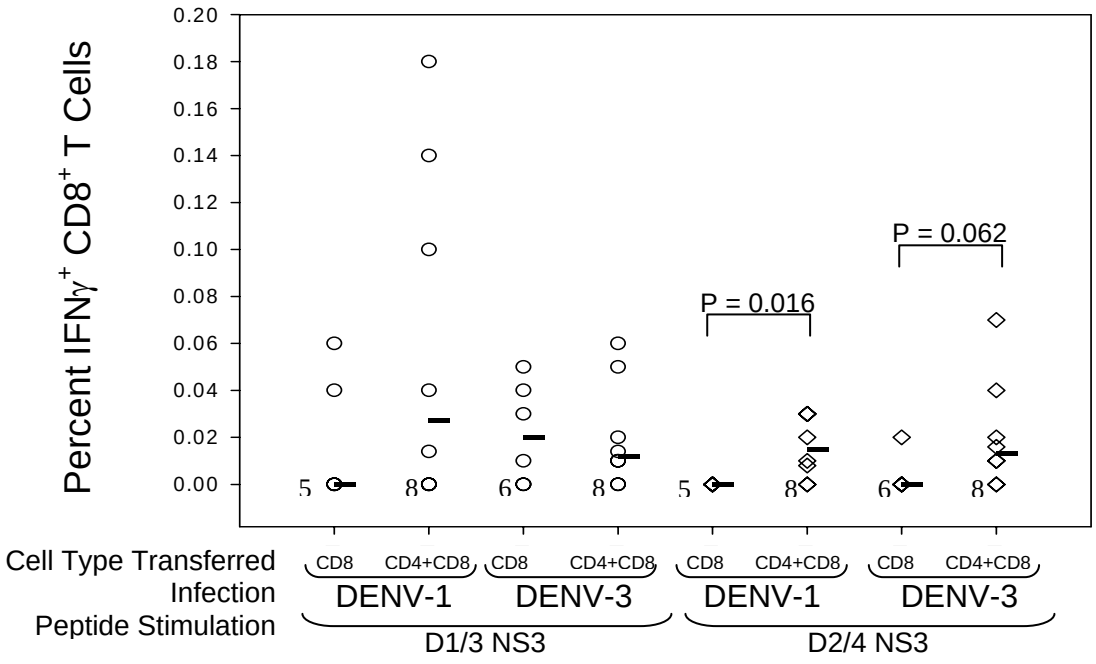


FIGURE IV-3: Transfer of memory CD4$^+$ T cells affects the CD8$^+$ T cell response to secondary dengue virus infections. CD4$^+$ and CD8$^+$ T cells were isolated from DENV-2-immune mice. The CD8$^+$ T cells alone or in combination with the CD4$^+$ T cells were transferred i.v. to naïve mice. The following day, mice were administered either DENV-1 or DENV-3. The splenocytes were harvested on day nine post infection. The peptide-specific CD8 T cell IFNγ response was measured by intracellular cytokine staining. *(a)* Representative intracellular cytokine staining. Cell type(s) transferred and serotype of virus infection are shown on the left. *(b)* Summary data. N values for each group are on the left to the dot plot for each group. Medians are represented by the horizontal bars. P values were calculated by the Mann-Whitney test are shown and those values <0.10 are shown. P values <0.05 were considered significant. N = 5 – 8 mice per group from a total of 3 experiments.

was the primary infection in both groups of mice. These results indicate that there was a differential $CD4^+$ T cell response between secondary DENV-1 and secondary DENV-3 infections, especially to the DENV-2 antigen.

B. Memory $CD4^+$ T cells augment the memory $CD8^+$ T cell response

We hypothesized that serotype-crossreactive memory $CD4^+$ T cells generated by a primary DENV-2 infection provide help for $CD8^+$ T cells upon secondary infection. Therefore, we next examined whether the memory $CD4^+$ T cells affect the $CD8^+$ T cell response to secondary infection. $CD8^+$ T cells with or without $CD4^+$ T cells from DENV-2-immune mice were transferred into naïve mice. The mice then were challenged with either DENV-1 or DENV-3. $CD8^+$ T cell IFNγ responses were measured in response to stimulation with the immunodominant CD8 T cell epitopes D1/3 NS3 and D2/4 NS3. In response to the D2/4 NS3 peptide, both groups of mice that received memory $CD4^+$ T cells and either DENV-1 or DENV-3 infection had an increased $CD8^+$ T cell response over that of the corresponding group that were not administered the memory $CD4^+$ T cells (Fig. IV-3). Furthermore, the transfer of the memory $CD4^+$ T cells appeared to increase the frequency of responding $CD8^+$ T cells to D1/3 NS3 in mice that received the DENV-1 infection (Fig. IV-3); however, this difference was not statistically significant ($p=0.355$). As a control, a group of mice received $CD8^+$ T cells from DENV-2-immune mice in combination with $CD4^+$ T cells isolated from naïve mice. There was no increase of the $CD8^+$ T cell response after transfer of the naïve $CD4^+$ T cells. This control confirmed that the increase in the response by the $CD4^+$T cells was due to memory $CD4^+$ T cells and not solely to the presence of transferred $CD4^+$ T cells (data not

shown). Another control included mice that received memory $CD4^+$ T cells alone. This group was incorporated into the experiment to define whether it was the response of memory or naïve $CD8^+$ T cells that was boosted by the memory $CD4^+$ T cells (data not shown). There was no increase in the naïve $CD8^+$ T cell response to infection; therefore, this group verified that the memory $CD8^+$ T cell response was the response affected by transfer of the $CD4^+$ T cells. These data show that memory $CD4^+$ T cells enhance the frequency of IFNγ producing memory $CD8^+$ T cells during secondary DENV infection.

C. DENV-2-specific memory $CD4^+$ T cells are more crossreactive to DENV-1 than DENV-3

A potential mechanism to explain the phenomenon that DENV-2-specific memory $CD4^+$ T cells amplify the frequency of responding $CD8^+$ T cells more in a secondary DENV-1 infection than in a secondary DENV-3 infection is that the memory $CD4^+$ T cells are more crossreactive to antigen from DENV-1 than that from DENV-3. To test this hypothesis, bulk culture lines were generated from splenocytes from mice that were in memory phase after DENV-2 infection. The cultures were stimulated with inactivated whole virus antigen from either DENV-1 or DENV-3. The cells were then assayed for IFNγ production on day 7 and day 14 post stimulation by intracellular cytokine staining in which they were stimulated with antigen from all four serotypes. Responses were generated to all four antigens on both days, though the response to DENV-2 antigen was highest in magnitude (Figs. IV-4 and IV-5). The response to DENV-2 antigen was significantly higher in cultures stimulated with DENV-1 antigen than in cultures stimulated with DENV-3 antigen. This difference was most striking day

14 post stimulation (Figs. IV-4 and IV-5). These results suggest that DENV-2-specific memory $CD4^+$ T cells expanded to a greater extent in vitro in response to stimulation with DENV-1 than DENV-3, and suggest a greater degree of crossreactivity to DENV-1 than DENV-3. In addition, these data point to a mechanism to explain the differential $CD8^+$ T cell response after secondary DENV-1 infection compared to secondary DENV-3 infection, in which DENV-2-specific memory $CD4^+$ T cells respond to a greater degree and therefore provide more help to memory $CD8^+$ T cells, resulting in an enhanced immune response during secondary infection.

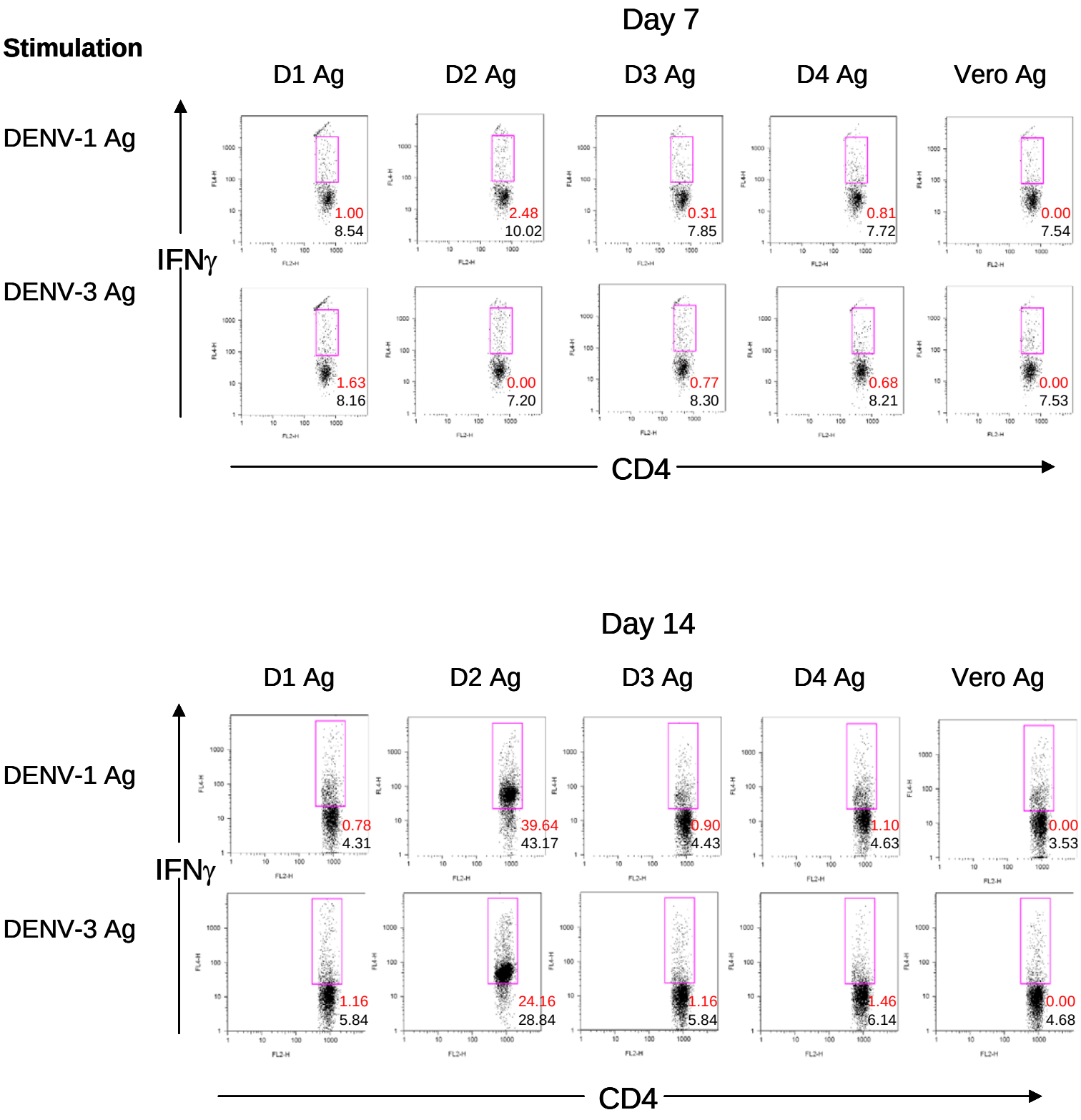


FIGURE IV-4: Representative intracellular cytokine staining of bulk culture CD4 T cells. Splenocytes from mice immunized 28 days previously with DENV-2 were stimulated in vitro with inactivated DENV-1 or DENV-3 antigens. The top panel shows representative intracellular cytokine staining of the T cell lines after seven days of culture. Cells were stimulated with DENV-1 antigen, DENV-2 antigen, DENV-3 antigen, DENV-4 antigen or control antigen (uninfected Vero cell lysate). The bottom panel shows intracellular staining after 14 days of culture. Frequencies in black type are before subtraction of the control values. Frequencies in red type are after the subtraction of the control

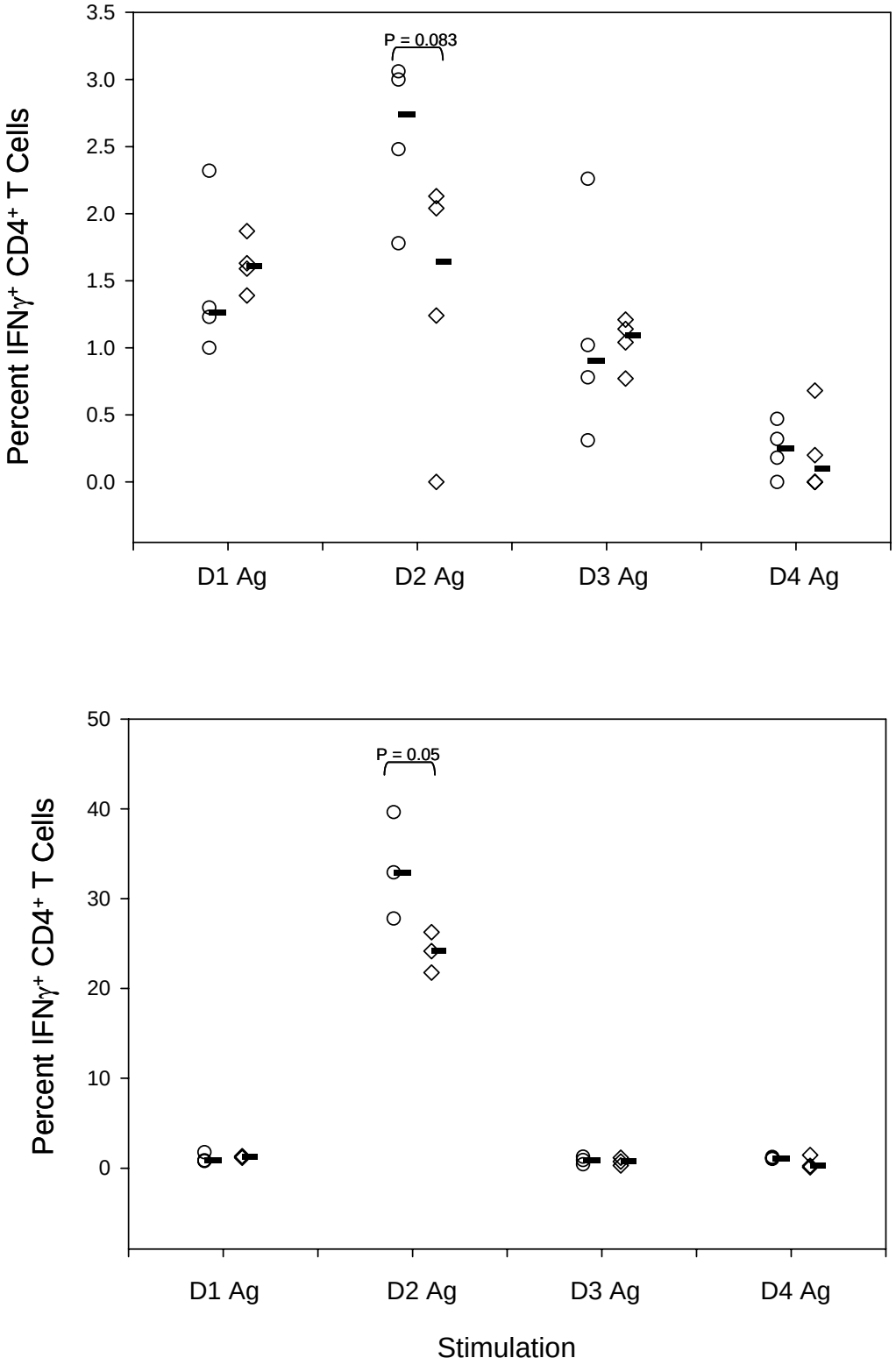


FIGURE IV-5: Crossreactivity of memory CD4 T cell responses from DENV-2–immune mice. Bulk culture T cell lines were generated from splenocytes from mice that were administered DENV-2 twenty-eight days prior as described in the Figure IV-4 legend. Cells were stimulated with inactivated whole virus antigens from either DENV-1 (circles) or DENV-3 (diamonds). Day 7 *(a)* and day 14 (*b*) post stimulation, cells were tested for IFNγ production to whole virus antigen for all for serotypes by intracellular cytokine staining. The median responses are represented by the black bars. P values were calculated by the Mann-Whitney test and those <0.10 are shown. P values <0.05 were considered significant. One experiment was performed with 4 mice per infection group. One culture from each mouse was established for each *in vitro* stimulation (D1 Ag, D3 Ag, and control Vero Ag) resulting in a total of 12 cultures.

CHAPTER V

DISCUSSION

To our knowledge, this is the first study to analyze immune responses in mice after heterologous sequential DENV infections. Our focus was on several aspects of the T cell immune response: specificity, kinetics, frequency and cross-reactivity. We performed ELISPOT assays, intracellular cytokine staining, and MHC-peptide tetramer staining to measure DENV-specific CD4 and CD8 T cell responses in immunocompetent Balb/c mice. Adoptive transfer of memory $CD4^+$ and $CD8^+$ T cell populations confirmed that memory cells were preferentially recruited to respond, influence and augment the immune response during heterologous secondary dengue virus infections.

A. KINETICS OF THE T CELL RESPONSE TO PRIMARY AND SECONDARY DENGUE VIRUS INFECTIONS

After both primary and secondary DENV infections, peptide-specific IFNγ responses were detected. Previous studies of the DENV-specific T cell response in mice had focused on the memory phase of the immune response, and had not quantitated cells directly ex vivo. Furthermore, the response to epitopes on nonstructural proteins was boosted after secondary infection; this showed that primary infection did not prevent the secondary infection.

When we examined the kinetics of the IFNγ response after primary and secondary infections, we found that the timing of the peak responses was similar after both immunizations, between days 8 - 10 (Table III-1). Though it was initially expected that

the onset and peak of the memory response might occur earlier than that after primary infection, the kinetics of the response may reflect the low antigen load in this animal model. DENV does not replicate well in immunocompetent mice (Shresta, Kyle et al. 2004); therefore, the timing of the T cell response may be accounted for by equally slow production of antigen in both primary and secondary DENV infections. Studies infecting IFNα/β/γ receptor knockout mice with DENV have shown development of viremia and dengue-induced disease (Roehrig, Bolin et al. 1998). Therefore, it might be possible to test if viremia affects the kinetics of the immune response to secondary infection by temporarily blocking IFNα signaling to allow viral replication and subsequently comparing the kinetics of immune response in primary versus secondary infected mice.

B. ENHANCEMENT OF T CELL RESPONSE AFTER SECONDARY INFECTION

After heterologous secondary DENV infections, an increase in the frequency of peptide-specific T cells was observed, when compared to that during acute and memory phases following primary infection (Figures III-1 and III-2). The boost in the immune response after heterologous secondary infection in this mouse model is consistent with findings in humans. In humans, increased T cell activation is also associated with more severe dengue disease (Green, Pichyangkul et al. 1999; Green, Vaughn et al. 1999).

Interestingly, the magnitude of the T cell response in secondary infection appeared to depend on the sequence of infections. This influence of sequence of infection has been seen in human studies, where secondary DENV-2 infections were associated with a higher risk for DHF (Sangkawibha, Rojanasuphot et al. 1984; Vaughn, Green et al. 2000). In Balb/c mice, secondary DENV-2 infection following primary DENV-3

immunizations resulted in the most pronounced boost in T cell response to the peptides studied (Figures III-1 and III-2). However, because of differences in epitopes recognized by humans and mice, the specific sequence in mice is not necessarily indicative of the risk in humans. In humans, certain DENV-2 strains have been associated with increased virulence and DENV-3 strains have been linked to increased disease severity (Leitmeyer, Vaughn et al. 1999; Endy, Nisalak et al. 2002), it is possible that sequential DENV-3 and DENV-2 infections may promote immunopathogenesis by resulting in an enhanced immune response.

We also saw that the D2/4 NS3 peptide elicited the largest response compared to the other peptides tested, regardless of the infecting serotype (Fig. III-1). A similar phenomenon was seen in DENV-immune humans, where particular epitope sequence variants induced a larger response *in vitro* regardless of the vaccination serotype (Bashyam, Green et al. 2006). Since a heterologous peptide can be more immunogenic than the homologous version, one can envision a situation in which a secondary heterologous infection can stimulate activation of cross-reactive memory T cells and result in an enhanced immune response.

The observed increased frequency of IFNγ-producing T cells in secondary DENV infection suggested that cross-reactive memory cells from the primary infection were being preferentially recruited during secondary infection. This is very difficult to demonstrate in a definitive way in humans as pre-infection samples are rarely available for study. Though there are limitations in using this system to model human infections, since the animals did not become ill. Also, no virus was detected by quantitative polymerase chain reaction (PCR) at days 4 and 9 post primary and secondary infections

in the sera, livers, or spleens. However, since $CD8^+$ T cell responses do exist to non-structural proteins, suggesting replication, there may be low levels of viral replication occurring at earlier time points after infection. Despite these limitations, we confirmed by adoptive transfer experiments that the DENV-specific cross-reactive memory $CD8^+$ T cells are, in fact, being preferentially enlisted to respond to secondary infection. It was found that the frequency of transferred $CD8^+$ T cells from DENV-immune mice responding was significantly larger than host naïve $CD8^+$ T cells, showing that the memory population was the main cell population responding to the secondary infection (Figure III-4). Our data are in agreement with data from human clinical studies, where T cell responses after secondary infection have been shown to be mostly serotype-cross-reactive (Kurane, Meager et al. 1989; Mathew, Kurane et al. 1998; Bashyam, Green et al. 2006). Our findings of serotype-cross-reactive immune responses *in vivo* are also consistent with data from Monkongolsapaya *et al*, who showed that cells from patients with secondary DENV-2 had a higher affinity to HLA-peptide tetramers corresponding to other serotypes, presumably the serotype of primary infection (Mongkolsapaya, Dejnirattisai et al. 2003).

C. THE ROLE OF ANTIBODY-DEPENDENT ENHANCEMENT OF INFECTION

The theory of antibody-dependent enhancement (ADE) proposes a mechanism for DHF in secondary DENV infection. Crossreactive, non-neutralizing, anti-envelope antibodies generated from the primary infection attach to virions from the secondary DENV infection and, through uptake of virus into cells via Fcγ receptors, enhance infection (Halstead, Nimmannitya et al. 1970). In addition to the epidemiological studies

by Kliks *et al* that demonstrated that maternal antibodies were sufficient to enhance viral infection by infants (Kliks, Nisalak et al. 1989), Huang *et al* observed in a cohort of infant patients that there were elevated TNFα, IL-6, IFNγ, and IL-10 levels (Huang, Lei et al. 2000). They hypothesized that since these infants had only a primary infection, that ADE was the explanation of elevated cytokine levels. To determine if ADE was the cause of the enhanced immune response that we observed, we passively immunized mice with DENV-3-immune sera prior to DENV-2 challenge. Rather than a boosting of the immune response, as would be expected if ADE played a role, passive immunization actually suppressed the IFNγ response (Figure III-3). We therefore concluded that antibody-dependent enhancement was not the driving factor of the increased $CD8^+$ T cell response seen.

Caveats do exist for our antibody dependent enhancement experiments. ADE is dependent upon the antibody concentration (Halstead 1979). It was found that ADE occurred at more dilute concentrations of antibodies, where the neutralizing antibody titers had waned, resulting in the serum being more concentrated with cross-reactive non-neutralizing antibodies (Halstead, Nimmannitya et al. 1970; Halstead 1979; Halstead 1982; Kliks, Nimmanitya et al. 1988; Kliks, Nisalak et al. 1989). We did not use different dilutions of serum in our experiments, nor did we measure the neutralizing antibody titers. Therefore, in addition to crossreactive memory T cells, it is a possibility that antibody dependent enhancement also may function to augment the T cell response that we observe after secondary infection in our system.

Though not directly related to our studies, the result that cross-reactive antibodies may suppress the immune response is interesting. A potential mechanism that would account for our findings would be that crossreactive neutralizing antibodies are present which decreased the viral load, thereby resulting in diminished IFNγ production. To follow up on this observation and determine if antibodies directed to the primary infection suppresses the IFNγ response to the secondary, it would be possible to employ recombinant chimeric DENV. Such a chimeric virus could consist of a DENV-3 genome with the E gene replaced with that of another flavivirus such as WNV. The DENV E protein is the protein to which the majority of the neutralizing antibody response is directed (Roehrig, Bolin et al. 1998). Using the chimeric virus as a primary infection, the antibody response elicited would presumably be to the WNV E protein. Following with a secondary DENV-2 challenge, it would be possible to see if the suppression of the immune response occurred with the lack of presence of cross-reactive anti-DENV-3 E protein antibodies. It also may be of interest to use a chimeric virus containing the WNV NS1 protein, since DENV NS1 is shown to elicit a non-neutralizing but complement-fixing antibody response.

These chimeric DENV are a valid tool to study the nature of the virus-immune system interactions since they are also currently being employed as potential vaccine candidates. For example, Calvert *et al*, vaccinated AG129 mice with a chimeric vaccine that was composed of a West Nile Virus backbone and the capsid and nonstructural proteins from DENV. Though this vaccine was not able to completely protect the mice from lethal DENV challenge, it increased the mean survival times of the animals (Calvert, Huang et al. 2006). Also, Blaney *et al*, (Blaney, Sathe et al. 2007) used a

vaccine with a DENV-4 backbone and replaced either the capsid, membrane, or envelope proteins (or combinations thereof) with those of DENV-1. Immunization with the virus containing the membrane and envelope proteins from DENV-1 resulted in 66% seroconversion in rhesus monkeys and protected the animals against DENV-1 challenge (Blaney, Sathe et al. 2007). Since the most successful DENV vaccine will most likely be a tetravalent vaccine, one that will elicit a concurrent immune response to all four serotypes, it will be important to the evaluation of the efficacy of this vaccine to determine the generation of the serotype-specific immune response. Since chimeric viruses have become a means by which the antibody and other immune responses to individual elements of DENV can be assessed, they are potentially an ideal vaccine candidate due to the ease of testing vaccine efficacy and ability to elicit a tetravalent immune response.

D. AUGMENTED TNFα RESPONSE AFTER SECONDARY CHALLENGE

The importance of TNF-α in human DENV infections and its implication in disease severity have been previously described. Using immunoenzymatic assays, Hober *et al*, found that serum samples from patients suffering from DHF in Tahiti in 1989-1990 all contained high levels of TNFα, with the highest levels found in the most severe grades of DHF (Hober, Poli et al. 1993). Another report demonstrated that TNFα was more frequently detected in the plasma of patients with DHF as opposed to DF and other febrile illnesses (OFI) (Green, Vaughn et al. 1999). Mangada *et al* measured the TNFα production of pre-infection PBMCs from DENV-infected patients to viral antigens. TNFα responses were found exclusively in patients that were hospitalized,

suggesting a correlation between TNFα production and severe disease (Mangada, Endy et al. 2002). These data suggest that serotype-cross-reactive memory T cells produce increased amounts of TNFα during secondary infection, resulting in more severe disease. Our data in mice also show that the frequency of DENV-specific $TNF\alpha^+$ $CD8^+$ T cells during the acute phase after heterologous secondary DENV-2 infection is greater than that during the acute phase of either primary DENV-3 or primary DENV-2 infections and also during the memory phase after a primary DENV-3 inoculation. This enhanced response was seen after stimulation with all peptides tested, though the degrees of augmentation varied (Figure III-5). These results correlate with human studies since the data in both human samples and this murine system demonstrate an elevated frequency of $TNF\alpha^+$ $CD8^+$ T cells after secondary DENV infection. These findings also suggest that secondary infection activates T cells with an altered functional profile.

The possibility should be considered that the increased amounts of TNFα seen were not due to activated DENV-specific cross-reactive memory T cells but rather that it is the nature of memory T cells to produce more TNFα than cells from acute infection. Slifka and Whitton found that $CD8^+$ T cells from Balb/c mice that were infected 295 days prior with LCMV produced significantly more TNFα in response to the immunodominant LCMV peptide $NP_{118-126}$ than $CD8^+$ T cells from mice infected only eight days prior (Slifka and Whitton 2000). To address this possibility, we examined whether $CD8^+$ T cells from mice that were in the memory phase after primary DENV infection produced more TNFα than those from mice in the acute phase of primary DENV infection in response to a non-specific stimulation, PMA/Ionomycin, directly *ex vivo* (media stimulation alone), or in response to the DENV peptides. We found no

significant differences in TNFα responses in any of these stimulation conditions between the acute and memory cell populations (data not shown). These results indicate that the augmented TNFα response seen after heterologous secondary DENV infection is DENV-specific and not a general phenomenon of the memory cells. The differences between our results and those of Slifka and Whitton may be explained by the different viruses studied. LCMV generates a much more potent immune response than that induced by DENV (Slifka and Whitton 2000). As a result, the memory T cells may be programmed differently after LCMV infection than those generated during DENV infection. Also the time point measured for the memory phase by Slifka and Whitton was 295 days post infection, where our studies examined the memory phase at 28 days post infection. To further examine this possibility, the memory TNFα response after DENV infection could be measured at the later time point; however, this would not affect the interpretation of our findings during secondary infection at an interval of 28-56 days.

E. ALTERED SPECIFICITY OF CYTOKINE AND CYTOTOXIC RESPONSES AFTER SECONDARY CHALLENGE

We hypothesized that the altered CD8 T cell response to secondary DENV infection would also be reflected by altered effector functions, such as cytolysis and cytokine secretion. We attempted to quantify cytotoxic activity using an *in vivo* cytotoxicity assay. However, we were unable to detect specific clearance of DENV peptide-pulsed at 4 or 16 h after injection (data not shown). We speculate that this low frequency of DENV-specific cytotoxic T lymphocytes (CTLs) made the *in vivo* cytotoxicity assay infeasible. Therefore, we examined DENV-specific CTL activity and

IFNγ and TNFα responses post primary and secondary infections *ex vivo* using bulk culture T cell lines. After five days of culture, we found that cell lines derived from mice with primary DENV-2 infection and stimulated with the D2/4 NS3 peptide were mostly specific for the D2/4 NS3 peptide based on tetramer staining and CTL activity (Figure III-6), indicating the expansion of a DENV-2-specific cell population. Cell lines from the same mice generated by stimulation with the D1/3 NS3 peptide showed equal cytotoxic activity towards targets pulsed with either peptide, indicating that cells expanded with a D1/3 NS3-specificity can be cross-reactive to peptides from other serotypes. In comparison, cell lines generated from mice that received a secondary DENV challenge, whether stimulated with D1/3 NS3 or D2/4 NS3 peptide, were predominantly serotype-crossreactive based on both tetramer staining and CTL activity (Figure III-6). These data from cultures derived from secondary infection suggest that serotype-crossreactive T cells from the primary infection expanded *in vivo* in secondary infection, and were the predominant cell type *in vitro* after stimulation with either the homologous or heterologous peptide. These results suggest that the primary infection altered the specificity cytotoxic responses of the cells responding to the secondary infecting virus.

Several of the DENV-specific cell lines could not be maintained in culture, especially those generated from mice with primary DENV-3 infection. Cell lines from mice with primary DENV-2 infection could be expanded in vitro; after three rounds of stimulation these cell lines became highly skewed, showing high frequencies of staining with the D2/4 NS3 tetramer but low staining with the D1/3 NS3 tetramer (Figure III-8). However, these cells were functionally crossreactive to D1/3 NS3 as demonstrated by IFNγ and TNFα production. In comparison, cell lines from mice receiving a secondary

DENV-2 infection, had low frequencies of TNFα^+ and IFNγ^+ cells, despite showing similar patterns of tetramer staining as the cell lines from primary DENV-2 infection (high levels of staining with the D2/4 NS3 tetramer and low staining with the D1/3 NS3 tetramer, Figure III-8). These findings lead us to speculate that the T cells from mice with secondary DENV-2 infection were functionally similar to the T cells generated during primary DENV-3 infection.

F. ROLE OF CD4$^+$ T CELLS IN SEQUENCE DEPENDENCE OF THE IFNγ RESPONSE

During studies of the IFNγ response of CD8$^+$ T cells in mice after secondary infection, we found that response to the D1/3 NS3 peptide was augmented in mice that received primary DENV-2 infection followed by a secondary DENV-1 challenge, but not in animals that also received a primary DENV-2 immunization but were rechallenged with DENV-3 (Figure III-1). This result was surprising since the amino acid sequence of this epitope was the same in both DENV-1 and DENV-3. This scenario gave us an opportunity to further investigate why the sequence of infection strongly influences the immunologic response. DENV-1 and DENV-3 both elicited comparable (and weak) CD8 T cell responses as primary infections (Figure III-1). We, therefore, concluded that the differences in the memory CD8$^+$ T cell response could not be explained by the levels of presentation of the D1/3 NS3 epitope. We hypothesized that serotype-cross-reactive memory CD4$^+$ T cells might provide help to the memory CD8$^+$ T cells thereby augmenting the CD8$^+$ T cell response, and that the degree of cross-reactivity of the memory CD4$^+$ T cells from DENV-2-immune mice for DENV-1 versus DENV-3 might

be different. We found that the DENV-specific CD4 T cell IFNγ response was larger after secondary DENV-1 challenge than secondary DENV-3 infection, particularly to DENV-2 antigen (Figure IV-2). The adoptive transfer of memory $CD4^+$ T cells from DENV2-immune mice along with memory $CD8^+$ T cells into naïve animals enhanced the DENV-specific $CD8^+$ T cell response to DENV-1 > DENV-3 (Figure IV-3). This phenomenon did not occur with adoptive transfer of memory $CD8^+$ T cells alone or with $CD4^+$ T cells from naïve mice. These results showed that memory $CD4^+$ T cells affected the peptide-specific IFNγ responses of $CD8^+$ T cells. This hypothesized role for memory DENV-specific CD4 T cells in secondary infections could potentially be explored further by immunizing mice with a primary infection and then depleting the CD4 T cells just prior to a secondary infection.

Different levels of CD4 T cell cross-reactivity to DENV1 and DENV3 represented a potential mechanism to explain this differential response to secondary infection *in vivo*. We evaluated this possibility by generating short-term cell lines from DENV-2-immune mice by *in vitro* stimulation with DENV-1 or DENV-3 antigen. We observed that expansion occurred after stimulation with either antigen. Cell lines showed the highest responses to DENV2 antigen (Figs. IV-4 and IV-5). In addition, the frequency of DENV-specific IFNγ $CD4^+$ T cells was significantly higher in DENV1 antigen-stimulated cultures than in DENV3 antigen-stimulated cultures (Figs. IV-4 and IV-5). These results suggest that DENV2-specific memory $CD4^+$ T cells are more crossreactive to DENV1 than DENV3. Surprisingly, by day 14, there was minimal IFNγ response to DENV1 and DENV3 antigens, which were used for stimulation of the lines (Figs. IV-4 and IV-5). This suggests that the responding cells had higher avidity for

DENV-2 than other serotypes, even those used for *in vitro* expansion. However, one caveat for interpreting these data arose from the use of crude viral antigen stocks made from cell lysates. It is possible that amount of viral antigen present in the stocks may vary between the DENV-1 and DENV-3 antigen preparations. Therefore, the differences in the IFNγ $CD4^+$ T cell responses may be a result of varying amounts of antigen that cell lines were cultured with as opposed to differences in crossreactivity of the DENV-2-specific memory $CD4^+$ T cells. Normalizing the amount of antigen in the different antigen preparations would allow for more accurate interpretation of our results. Also, once $CD4^+$ T cell epitopes are defined for the Balb/c mice, a known concentration of stimulation could be utilized for these experiments.

An effect of $CD4^+$ T cell help on memory $CD8^+$ T cell responses has also been demonstrated in other experimental systems such as LCMV and *Listeria monocytogenes* infections. In these systems it was found that a lack of $CD4^+$ T cell help resulted in a diminished number of memory $CD8^+$ T cells. Evidence has been presented from many groups that $CD4^+$ T cell help during the initial priming of a naïve $CD8^+$ T cell was dispensable for the primary $CD8^+$ T cell response. However, $CD4^+$ T cell help during this initial phase was required for the generation of an effective $CD8^+$ T cell memory response (Shedlock, Whitmire et al. 2003; Sun and Bevan 2003). Though there appears to be general consensus that $CD4^+$ T cell help is required for the production of an operative $CD8^+$ T cell memory response in many systems, the mechanism by which this phenomenon occurs has remained controversial. Sun *et al* provided data that indicated that $CD4^+$ T cell help was required for the maintenance of the pool of memory $CD8^+$ T cells (Sun and Bevan 2003). Others presented data that $CD4^+$ T cell help was required to

program the $CD8^+$ T cell for memory (Bevan 2004). Most recently, Novy *et al*., using vaccinia virus, determined that the presence of $CD4^+$ T cells was crucial for the expansion and survival of memory $CD8^+$ T cells during rechallenge (Novy, Quigley et al. 2007). Our data support these aforementioned studies that conclude that memory $CD4^+$ T cells influence the $CD8^+$ T cell response during rechallenge. It also identifies a potential mechanism for how the serotype sequence of heterologous DENV infection influences disease severity.

G. MURINE MODELS OF HETEROLOGOUS IMMUNITY AND IMMUNOPATHOLOGY

Other viral systems have displayed heterologous immunity and their roles in immunopathogenesis in mice. Studies of LCMV infected mice showed that a heterologous infection decreases the numbers of T cells specific for different pathogens from the memory $CD8^+$ T cell pool (Selin, Vergilis et al. 1996). However, if these memory cells are cross-reactive to the heterologous infection, then these cells do not disappear from the memory T cell population. These cells may actually increase in number (Brehm, Pinto et al. 2002).

It had also been demonstrated that immunity from a primary infection may offer protection from a heterologous secondary infection. They showed that immunity generated from LCMV, Pichinde Virus (PV) and MCMV infections was able to protect mice from a lethal vaccinia virus (VV) challenge. These results were not reciprocal, in that VV infection did not confer protection to challenge with the other viruses. Protection appeared to depend on changes in the epitope hierarchy and differential IFNγ and cytotoxic responses (Selin, Varga et al. 1998). Our model of heterologous

dengue infections shows similar consequences since different patterns of immune responses are seen depending on sequence of infection and also these patterns were not necessarily seen in the reciprocal serotype sequences.

The LCMV-VV combinations of infections were also able to cause an immunopathology. Secondary VV infection given i.p. resulted in lesions on the fat pads, were intranasal (i.n.) inoculations caused lung pathologies (Selin, Varga et al. 1998; Chen, Fraire et al. 2001). These studies give examples of heterologous immunity causing pathogenic effects, much like what is suggested to occur in heterologous secondary dengue virus infections.

H. THE SIGNIFICANCE OF OUR NOVEL MURINE SYSTEM

Though there have been many mouse models established for the study of dengue infections, our murine system offers distinct advantages. Many laboratories have utilized immunodeficient mouse strains such as AG129, SCID, and STAT-1 deficient $STAT1^{-/-}$ 129/Sv/Ev mice (Table I-1). The AG129 and $STAT1^{-/-}$ 129/Sv/Ev models have given invaluable insight for the role in which interferons play in controlling DENV infection. The engrafted-SCID mice have allowed for the establishment of DENV infections in human cells in a small animal model. However, the use of immunodeficient mice does not allow for the study of the T cell responses in relation to DENV infection, as our model does. One limitation to our murine system is that there is little or no viral replication and our mice do not fall ill. However, the DENV-induced encephalitis and paralysis seen in most dengue disease models are not clinically relevant findings in that they do not accurately reproduce what is seen in humans. Studies in immunocompetent

mouse models have reported elevations of serum cytokine levels upon infection, neuropathologies, TNFα-mediated death, and transient liver enzyme elevations and thrombocytopenia (Table I-1). Though these models recapitulate some features of dengue disease seen in humans, a very high (likely non-physiological) titer of virus has been required. The use of these high doses calls into question the relevance of these models to human infections. Our experiments used a much smaller challenge dose than other models to generate an immune response.

Our model is also the first to study the immune response to sequential heterologous DENV infections. We have shown that serotype-cross-reactive DENV-specific memory T cells preferentially respond *in vivo* during secondary infection. These cells enhance the IFNγ and TNFα responses over what is seen in primary infection. In humans, this phenomenon has been hypothesized to cause dengue immunopathology (V-1).

The advantage of an animal model for dengue, especially inbred mice, is the ability to manipulate the immune system to dissect physiological mechanisms. We demonstrated that serotype-cross-reactive memory $CD4^+$ T cells enhanced the $CD8^+$ T cell cytokine response to secondary DENV infection. The degree to which these cells enhanced the immune response varied depending on the serotype of infection, potentially lending some insight into the mechanism of how different sequences of infection result in different immunological and clinical outcomes in humans.

Our system for heterologous dengue infections can be further exploited. Due to its apparent effect on the $CD8^+$ T cell response, the DENV-specific $CD4^+$ T cell response needs to be further characterized. Once MHC Class II-restricted epitopes are defined, it

would be possible to investigate the memory $CD4^+$ T cell population and its effects on the $CD8^+$ T cell response to secondary infection in greater detail. For example, recombinant vaccinia viruses could be produced that express a dominant dengue $CD4^+$ T cell epitope. After primary immunization with this recombinant virus, a subsequent DENV challenge can be administered. With this experiment, the effect of the $CD4^+$ memory T cells on the $CD8^+$ T cell response can be more definitively evaluated.

The findings of our study on the immunological response to secondary DENV infection suggest several new strategies that might be tested to develop a dengue disease model. In humans, high level viremia appears to be necessary, in addition to T cell activation, for the development of DHF. Temporarily abrogating the interferon–α response in mice previous immunized with a primary DENV infection by administering a blocking anti-interferon-α antibody may lead to robust viral replication in the setting of an altered T cell response. Disease could be measured by visual inspection for lethargy, ruffled fur, and neurological signs. Blood tests would be useful to test for thrombocytopenia, hemoconcentration, and cytokine levels. In addition, vascular leakage could be measured using Evan's blue dye (Huang, Li et al. 2000).

I. A MODEL FOR THE IMMUNOPATHOGENESIS OF DENGUE VIRUS

We and others have proposed that the increased risk for DHF/DSS after heterologous secondary dengue infections is due to immunopathological mechanisms. It is hypothesized that an increased production of inflammatory cytokines during the immune response resulting from cross-reactive memory cells causes the increased vascular permeability and plasma leakage characteristic of severe dengue disease (Hober, Poli et al. 1993; Green, Vaughn et al. 1999; Mangada, Endy et al. 2002). Also, it has

been previously demonstrated that altered peptide ligands change the functionality of the T cell populations, which may then have a potential immunopathogenic effect (Bashyam, Green et al. 2006). Since the four serotypes of dengue do not share perfect homology, it is conceivable that secondary infections of dengue could lead to stimulation of cross-reactive memory T cells with naturally occurring altered peptide ligands. This stimulation could then change the functional profile of the cells leading to an immunopathology.

The mechanism by which this altered functional response due to cross-reactivity occurs is referred to original antigenic sin (Francis 1953; Klenerman and Zinkernagel 1998). In dengue infections, a cell with the highest affinity to one particular serotype, DENV-2 for example, will be activated upon primary infection with that serotype. These T cells however may be cross-reactive, that is, having an affinity, albeit a lower one, for another antigen. After the infection is cleared, the majority of these effector cells will die off, leaving a minority of these cells in the memory pool. (Fig. V-1) These memory cells may have a highest affinity for antigen from DENV-2, but may be cross-reactive and also have a partial affinity for antigens from another serotype of dengue.

Upon a heterologous secondary infection, for example DENV-1, not only will naïve T cells with the highest affinity for DENV-1 antigen be activated, these cross-reactive DENV-2 specific memory $CD4^+$ and $CD8^+$ T cells will as well. Since these memory cells have a lower threshold of activation and are presumably more numerous than the naïve DENV-1 specific cells, the memory cells will become activated more quickly and outnumber the activated DENV-1 specific cells. In addition, cross-reactive memory $CD4^+$ T cells may provide help to the activated $CD8^+$ T cells. As a result, there

will be a larger number of cells responding during this DENV-1 secondary infection, more so than would be in a primary DENV-1 infection, resulting in a massive amount of inflammatory cytokines being secreted, such as IFNγ and TNFα (Fig. V-2). In humans, these cytokines can induce a receptor-mediated change in vascular endothelial cells resulting in an increase in vascular permeability, leading to DHF and DSS. (Fig. V-3)

Our data shows that after primary infection with dengue, memory $CD8^+$ and $CD4^+$ T cells are generated. These cells may then be cross-reactive with certain other heterologous dengue serotypes and not to others. If the memory T cells are not cross-reactive, then the immune response to a secondary dengue infection will not be affected by the memory T cell pool and this response would mirror that of a primary infection with that serotype (Fig. V-1). However, if the cells are cross-reactive, then the memory T cells become activated. We show that these cross-reactive memory $CD8^+$ T cells respond by producing IFNγ and TNFα, ultimately enhancing the overall cytokine response. We also demonstrate that the activated cross-reactive $CD4^+$ T cells are able to provide help to the $CD8^+$ T cells, further augmenting the cytokine production. Our novel mouse model allows for the examination of the immunological aspects of dengue infections. Therefore, this murine system is a useful model to further study other immunological aspects after dengue infection.

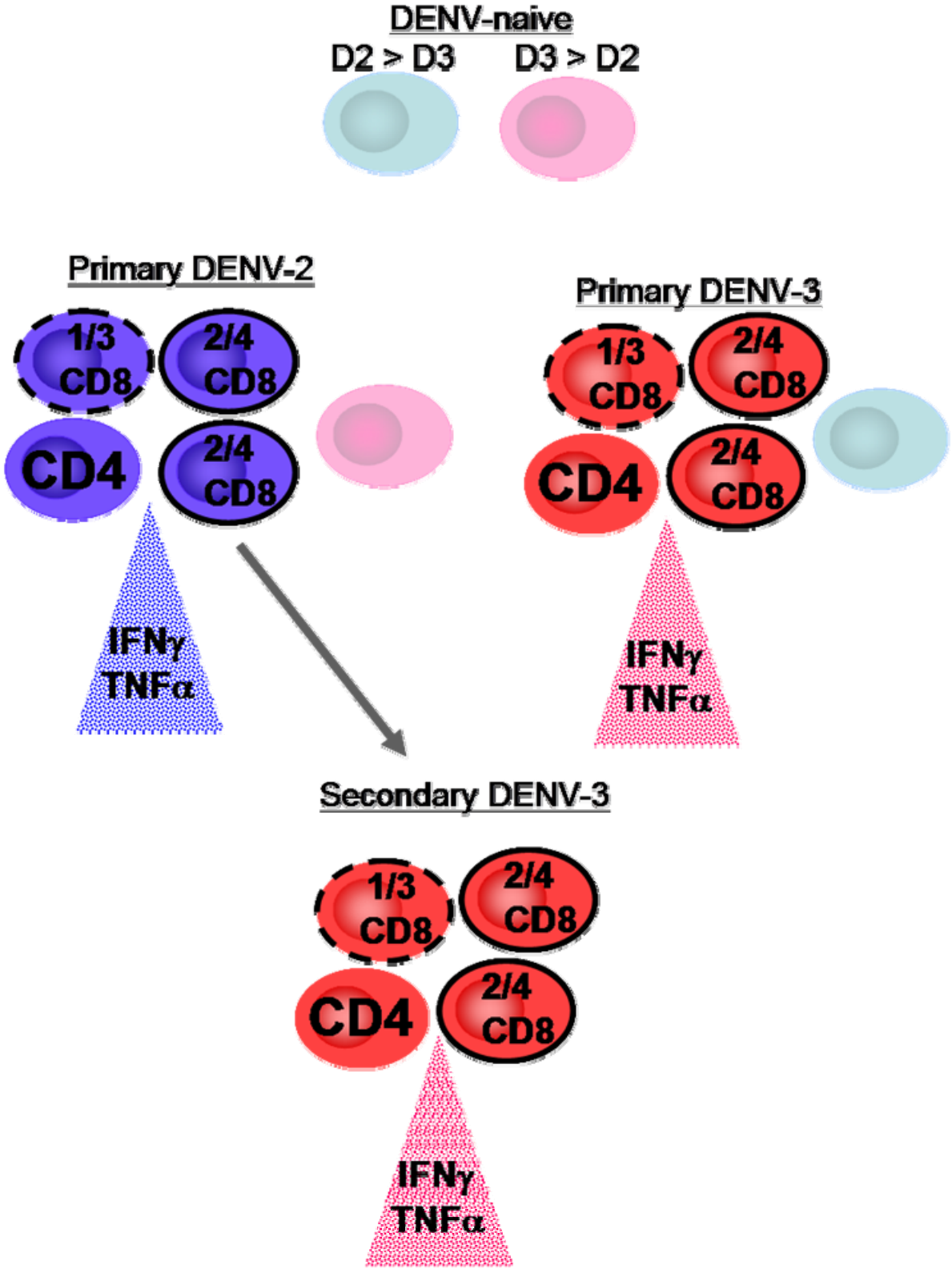


FIGURE V-1: A model for the role of T cells in the immunopathology of non-cross-reactive heterologous dengue virus infections. In primary infection, the T cells with the highest affinity for infecting virus, here DENV-2 or DENV-3, expand and enter the memory pool (cells with the darker colors). Upon heterologous secondary infection where no or few cross-reactive memory CD4 or CD8 T cells are stimulated, here DENV-2 followed by DENV-3, the cytokine response is similar in magnitude to primary infection.

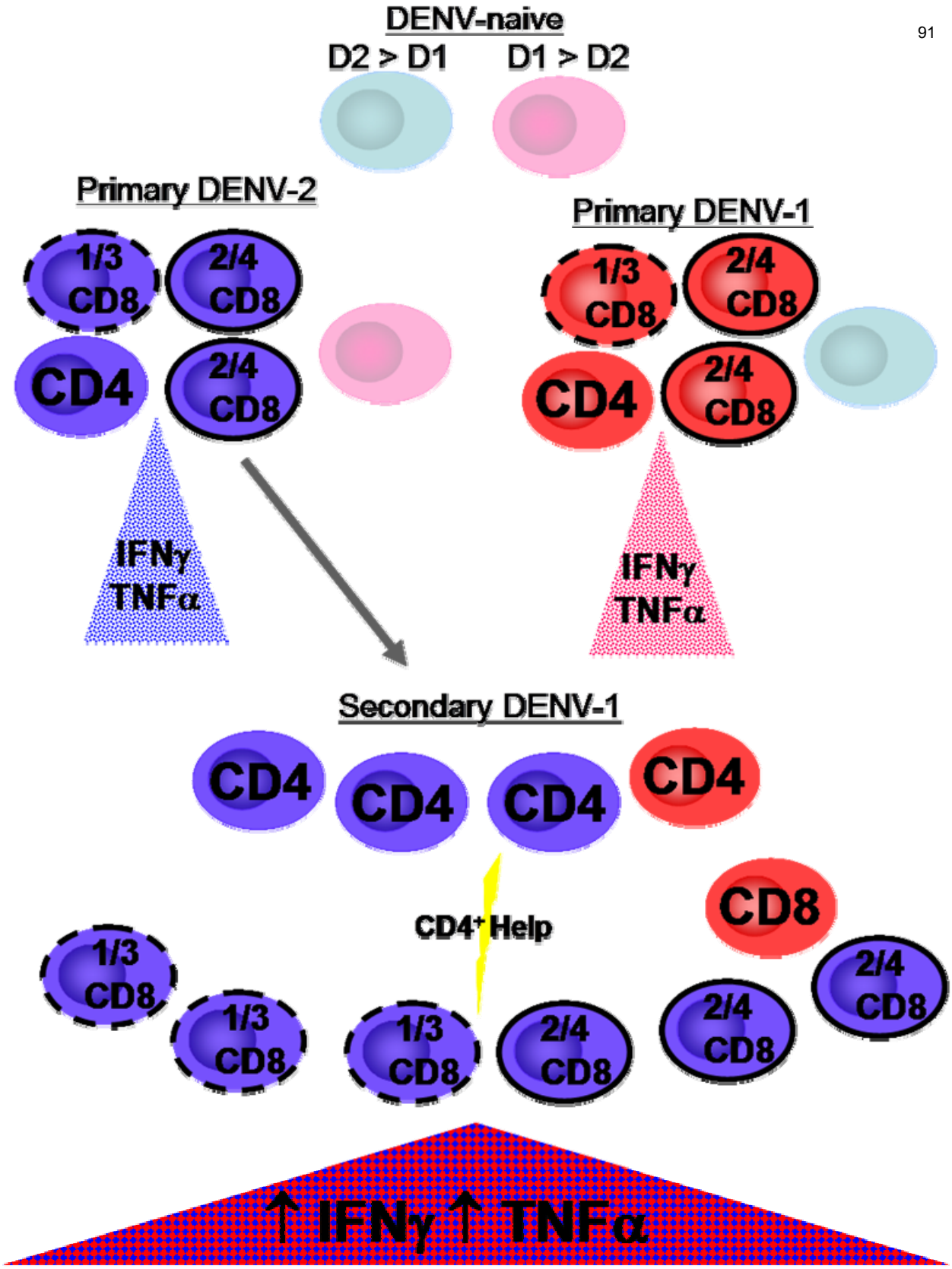


FIGURE V-2: A model for the role of T cells in the immunopathology of cross-reactive heterologous dengue virus infections. In primary infection, the T cells with the highest affinity for infecting virus, here DENV-2 or DENV-1, expand and enter the memory pool (cells with the darker colors). Upon heterologous secondary infection, here DENV-2 followed by DENV-1, serotype-cross-reactive memory CD4+ T cells are activated to provide help to the CD8+ T cells, the lower affinity memory CD8 T cells expand preferentially, and the epitope hierarchy changes; in the case shown, the proportion of cells responding to peptide variant D1/3 NS3 increases relative to cells specific for variant D2/4 NS3. This expansion of the memory population results in an increase in inflammatory cytokine secretion.

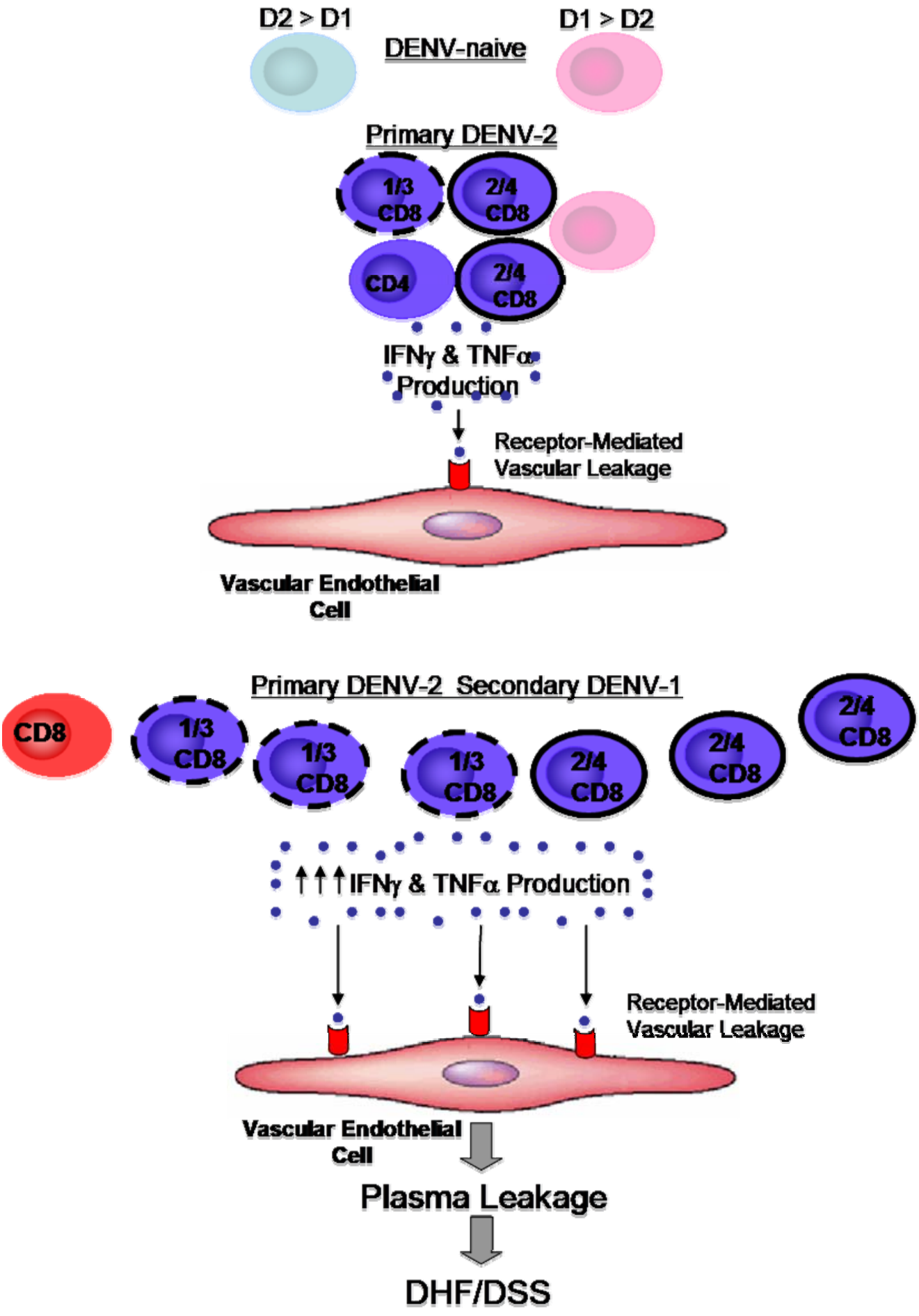

D2 > D1
D1 > D2
DENV-naive
Primary DENV-2
1/3 CD8
2/4 CD8
CD4
2/4 CD8
IFNγ & TNFα Production
Receptor-Mediated Vascular Leakage
Vascular Endothelial Cell
Primary DENV-2 Secondary DENV-1
CD8
1/3 CD8
1/3 CD8
1/3 CD8
2/4 CD8
2/4 CD8
2/4 CD8
IFNγ & TNFα Production
Receptor-Mediated Vascular Leakage
Vascular Endothelial Cell
Plasma Leakage
DHF/DSS

FIGURE V-3: A Model for Dengue Immunopathogenesis in Humans. During a primary DENV infection, T cells specific to that particular serotype, here DENV-2, are activated and secrete moderate amounts of inflammatory cytokines such as IFNγ and TNFα. These cytokines induce receptor-mediated changes in the endothelial cell junctions and cytoskeleton, resulting in some increase in vascular permeability, though the endothelium will remain largely intact and plasma leakage is not clinically evident. However, during heterologous secondary dengue virus infections, here DENV-2 followed by DENV-1, cross-reactive memory T cells from the primary infection become preferentially activated and the epitope hierarchy changes. Activated cross-reactive cells $CD4^+$ T cells provide help to $CD8^+$ T cells. These events lead to an increase in $CD8^+$ T cell activation, and greater IFNγ and TNFα secretion. Vascular permeability drastically increases, resulting in plasma leakage and dengue hemorrhagic fever/dengue shock syndrome.

CHAPTER VI

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