SEROTYPE CROSS-REACTIVE $CD8^+$ T CELL RESPONSE TO HETEROLOGOUS SECONDARY DENGUE VIRUS INFECTION IN HUMANS

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HEMA SUNDARA BASHYAM

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HEMA SUNDARA BASHYAM

Approved as to style and content by:

Raymond M. Welsh, Ph.D., Chair of Committee

Leslie J. Berg, Ph.D., Member of Committee

Katherine Luzuriaga, M.D., Member of Committee

Madelyn Schmidt, Ph.D., Member of Committee

Christian Brander, Ph.D., Member of Committee

Alan L. Rothman, M.D., Thesis Advisor

Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

Program in Immunology and Virology

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THESIS ABSTRACT

The generation of memory T cells following primary exposure to a pathogen is a critical feature of the vertebrate immune system which has evolved as a protective mechanism in order to defend the host against repeated assaults by the pathogen. Memory T cells are long-lived, undergo rapid proliferation upon re-activation, mediate a robust secondary response and clear the pathogen much more efficiently. These aspects have made the generation of memory T cells an attractive goal for the production of both prophylactic and therapeutic vaccines. However, the degeneracy of the T cell receptor, whereby a given T cell recognizes more than one epitope, allows the T cell to be modulated by epitope variants which could be self-ligands, ligands related to the original epitope but altered in sequence, or completely unrelated epitopes. Experiments in both mice and humans show that such cross-reactive stimulation of memory T cells results in complete, partial, or no activation of T cells, and in some cases, even alters the functional identity of the T cell (for example, T helper 1 cells start secreting IL-4, IL-5 and become part of a T helper 2 response). In the context of secondary infection of immune organisms with pathogens containing mutated or related T cell epitopes, such alterations at the cellular level translate into drastic changes in the overall clinical outcome of the infection. Thus, the presence of cross-reactive T cells in the memory population implies that the protective or pathologic nature of the secondary immune response is a consequence of the host's infection history. Although several murine models of heterologous infection resulting in altered pathological outcome have been studied, the exact immune correlates of protection versus immunopathology are still unclear. This thesis addresses this issue in dengue virus infections in humans.

Dengue fever (DF) and Dengue Hemorrhagic Fever (DHF) are two disease manifestations caused by infections of humans by the dengue viruses. These are a group of 4 serologically distinct flaviviruses (D1-4) which often co-circulate among endemic populations. While primary infection with any of the four serotypes can result in the more severe clinical disease characterized by DHF, epidemiological data from several outbreaks show that 80% – 90% of DHF cases occur among individuals with secondary infection. This implies that prior immunity to dengue is actually a risk factor for developing severe disease. In these DHF cases, there are increased numbers of $CD69^+$ $CD8^+$ T cells in circulation, with increases observed in the frequency of epitope-specific T cells, and the serum levels of several T cell produced cytokines, chemokines, and immune receptors are highly elevated. Since the four serotypes share 65% - 75% amino acid sequence homology, the possibility that unconserved T cell epitope sequences stimulated cross-reactive responses was borne out in *in vitro* examinations. In these studies, peripheral blood mononuclear cells (PBMC) and cloned T cells from both vaccinated and infected donors contained large populations of memory T cells that were cross-reactive for heterologous viral serotypes in proliferation and CTL assays. These data suggest that the severity of disease seen in DHF patients can be attributed to an immunopathologic secondary response during heterologous infection, and highlight a role for serotype cross-reactive T cells in this process.

This thesis addresses the hypothesis that the recognition of the natural variants of dengue virus T cell epitopes by serotype cross-reactive $CD8^+$ T cells of a dengue-immune donor results in an altered secondary response profile, with the changes reflected in both the quantitative and qualitative nature of the response. In order to compare the functional

profile of the secondary response of dengue-immune PBMC re-activated with heterologous serotypes, we focused on a panel of 4 donors who were vaccinated with live attenuated monovalent vaccines corresponding to D1, D2, or D4 serotypes. We screened a panel of peptides predicted to bind to HLA-A*0201 for cytokine responses and identified 4 novel epitopes that were highly immunogenic in all four donors. Direct *ex vivo* stimulation of donor PBMC with the heterologous sequences of these epitopes also showed sizeable serotype cross-reactive T cell populations. CFSE- and intracellular staining for cytokines and chemokines showed that these cross-reactive T cells not only expanded but also produced IFNγ, TNFα, and MIP-1β. Multi-parameter staining revealed functionally diverse populations comprised of single cytokine ($IFN\gamma^+$, $TNF\alpha^+$, $MIP\text{-}1\beta^+$), double cytokine ($IFN\gamma^+TNF\alpha^+$, $IFN\gamma^+MIP\text{-}1\beta^+$, $TNF\alpha^+MIP\text{-}1\beta^+$), and triple cytokine ($IFN\gamma^+TNF\alpha^+MIP\text{-}1\beta^+$) secreting sub-sets. Stimulation with the epitope variants altered the magnitude of the overall response as well as the relative sizes of these sub-sets. The patterns of responses revealed the effects of epitope immunogenicity, infection history and donor-specific variability. All 4 donors showed the highest cytokine response to a single epitope (NS4b 2353). The same two peptide variants (D2 NS4a 2148 and D3 NS4b 2343) induced the highest response in all 4 donors regardless of the serotype of primary dengue infection. Interestingly, the epitope variants which showed the highest immunogenecity in our donors corresponded to the D2 and D3 serotypes which have been documented as being more virulent as well as a viral risk factor for DHF. In one donor, the response to all peptide variants was dominated by the same cytokine sub-sets. These data suggested that the dengue-immune memory T cell repertoire was functionally diverse and underwent alterations in size after secondary stimulation. Therefore, we also

investigated the effect of epitope variants on dengue-specific $CD8^+$ T cell clones isolated from vaccinated and infected donors in order to determine if epitope variants induced altered functional outcomes at the clonal level. The epitope variants functioned either as strong agonists (particularly the D2 and D3 sequences), partial agonists, or null ligands. Some variants were able to induce cytolysis but not other effector functions at low concentrations. The variant ligands also influenced the hierarchy of cytokine responses within each clone.

The third part of this thesis focused on the characterization of the frequency and phenotypic profile of epitope-specific $CD8^+$ T cells in patients with DHF and DF at different times in the disease course in order to better understand the kinetics of the response and delineate any differences between the immune profile of severe vs. moderate disease. Tetramer staining for a previously identified HLA-B*07 restricted epitope was combined with staining for activation markers (CD69, CD38, HLA-DR), homing receptors (CCR7, CD62L), and programmed death receptor 1 (PD-1). The DHF subjects had early T cell activation with higher frequencies of $tetramer^+CD69^+$ cells as compared to DF subjects, in whom T cell frequencies peaked around the time of defervescence. While each subject had a unique phenotypic profile of $tetramer^+$ cells, there was a difference between DF and DHF subjects in terms of CCR7 expression; all subjects expressed low levels of CCR7 during acute illness but only the DHF subjects did not show upregulation of CCR7 on $tetramer^+$ cells during convalescence. These data suggest that there is a sustained alteration in memory phenotype in those who recovered from severe dengue disease. A majority of the $tetramer^+$ cells also expressed PD-1 during acute illness but not during convalescence. Double-staining with variant tetramers

allowed us to directly visualize serotype cross-reactivity of the epitope-specific population, and showed that secondary stimulation did induce the expansion of cells with low avidity for that secondary serotype and higher avidity to the variant. Furthermore, the ratios of these sub-sets changed during the course of the response.

Taken together, these studies suggest that the immune response to heterologous secondary dengue infection is mediated by a heterogeneous population of serotype-cross reactive T cells that have different functional avidities to epitope variants and is influenced by the serotype of the secondary infection as well as the prior infection history of the individual. The preferential expansion of clones which secrete IFNγ but not inflammatory MIP-1β or TNFα or a repertoire characterized by a higher ratio of cytolytic to cytokine producing clones could limit immune mediated damage while efficiently clearing the virus. This information will be useful in the design of vaccine strategies aimed at inducing protective cross-reactive responses against all 4 dengue serotypes while preventing immunopathological outcomes following secondary infection.

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ABBREVIATIONS

ADCC	antibody dependent cell cytotoxicity
ADE	antibody dependent enhancement
APC	allophycocyanin
APC	antigen presenting cell
APL	altered peptide ligand
BCG	Bacillus Calmette Guerin
BLCL	B-lymphoblastoid cell line
CCR	CC chemokine receptor
CFSE	carboxyfluorescein diacetate succinimidyl ester
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DC-SIGN	dendritic cell - specific ICAM-3-grabbing non-integrin
DHF	dengue hemorrhagic fever
DF	dengue fever
DSS	dengue shock syndrome
DV	dengue virus
EBV	Epstein-Barr virus
ELISPOT	enzyme-linked immunospot
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen

IFN	interferon
IL	interleukin
JEV	Japanese encephalitis virus
LCMV	lymphocytic choriomeningitis virus
LTNP	long term non-progressors
MCMV	murine cytomegalo virus
MIP	macrophage inflammatory protein
NK	natural killer
NS	non-structural
PBMC	peripheral blood mononuclear cells
PD-1	programmed death receptor-1
PV	Pichinde virus
RANTES	regulated on activation, normal T-cell expressed and secreted
RSV	respiratory syncytial virus
TBE	Tick-borne encephalitis
TCR	T cell receptor
TNF	tumor necrosis factor
TRAIL	tnf-related apoptosis inducing ligand
WNV	West Nile virus
YFV	Yellow fever virus

CHAPTER I

INTRODUCTION

Dengue is an arthropod-borne viral illness which threatens about 2.5 billion people worldwide. About 50 to 100 million people each year are estimated to suffer from dengue fever (DF) or the more severe and potentially fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Gubler, 1997). These illnesses are caused by a group of 4 antigenically related viruses referred to as dengue serotypes 1-4 (Sabin, 1952). Infection with one serotype provides life-long immunity to re-infection by only that serotype but not the others, and sequential infection with a heterologous dengue serotype frequently results in DHF/DSS. Hence inhabitants of those areas where dengue is endemic and where multiple serotypes are in co-circulation are susceptible to multiple infections over their life-time and are at risk for developing severe dengue disease. Dengue infections are one of the biggest public health problems in tropical areas such as South/South-East Asia, South America, the Caribbean countries, and Africa (Halstead, 2002). In the United States, dengue outbreaks have been reported since the 1980s in communities along the U.S-Mexico border and also in Hawaii in 2001-2002 (MMWR, 2001; Effler et al., 2005). The mosquito vector of dengue – *Aedes aegypti* – is widely found in the Southern States and around 4000 cases of imported dengue have been reported in the last 3 decades (CDC, 2006).

There is currently no licensed vaccine against dengue. The lack of success in this conventional solution to the problem can be partly attributed to the lack of appropriate animal experimental models and the necessity of inducing protective immunity against all

four dengue serotypes while avoiding potentially harmful virus-specific immune responses. Aside from studies aimed at designing and clinically testing dengue vaccine candidates, a major focus of dengue research is to understand why secondary dengue infections cause severe disease and how the mechanisms of secondary immune response may cause immunopathology as opposed to offering protective immunity.

Based on earlier research, our group had hypothesized that the re-activation of memory serotype cross-reactive T cells during secondary dengue infection and their suboptimal activation by the epitope variants of the heterologous serotype contributed to the enhanced immunopathology seen in DHF. The goal of this thesis was to test this hypothesis by studying the breadth and patterns of functional heterogeneity among serotype cross-reactive $CD8^+$ T cell subsets. We identified novel T cell epitopes restricted by the commonly found class I HLA-A*02 allele which is associated with high risk for DHF, by screening PBMC from immunized individuals and studying the functional cross-reactivity of dengue virus-specific T cells at bulk and clonal levels. We also attempted to understand the kinetics of the memory response and the differentiation of dengue-specific T cells during the acute and convalescent stages of viral infection by tracking $tetramer^+$ T cells in PBMC samples from dengue-infected patients.

This Introduction is divided into sections that pertain to various aspects of this thesis. An overview of the viral genome and polyprotein is followed by sections covering clinical descriptions of dengue disease, DHF risk factors, dengue-specific immune responses, and evidence of DHF immunopathology. The next sections are specifically devoted to the discussion of serotype-cross-reactive T cells, their contributions to the phenomenon of heterologous immunity, and the biological implications of their

activation by altered peptide ligands. The final sections include an overview of $CD8^+$ T cell differentiation during acute viral infection and a description of various T cell memory subsets as characterized by phenotypic and functional markers.

A. DENGUE VIRUS – GENOME, STRUCTURE, AND LIFE CYCLE.

Dengue is a member of the *Flaviviridae* family and belongs to the *Flavivirus* genus in which there are more than 70 viruses including yellow fever virus (YFV), West Nile virus (WNV) and Japanese encephalitis virus (JEV). The dengue virion is composed of a single, positive-sense ~11kb RNA genome packaged by a viral capsid (C) protein and enclosed within a host-derived lipid bilayer studded with two viral structural proteins – the envelope (E) protein and the membrane (M) protein. Seven other non-structural proteins are encoded by the genome, and these are designated as NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. The genome is translated as a single open reading frame. The polyprotein is processed by a host enzyme to yield the pre-M, E, and NS1 proteins, and the rest of non-structural proteins are produced as a result of cleavage by the viral protease encoded by NS3 (reviewed in Lindenbach, 2001).

Dengue virus enters the host cell by receptor-mediated endocytosis. Proteins such as DC-SIGN, GRP78, and CD14-associated molecules have been proposed as the host cell receptors (Lozach, et al., 2005; Tassaneetrithep, et al., 2003; Jindadamrongwech, et al., 2004; Chen, et al., 1999), while glycosaminoglycans such as heparan sulfate have been suggested as low-affinity co-receptors (Chen, et al., 1997). Fusion of the virion and the host cell is followed by the release of the nucleocapsid into the cytoplasm, dissociation of the RNA genome from the capsid protein, initiation of translation and

replication of the viral genome, followed by virion assembly (reviewed in Mukhopadhyay, et al., 2005). Work done on tick-borne encephalitis (TBE) virus shows that the structural genes C, pre-M, and E, which are at the 5' end of the genome, are the first to be synthesized at the ER surface. A signal sequence at the carboxyl terminus of the C protein allows the pre-M and E to be translocated into the ER lumen, undergo late cleavage by furin proteinase to form mature M and E proteins (Lorenz, et al.,2002). This step is crucial for the formation of fully infectious virion particles since virions containing uncleaved prM have low infectivity (Randolph, et al.,1990).

The E protein is essential for receptor binding and membrane fusion, since it contains several receptor binding sites and encodes a fusion peptide (Lorenz, et al., 2002; Allison, et al., 2001). This protein also contains an assortment of epitopes for neutralizing and non-neutralizing antibodies. Mutations at these epitopes have been shown to affect viral infectivity and pathogenesis (Monath, et al., 2002). An array of monoclonal antibodies (mAbs) against E protein epitopes have been developed and tested for their protective efficacy (reviewed in Roehrig, et al., 2001). Non-neutralizing mAbs and neutralizing mAbs at sub-neutralizing concentrations have been shown to enhance the infection of susceptible cells via a process known as antibody dependent enhancement (ADE) (reviewed in Morens, 1994). This is an *in vitro* phenonmenon in which the infection of cells such as monocytes and macrophages which bear Fc gamma receptors is modified by the addition of virus-reactive antibodies at certain concentrations.

The 7 non-structural genes, which represent two-thirds of the dengue genome, are found at the 3'end. The NS proteins are generally more conserved among the dengue serotypes and other flaviviruses. The NS2a and the NS3 proteins have proteolytic

activity. NS2a mediates cleavage between NS1 and NS2b. The NS1 protein has complement fixing activity and can exist in cell-associated, cell-surface, or secreted forms (Lee, et al., 1989). Two recent studies in dengue patients showed that high levels of circulating NS1 correlate with high viral titers, complement activation and increased disease severity, and the ability of NS1 to mediate complement activation has been proposed to contribute to vascular leakage in DHF and DSS (Libraty, et al., 2002; Avirutnan, et al., 2006).

The NS3 protein is the second largest and most highly conserved of the dengue virus proteins and is a multi-functional enzyme. It has N-terminal sequence homology to trypsin-related serine proteases and C-terminal homology to RNA helicases and is involved in viral RNA replication and capping (Bazan and Fletterick, 1989; Benarroch, et al., 2004). The NS4a and NS4b proteins are hydrophobic proteins and have both been shown to contribute to the inhibition of interferon signaling and IFNβ-stimulated gene expression (Munoz-Jordan, et al., 2005). The NS5 protein is the largest dengue protein and is an RNA dependent RNA polymerase. Its N-terminal portion includes a methyltransferase core and a nearby GTP-binding site, which enable this protein to mediate RNA capping (Engloff, et al., 2002). An additional role for this protein has been revealed in a recent study which showed that the expression of NS5 in HEK-293 cells induces IL-8, a chemokine found at high levels in sera from patients with severe dengue disease (Medin, et al., 2005).

B. CLINICAL MANIFESTATIONS OF DENGUE DISEASE.

A majority of primary dengue virus infections results in asymptomatic infections. Clinical dengue disease ranges from a relatively mild DF to the potentially fatal

hemorrhagic disease DHF and its most severe form, DSS (Burke, et al., 1988, Nimmanitya, 1987). DF is an acute, self-limited, febrile illness characterized by fever, malaise, muscle aches, headache, nausea, and vomiting which lasts for about 5-7 days, and can occur after infection with any of the four dengue serotypes. Patients with these symptoms sometimes also have a macular or maculopapular rash. Other characteristics of the disease include neutropenia, leucopenia, thrombocytopenia, and the presence of atypical lymphocytes. Most patients recover from DF.

In about 5-30% of cases, these symptoms are followed by the development of increased capillary permeability and vascular leakage during the time of defervescence, which are characteristic of DHF. According to guidelines established by the WHO, the clinical definition of DHF includes fever, a positive tourniquet test, hepatomegaly, hemoconcentration (>20% increase in hematocrit), and thrombocytopenia ($<100{,}000/mm^3$). These minimal criteria are classified either as grade 1 DHF or, if spontaneous bleeding is also present, as grade 2 DHF. Grades 3 and 4 DHF, in which shock develops, are defined as DSS. As many as 95% of DHF/DSS patients (in South East Asia) are children under the age of 15. Most patients recover from these symptoms following fluid replacement therapy. However, ~50% of DSS cases will result in mortality in the absence of treatment (Guzman and Kouri, 2002). Severe gastrointestinal bleeding and hepatic failure can be direct causes of death.

C. RISK FACTORS FOR DHF.

1. Viral factors:

While it is known that there are genetic differences between the various strains of dengue virus, the difference in virulence and its impact on clinical disease was highlighted by the Cuban epidemic of 1981. Although several dengue viral strains from the American genotype of D2V were found to be in co-circulation in South American countries by the 80s, the occurrence of DHF cases during the epidemic coincided with the appearance of the Asian genotype of D2V (Rico-Hesse, 1990). This notion of strain-dependent virulence as a DHF risk factor has been borne out by other epidemiological findings from outbreaks in Peru and Sri Lanka. These studies showed that the presence of DHF cases was not associated with the American D2V genotype, and coincided with circulation of the more virulent Asian D2V genotype (Watts, et al., 1999; Messer et al., 2002). Another study which examined the relationship between dengue disease and circulating serotypes in Bangkok found that, although yearly epidemics were associated with different serotypes, the years with severe outbreaks were solely associated with the D3 serotype (Nislalak, et al., 2003).

Other studies have tried to pinpoint the specific molecular determinants of virulence. These investigations were carried out *in vitro* in cloned or cell-culture passaged wild or attenuated viruses. Sequence changes that altered virulence were shown to map to regions in the prM, E, NS1, and NS3 genes and also to the 5' and 3' non-translated regions (NTRs) (Kinney, et al., 1997; Mangada, et al., 1997, Mangada, et al., 1998).

2. Host factors:

The factors which may contribute to predisposing an individual to severe dengue disease include sex, race, nutritional status, immune status (occurrence of chronic

infections), and age. Epidemiological surveys of Thai and other South East Asian epidemics have shown that children under 1 year and those between 3 – 5 years of age are at higher risk for DHF (Nimmanitya, 1987). Genetic polymorphisms have also been shown to influence susceptibility to DHF. A mutation leading to the change of a single amino acid in the FcγR IIA receptor is believed to reduce antibody-dependent enhancement of infection by decreasing the level of opsonization by IgG2 antibodies and thus offers protection from DHF (Loke, et al, 2002). Similarly, certain variants of the vitamin D receptor associated with a stronger T helper response and a variant in the DC-SIGN promoter CD209 thought to result in lower transcription and expression levels of the receptor are associated with decreased risk of severe disease and protective immunity (Loke, et al., 2002; Sakuntabhai, et al., 2005).

Several groups have also proposed HLA haplotype as a determinant of disease outcome. The earliest study carried out on Thai subjects in the 80s showed an increased frequency of HLA-A*02 among DHF patients (Chiewsilp, et al., 1981). Recently, Loke et al, showed that susceptibility to DHF among Vietnamese subjects was significantly associated with polymorphism at the HLA-A locus, and that the HLA-A*33 allele was associated with a reduced risk of developing DHF (Loke, et al., 2001). Another study compared HLA class I allele profiles of Thai subjects both at the phenotypic and molecular level and found that HLA-A*0203 was associated with less severe DF regardless of the secondary infection serotype while HLA-A*0207 was associated with the more severe DHF in patients with secondary D1 or D2 only (Stephens, et al., 2002). That study also looked at HLA-B alleles and identified HLA-B44, B62, B76, and B77 as alleles that appeared to protect against clinical disease following secondary infection,

while showing an association of HLA-B51 with severe DHF and HLA-B52 with DF. These findings imply that the presentation of CD8 T cell epitopes by the disease susceptible alleles is a risk factor for DHF and this must therefore be taken into consideration during the design of vaccines to provide protective immunity. Finally, the previous infection history of the host may also predispose him or her to severe DHF via the action of pre-existing dengue-specific antibodies and serotype cross-reactive dengue-specific T cells. The mechanism of action and immunopathological contributions of both these immune response components will be explained in the following sections.

3. Sequential dengue infections:

A minority of DHF cases occur as a result of primary infections particularly in infants 6-12 months of age whose mothers had two or more previous dengue infections (Kliks, et al., 1988). These infants showed DHF/DSS symptoms only during the time when maternal antibodies had degraded to titers where they were no longer neutralizing virus but enhancing infection. Supporting this idea, that infants only became vulnerable to severe disease during the particular time-frame when maternal antibodies were no longer protective, another study found that infants under 3 months of age were in fact symptom-free despite being seropositive for dengue infection (Halstead, et al., 2002). This study also found that there were very few DHF/DSS cases among children older than 1 year and younger than 4. In these children, maternal antibodies had been completely degraded to concentrations below that required to enhance infection.

However, early studies of dengue epidemics in Thailand in the 80s found that DHF and DSS were 15-80 times more likely to occur following secondary dengue

infections than primary infections (Halstead, et al., 1982; Sangkawibha, et al., 1984). Similar findings were reported by another group which studied an epidemic caused by D2V in Cuba in 1981 following a primary D1V epidemic in 1977 (Guzman, et al., 1987; Bravo, et al.,1987; Guzman, et al., 1991). While children between 1-3 years of age who had not been previously exposed to D1V developed DF, most of the DHF cases occurred in those patients older than 4 years who had previously had a primary D1V infection. These studies, along with Halstead et al's observation that the Thai secondary DHF cases showed dengue-specific antibodies, supported the hypothesis that the immune response to the secondary infection played a role in the pathogenesis of DHF. It is now accepted that antibody-dependent enhancement mediated by cross-reactive sub-neutralizing antibodies and the immune response mediated by serotype cross-reactive T cells are two mechanisms by which secondary dengue viral infection could result in severe disease.

D. IMMUNE RESPONSES TO DENGUE VIRUS.

The host response to dengue virus infection involves a rapid induction of innate immune mechanisms followed by humoral as well as T cell mediated immune responses.

1. Innate immunity:

The infection of immature dendritic cells (DCs) via the DC-SIGN receptor induces the production of anti-viral cytokines such as type-I interferons α and β, and TNF-α, (Libraty, et al., 2001; Palmer, et al., 2005). The early release of the type I interferons as well as IFNγ is crucial in mediating resistance to DV infection; mice deficient in type-I interferon receptors do not survive intraperitoneal inoculation of DV (Shresta, et al., 2004; Johnson et al., 1999). DV-stimulated DCs upregulate the expression

of maturation markers such as HLA-DR, CD83, and CD11b. Several studies, however, show that they differentiate into functionally impaired antigen-presenting cells which secrete IL-10 and are unable to efficiently activate T cells (Mathew et al., 1999; Palmer, et al., 2005). Considered along with the previously discussed ability of DV to suppress type-I interferon signaling, these findings highlights the ability of DV to evade innate immune mechanisms. Another aspect of the early response against DV infection is the activation of NK cells which can clear infected cells by direct cytolysis or antibody-dependent cell cytotoxicity (ADCC). While information on the importance of NK cell activity in human infections is limited, studies in mice have shown that NK cell activity may be important for clearing primary DV infection (Shresta, et al., 2004).

2. Antibody-mediated immunity:

The dengue virus E protein is the predominant target of antibodies that can prevent viral binding and fusion and neutralize viral infectivity *in vitro* (Roehrig, et al., 1998). Another target of the antibody response is the NS1 protein, which is present as both cell surface and secreted forms. Anti-NS1 antibodies can fix complement and mediate the lysis of dengue-infected cells. *In vivo* evidence of antibody-mediated protection against dengue infection comes from studies where passively transferred antibodies against the E, prM , and NS1 proteins protected mice against lethal infection (Kaufman, et al., 1987; Kaufman, et al., 1989; Schlesinger, et al., 1987). In humans, however, anti-NS1 antibodies (against amino acids 1-15) have been shown to have detrimental effects due to their cross-reactivity with endothelial cell surface antigens, thereby inducing nitric-oxide (NO) mediated apoptosis, which is a possible cause for the vascular damage seen in DHF/DSS patients (Lin, et al., 2003).

Another study that highlighted the pathologic effect of the antibody response was published by Endy et al. Pre-secondary infection plasma obtained from dengue-immune children contained serotype-cross-reactive antibodies which seemed to protect against disease severity following secondary D3 infection but not secondary D1- or D2 infections (Endy, et al., 2004). However, the downside to inducing antibody responses is of course ADE, which occurs when antibodies present at sub-neutralizing concentrations and those generated against non-neutralizing epitopes enhance viral uptake into FcRII bearing cells. Pre-existing antibodies thereby increase the viral load, which in some instances has coincided with disease severity (Littaua, et al., 1990; Vaughn, et al., 2000; Kliks, et al., 1989).

3. T cell-mediated immunity:

The $CD4^+$ and $CD8^+$ T cell response to DV is directed primarily against non-structural proteins with the immunogenic NS3 protein containing most of the epitopes identified to date (Lobigs, et al., 1994; Mathew, et al., 1996). Following their interaction with DV-infected antigen presenting cells, DV-specific T cells are activated to proliferate, produce high levels of IFNγ, TNFα, TNFβ, and MIP-1β, and lyse DV-infected targets via perforin and granzyme mediated mechanisms (Rothman, 2003). Both serotype-specific and serotype-cross-reactive memory T cells have been detected in dengue-immune PBMC following primary infection (Kurane, et al., 1989). While earlier studies showed that the highest T cell responses against whole dengue antigen or virus were against the primary infecting serotype, more recent studies have shown that the level and pattern of cross-reactivity depend on the extent of homology between the

epitope sequences among the four serotypes. Analysis of cross-reactive responses at the clonal level shows that these differences in sequence might have functional consequences. For example, a $CD8^+$ T cell clone has been shown to have partial agonist responses against a variant epitope sequence from a heterologous serotype by lysing target cells while being unable to proliferate or produce cytokines (Zivny, et al., 1999).

Other examples of altered immune responses following heterologous secondary infection have been reported in studies which examined epitope-specific T cell frequencies in dengue-infected patients using HLA tetramers bound to variant epitopes (Mongkolsapaya, et al., 2003; Mongkolsapaya, et al., 2006). In patients with severe disease, frequencies of $CD8^+$ T cells specific for the known secondary serotype were lower than the frequencies of cells specific for presumably previously encountered heterologous serotypes.

In terms of responses on a larger scale, *in vitro* activation by whole dengue antigen resulted in higher frequencies of $IFN\gamma^+$ $CD4^+$ T cells in response to homologous stimulation while stimulation with heterologous antigens resulted in a higher ration of TNFα: IFNγ producing cells (Mangada, et al., 2005). In a clinical study which examined cytokine responses to all 4 serotypes in PBMC from dengue-immune individuals prior to their secondary infections, a broadly serotype-cross-reactive IFNγ response was weakly associated with the occurrence of mild disease (Mangada, et al., 2002). While there remains uncertainty as to how T cell mediated responses specifically contribute to protective immunity, the findings of these two studies seem to suggest that particular T cell functions performed by a subset of cells among the heterogeneous dengue-specific population could potentially result in a beneficial outcome.

E. IMMUNOPATHOLOGICAL MECHANISMS IN DHF.

1. Evidence of immune-mediated cellular damage:

The capillary permeability and vascular leakage seen in DHF patients occurs at the time of defervescence following viral clearance, is reversible, and does not result in permanent organ failure. This suggests that the damage is not due to cytopathic effects of the virus itself. *In lieu* of animal models that faithfully reproduce the characteristics of human dengue virus infection, investigators have used human endothelial cell lines infected *in vitro* to track the mechanisms that lead to vascular permeability. Upon DV infection, these cell lines produce pro-inflammatory cytokines such as IL-6, IL-8, and RANTES, undergo changes in their actin cytoskeleton, and show increased permeability to small molecules (Peyrefitte, et al., 2006; Talavera, et al., 2004). Treatment of endothelial cells with dengue-immune sera from patients with acute infection activates them and renders them vulnerable to apoptosis (Cardier, et al., 2005). However, the addition of anti-TNFα monoclonal antibodies can partially reverse these effects indicating a role for this cytokine in initiating plasma leakage.

Liver injury as indicated by elevated serum levels of liver enzymes, and detection of viral antigen in liver hepatocytes in a small number of cases are other characteristics of DHF (Kuo, et al., 1992; Couvelard, et al., 1999). HepG2 cells infected *in vitro* with DV produce various inflammatory cytokines and chemokines and undergo TRAIL-mediated apoptosis (Matsuda, et al., 2005). Further evidence of immunopathology was seen in immunocompetent D2V-infected mice in which the kinetics of liver enzyme elevation correlated with the kinetics of cellular infiltration and T cell activation (Chen, et al., 2004). In D3V infected Thai subjects, liver enzyme levels correlated with plasma levels

of soluble IL-2 receptor, which is an indicator of T cell activation and proliferation (Libraty, et al., 2002). This suggests that liver injury may be a result of T cell activation.

2. Evidence for T cell-mediated pathology:

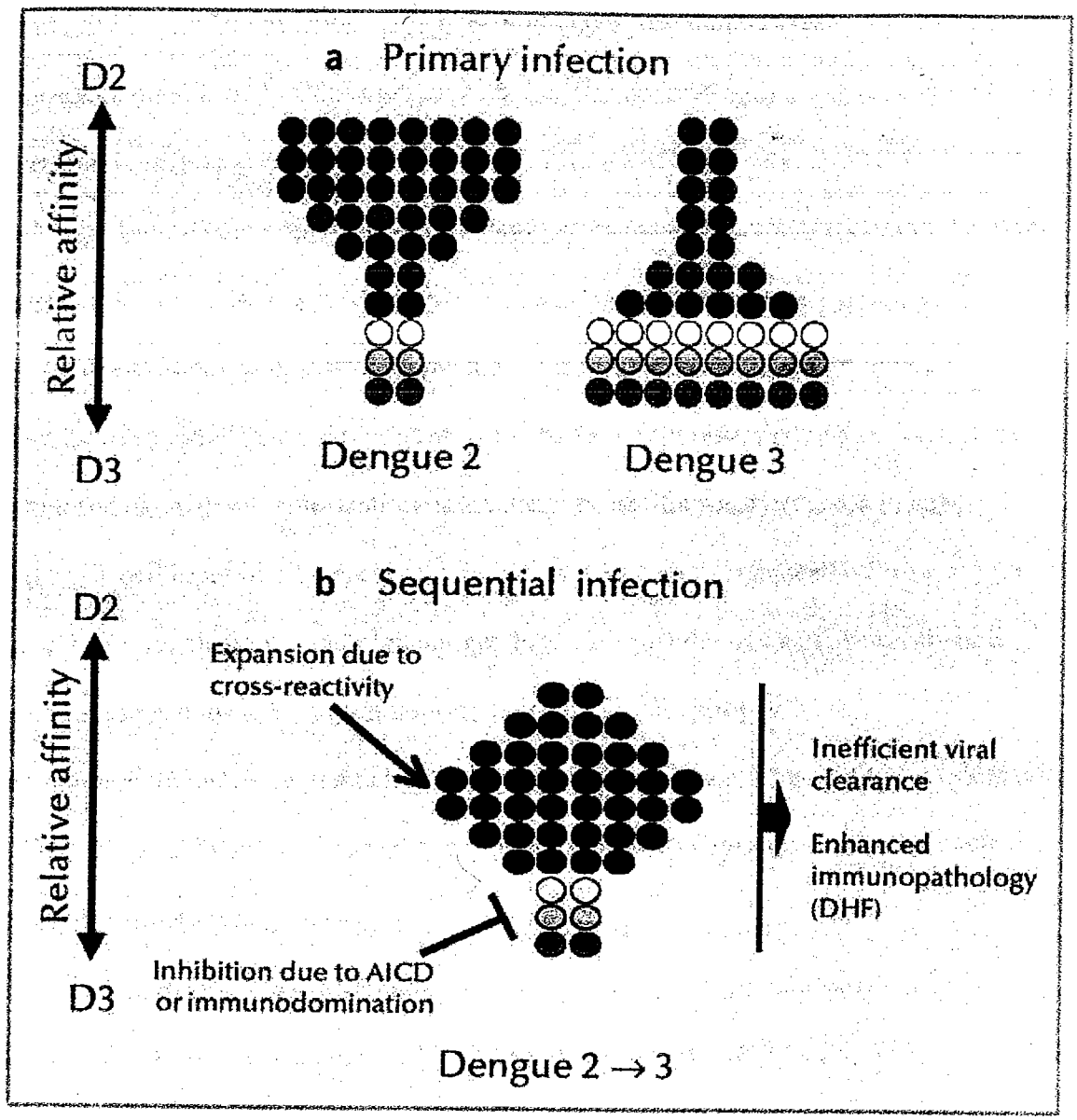
The epidemiological data which show increased occurrence of DHF after secondary dengue infections and the detection of serotype cross-reactive T cells in patient PBMC following primary infections suggest a role for memory T cells in DHF immunopathology. Elevated levels of T cell-produced cytokines such as IFNγ, TNFα, IL-2, IL-10, and other cytokines such as IL-8 are seen during acute dengue infection even before the onset of plasma leakage (Kurane, et al., 1991). A higher level of immune activation is seen in DHF cases as compared to subjects with mild disease as shown by studies in which elevated levels of soluble TNF receptors, soluble IL-2 receptor, and soluble CD8 correlated with disease severity (Green, et al., 1999). Other observations in DHF subjects include an earlier and increased expression of CD69 both on NK cells and $CD8^+$ T cells (Green, et al., 1999), and earlier peak levels of IFNγ as compared to that seen in DF cases (Libraty, et al., 2002). High levels of TNFα have also been associated with disease severity (Hober, et al., 1993; Mangada, et al., 2003).

The increased immune activation in DHF is accompanied by an overall decrease in the sizes of lymphocyte subsets, including $CD4^+$ and $CD8^+$ T cells, NK cells, and γδ T cells (Green, et al., 1999). It is possible that DV infection results in the apoptosis of PBMC via Fas signaling; alternately, the cells may be undergoing activation-induced cell death following their interaction with functionally impaired DV-infected APCs (Myint, et al., 2006; Mathew et al., 1999).

In more recent studies which examined epitope-specific T cell responses, higher frequencies of epitope-specific T cells correlated with severe disease (Zivna, et al., 2002; Mongkolsapaya, et al., 2003; Mongkolsapaya, et al., 2006). While Zivna et al used ELISPOT assay to track HLA-B*07 restricted epitope-specific responses, the latter studies used HLA tetramers to identify T cells specific for an HLA-A*11 restricted epitope.

Based on these findings, our group initially proposed a model for DHF immunopathology in which serotype cross-reactive T cells generated during the immune response to the primary infection were preferentially expanded early during the secondary immune response to a heterologous serotype. The partial activation of these cells by variant heterologous T cell epitopes could produce an enhanced proinflammatory response while only providing a suboptimal antiviral effect, thereby resulting in poor viral clearance and severe disease outcomes (Kurane, et al., 1994; Zivny, et al., 1999). We have since attempted to further characterize the specific nature of this altered secondary immune response by examining an array of *in vitro* T cell functions following heterologous stimulation.

Mongkolsapaya et al approached this issue by examining the avidities of dengue-specific T cells to epitope variants from the secondary serotype vs heterologous serotypes in PBMC from patients with acute dengue infection. They found that serotype cross-reactive T cells had a low avidity for the secondary serotype while higher frequencies of cells were specific for the heterologous serotype. These low affinity memory T cells were thought to dominate the secondary immune response because of their higher precursor frequencies or alternatively due to the death of high affinity T cells by apoptosis



Welsh and Rothman, 2003.

Figure 1: Model of 'original antigenic sin' and its impact on DHF immunopathology

The naïve T cell repertoire is composed of clones which are heterogeneous in their affinities for DV epitopes. (a) Primary infection with D2V or D3V induces the expansion of the clones that have highest affinity for that serotype and the immunodominance of this population is reflected in the memory pool that is established. (b) Secondary infection of a D2 immune subject by D3V may be followed by the preferential expansion of the memory population that had higher affinity for D2 as opposed to naïve cells that will be primed by D3V. An additional or alternative mechanism may be the deletion of D3V high-affinity cells due to activation-induced cell death (AICD). Under either circumstance, the result is a T cell repertoire that is composed of cells that have low avidity to the secondary serotype. This situation may lead to inefficient lysis of infected cells and enhanced secretion of inflammatory cytokines and thereby result in DHF.

following antigenic stimulation. This phenomenon of 'original antigenic sin' was proposed to contribute to DHF immunopathology by preventing the efficient clearance of the currently infecting serotype due to the low affinity of the T cells for the viral variant while still maintaining an output of proinflammatory cytokines and chemokines (Figure 1).

These findings altogether imply that (i) the outcome of secondary dengue infection in an individual is influenced by the history of previous dengue infections in that individual, (ii) the modification of the dengue-specific serotype cross-reactive memory T cell repertoire by secondary infection may lead to enhanced immune response due to low viral clearance and high antigen load, and (iii) there is a qualitatively altered immune response following stimulation by variant T cell epitopes. Thus, immunomodulation by sequentially infecting heterologous serotypes, which is referred to as 'heterologous immunity', can play a role in deciding the balance between protective immunity and immunopathology.

F. HETEROLOGOUS IMMUNITY: PROTECTIVE RESPONSES VS. IMMUNOPATHOLOGY.

1. Animal models of heterologous infection:

Studies of LCMV infection in mice have shown that the $CD8^+$ memory T cell pool remains remarkably stable until a heterologous viral infection, which results in a decrease in the numbers of T cells specific for previously encountered pathogens (Selin, et al., 1996). If, however, the sequentially infecting pathogens share cross-reactive epitopes, then those epitope-specific cross-reactive T cells are maintained in the memory

pool and may even be enriched by the second stimulation (Brehm, et al, 2002). When mice immune to LCMV, PV, MCMV, or an unrelated virus such as vaccinia were subjected to heterologous challenge by these viruses, partial protection or in some cases, non-reciprocal protection ensued (Selin, et al., 1998). For example, infection with LCMV, PV, and MCMV protected mice against infection with vaccinia virus whereas vaccinia virus immune mice were not protected during challenge by the other viruses. Similarly, primary infection of mice with LCMV protected them more efficiently against PV than the reverse sequence of infection. These patterns of heterologous immunity were influenced by mechanisms such as the altered hierarchy of epitope-specific responses, and the skewing of anti-viral responses towards IFNγ production or cytotoxicity.

The immunopathological effects of heterologous immunity were seen in experimental models of LCMV-immune mice injected with vaccinia virus intraperitoneally or intranasally. In the former instance, the mice developed immunopathological lesions in their abdominal fat pads, while in the latter model the mice displayed a distinctly altered lung pathology characterized by bronchiolar obstruction by inflammatory cells (Selin, et al., 1998; Chen, et al., 2001).

Heterologous immunity may also be a contributing factor in autoimmunity. Transgenic mice expressing LCMV antigens experience chronic inflammation of the CNS when infected with LCMV but not with PV or vaccinia virus. However, rechallenge of the mice with PV or vaccinia after the breaking of tolerance by LCMV results in exacerbation of the disease (Evans, et al., 1996). These data can be extrapolated to a situation known as 'molecular mimicry' where viral epitopes happen to be homologous to

self-antigens. Cross-reactive T cells that get activated by viral infection may then cause autoimmunity by targeting all the cells that express that self-antigen.

Immunity to subsequent infections by heterologous viruses is also influenced by the degree to which T cell functions of the memory pool are polarized to the T_H1 or T_H2 phenotype. For example, in mice immunized with a recombinant vaccinia virus expressing an RSV protein, the immune response to an RSV challenge is characterized by an aberrant T_H2 response which results in eosinophil infiltration and severe lung damage (Walzl, et al., 2000). If, however, the mice were first primed by influenza virus, and then immunized with the vaccinia-RSV recombinant, the subsequent challenge with RSV leads to an altered immune response characterized by reduced T_H2 cytokine secretion and mild eosinophila. Thus, the cytokine milieu established during a prior infection can influence not only the nature of the memory pool but also alter the immunopathology caused by subsequent infections.

2. Heterologous immunity in humans:

An interesting example of how deviation of a heterologous memory response to a secondary dengue infection might affect clinical outcome is seen in a recent study which examined T_H1/T_H2 transcription factor expression along with viral load and cytokine profiles in DF and DHF patients from Taiwan (Chen, et al., 2005). While both DF and DHF patients had similar viral loads, the DHF patients had lower IFNγ levels, higher IL-10 levels, and significantly lower levels of T-bet mRNA as compared to DF patients suggesting that a strong anti-viral T_H1 response spear-headed by IFNγ can mitigate disease whereas a predominantly T_H2 response may result in increased viral load and

immunopathology. Other studies that have associated dengue immunopathology with T_H2 responses have reported elevated levels of TGFβ, IL-13, and IL-18 in DHF patients (Agarwal, et al., 1999; Mustafa, et al., 2001).

Similar to the previously discussed murine models of heterologous immunity mediated by memory responses to cross-reactive epitopes, the outcome of human infections can also be altered by pre-existing memory to unrelated viruses. Patients who develop severe acute HCV infections have unusually strong $CD8^+$ T cell responses to a HCV NS3 epitope that is cross-reactive with a sequence from the influenza virus neuraminidase protein, suggesting that a prior history of influenza infection could be a risk factor for severe hepatic immunopathology following HCV infection (Urbani, et al., 2005).

Given their ability to proliferate faster, quickly acquire effector functions and dominate a recall response, it is also conceivable that pre-existing cross-reactive T cells can provide protective immunity or enhanced resistance to heterologous viral infection. While clear-cut examples of this scenario and the functional mechanisms that define protective immunity are still under debate, some studies that show a correlation between infection or vaccination patterns and decreased disease severity outcomes suggest such a role for heterologous immunity. For example, individuals immunized with the live attenuated BCG vaccine have a lower risk of developing asthma (Shirakawa, et al., 1997). Immunization with BCG or vaccinia during childhood (another live, attenuated vaccine) has also shown to decrease the risk of developing melanoma later on (Krone, et al., 2005). Most recently, a statistical analysis of mortality and disease progression in HIV patients co-infected with the GBV C flavivirus showed increased survival in those

who had GBV-C infection late in the timeline of their HIV-related disease as compared to those with early co-infections (Zhang, et al., 2006).

These clinical observations of the immunomodulatory effects of heterologous viral infections imply that the design of vaccines against dengue will have to include testing for cross-reactive T cell responses and their adverse effects. The specific challenge that heterologous immunity poses to dengue vaccine design is discussed in the following section.

3. Implications for dengue vaccine design:

A successful anti-dengue vaccine should be able to elicit cross-protective T cell responses while also raising a high titer of neutralizing antibodies against all 4 serotypes. Clinical studies which tested live attenuated tetravalent vaccines in humans reported good seroconversion rates in both adults and children but did not resolve potential pitfalls such as uneven immunogenicity to all four serotypes (Rothman, et al., 2001; Kanesa-thasan, et al., 2001). Other groups have developed genetically modified infectious clones which use the non-structural regions from Yellow Fever 17D vaccine and the structural genes from DV (reviewed in Seligman and Gould, 2004). Although this ChimeriVax-Dengue vaccine induces a strong antibody response against the E protein, the T cell responses that could be induced by the potentially cross-reactive epitopes in the YF backbone have not yet been studied. Other recombinant viral vectors studied include replication-defective attenuated adenoviruses that have been shown to induce both neutralizing antibodies as well as T and B cell responses against D2V in mice (Jaisawal, et al., 2003). However, more work is required to demonstrate that such viral vector or DNA plasmid

immunization approaches can induce serotype cross-protective responses. The recent descriptions of 'original antigenic sin' among epitope-specific T cell populations from patients with heterologous dengue serotypes suggest that a cross-protective response may be mediated by high-avidity cross-reactive T cells since these cells are more proficient than low-affinity T cells at clearing the virus via cytotoxic mechanisms (Alexander-Miller, et al., 1996). However, high-affinity T cells may also exacerbate disease by secreting large quantities of pro-inflammatory cytokines. Therefore, the identification of cross-reactive T cell epitopes on DV that stimulate appropriate T cell functional sub-sets is an important goal for dengue vaccine design.

G. IMPACT OF NATURAL VARIANT EPITOPES ON T CELL RESPONSE.

The 4 serotypes of DV share ~65% - 75% amino acid sequence homology and this varies among the different regions of the polyprotein (Fu, et al., 1992). Therefore, the T cell epitopes recognized by serotype cross-reactive T cells have incomplete sequence homology. This thesis tested the hypothesis that these variant sequences acted as altered peptide ligands *in vitro* and induced functional heterogeneity in the peptide-specific T cell population.

1. T cell activation by altered peptide ligands (APL):

APLs were originally defined as synthetic variants of a native peptide that altered T cell responsiveness by selectively triggering some but not all T cell functions (reviewed in Sloan-Lancaster and Allen, 1996). Ligands which stimulate a TCR to the most optimal or fullest extent are agonists, to a lesser extent are partial agonists, and those that do not

activate the TCR or inhibit agonist-induced stimulation when presented at the same time are referred to as antagonists. TCR signals generated by APLs are both quantitatively as well as qualitatively different from those stimulated by the native peptide. Partial agonists induce changes in CD3ϵ and ζ chain phosphorylation and defects in ZAP-70 phosphorylation (Madrenas, et al., 1995). Consequently, stimulation with APLs results in altered functional outcomes characterized by selective elicitation of proliferation, cytokine production, and cytotoxicity (Jameson, et al., 1993; LaFace, et al., 1997). The extreme sensitivity of TCR to APL binding, its impact on downstream signaling and the eventual outcome is demonstrated by the evidence that APLs can distinguish between fas- and perforin-mediated killing, and can selectively trigger T cell apoptosis but not cytotoxicity (Cao, et al., 1995; Wei, et al., 1999).

Biochemical and structural studies of TCR-APL binding suggest several models for variant ligand function that fall into three main categories – kinetic, conformational, and stereochemical (reviewed in Madrenas and Germain, 1996). Since APL-bound MHC have different affinities for their TCR as compared to native pMHC, the kinetic model proposes that the activation of downstream events depends on the rate of dissociation of the TCR and the ligand. The conformational model takes into account the sensitivity of the TCR for the structure of the ligand and proposes that only some ligands can induce the allosteric change that is necessary for TCR signaling independent of association or disassociation constants. In contrast, the stereochemical model postulates that it is the arrangement of the various sub-units of the TCR-oriented signaling complex and not a conformational alteration of the TCR itself which dictates the strength and quality of downstream signaling.

2. Biological effects of APL signaling:

The engagement of TCR by APL and the ensuing alterations in T cell functions can have profound biological consequences. APL signaling has consequences during thymic development via the regulation of positive and negative selection. While high affinity agonists delete thymocytes, a lower concentration of the same ligands can result in positive selection (Alam, et al., 1996). For mature peripheral T cells, engagement by antagonists can cause anergy while stimulation by partial agonists can result in an alteration in cytokine secretion patterns and a rescue from disease phenotype. This is seen to good effect in the autoimmunity model of experimental autoimmune encephalitis (EAE) in mice following the adoptive transfer of T_H1 cells specific for a self-epitope. In contrast, the transfer of T_H2 cells specific for an APL of this same epitope suppresses EAE via the secretion of IL-4, IL-13, and suppressive cytokines such as IL-10 and TGFβ (Young, et al., 2000).

Studies of chronic viral infections such as HCV, HBV, and HIV show that T cell responses to natural viral epitope variants also influence the development of an efficient immune response (Klenerman, et al., 1994; Bertoletti, et al., 1994; Kaneko, et al., 1997). Mutations which generate antagonist or partial agonist ligands lead to the evasion of cytotoxic T cell responses *in vitro*, which could conceivably result in viral persistence and prolonged infection. The effects of APL on viral clearance *in vivo* were seen in a study by Klenerman and Zinkernagel (1998), in which, the challenge of LCMV immune mice with a variant strain bearing a single APL resulted in the generation of CTL that

were predominantly specific for the original epitope and the reduced clearance of the variant virus.

Very limited information is available on the biological effects of natural viral variants during acute infection in humans. In a study published by our group, the *in vitro* stimulation of dengue-immune PBMC generated a set of serotype cross-reactive epitope-specific T cells which were only partially activated by a variant epitope (Zivny, et al., 1999). Although there was a single amino acid difference between D2 and D3 variants of the NS3 71-79 peptide, a CTL clone isolated from a D3V immunized donor after *in vitro* D3V stimulation did not proliferate or produce IFNγ upon stimulation with the D2 peptide and only recognized this variant in CTL assays when peptide was added at a high concentration. This D2 peptide was not recognized in bulk culture CTL assays or in IFNγ ELISPOT assays of uncloned PBMC suggesting that the CTL specific for this epitope are present in very low frequencies and are of low-affinity. This also suggests that there is a correlation between the strength of the T cell activating signal and the responses that follow. The pattern of cytotoxicity < IFNγ secretion < proliferation ('<' = requiring less peptide) seen in this study has been reported by others (Evavold, et al., 1993; Hollsberg, et al., 1995).

One of the aims of this thesis, therefore, was to further explore the breadth of this functional heterogeneity by simultaneously analyzing for IFNγ, TNFα, and MIP-1β responses by flow cytometry following variant peptide stimulation.

H. CHARACTERISTICS OF HUMAN $CD8^+$ T CELL MEMORY.

1. <u>T cell differentiation in acute infection:</u>

Virus-specific T cells have been studied at different stages of a viral infection by assessing the expression of various phenotypic markers in relation to effector function in order to not only understand the physiology of the immune response but to also determine why the responses against certain viruses fail to be protective. In humans, the panel of well-researched expression markers which link surface phenotype to function includes the two CD45R isoforms (CD45RA and CD45RO), receptors involved in lymphocyte migration and homing (CCR7 and CD62L), co-stimulation (CD27 and CD28), and activation (CD69, CD38, and HLA-DR). During acute infection, activated $CD8^+$ T cells upregulate the expression of CD69 within days of antigenic contact, undergo robust expansion, and sustain high levels of CD38 and HLA-DR late into the infection (reviewed by Wherry and Ahmed, 2004). T cell activation also induces a shift from CD45RA expression such that most of the antigen-specific cells are now $CD45RO^+$. A majority of the activated cells also down-regulate their expression of CCR7 and CD62L thereby transitioning into the peripheral circulation. CD27 and CD28 are also expressed in the early acute stage. The effector functions of these cells are characterized by rapid expression of perforin, granzyme, and secretion of IFNγ. This proliferative phase wanes after about 2 weeks following the clearance of antigen and this is accompanied by the apoptosis of about 95% of the antigen-specific T cells.

The memory population is established by a small pool of precursor cells which were recently identified by their high expression of surface CD127 (IL-7Rα) and CCR7, and loss of CD38 expression in human acute HBV infections (Boettler, et al., 2006). Further heterogeneity within the memory population is defined by changes in the expression of CD45R isoforms, CD62L, CCR7, CD27 and CD28. There are two main

models of memory cell differentiation based on studies of HIV, EBV, HCV, and CMV infections in humans. In the first model, antigen-specific $CD8^+$ T cells differentiate from naïve T cells into $CD45RA^-CD62L^+CCR7^+$ 'central memory' (T_{CM}) cells, which are lymph-node homing cells that lack perforin and other effector functions but show a high proliferative potential upon antigenic restimulation (Sallusto, et al., 1999; Champagne, et al., 2001). In contrast, $CD45RA^-CD62L^-CCR7^-$ cells are tissue-homing 'effector memory' (T_{EM}) cells. A further distinction within this subset is defined by the expression of CD45RA and perforin (T_{EMRA}) in cells that are terminally differentiated. In the second model, naïve T cells differentiate into $CD27^+CD28^+$ (early), $CD27^+CD28^-$ (intermediate), and $CD27^-CD28^-$ (late) memory T cells (Appay, et al., 2002).

2. Functional correlates of protective immunity:

Long-term persistence of immunological protection depends on both the size and quality of the memory T cell pool. Much information on the potential of various phenotypic sub-sets to contribute to protective immunity has been gleaned from the HIV infection model, particularly from studies that compare immune responses between HIV^+ patients with progressive disease and HIV^+ long term non-progressors (LTNP). The earliest studies suggested that a high frequency of antigen-specific cells and a broadly cross-reactive $CD8^+$ T cell response targeted at multiple viral targets correlated with better control of viral replication and slower disease progression (Harrer, et al., 1996; Ogg, et al., 1998). However, later studies which tracked $tetramer^+$ T cell functions over a period of time revealed that while the frequency of antigen-specific $CD8^+$ T cells remained high as measured in functional assays, there was a decline in the number of

tetramer$^+$ cells that secreted IFNγ, TNFα, and chemokines such as CCL4 and CCL5 in patients with progressive disease (Kostense, et al, 2001; Scott-Algara, et al., 2005). The importance of a diverse functional response has been further demonstrated in a recent study which simultaneously analyzed HIV-specific CD8$^+$ T cells for CD107a expression (a marker for degranulation) and secretion of IFNγ, TNFα, IL-2, and MIP-1β (Betts, et al., 2006). The LTNPs consistently showed a broad functional profile characterized by a high frequency of cells that could perform all 5 functions whereas the immune response in the progressors was characterized by the low frequency or complete absence of multi-functional subsets. There is some evidence that, in addition to the polyfunctionality of the memory T cells, a high frequency of a specific T_{CM} phenotype characterized by the consistent expression of CD127/IL-7Rα can also contribute to protective immunity (Paiardini, et al., 2005). In comparison with CD8$^+$ T cells specific for viruses such as vaccinia, HBV, and influenza, which are quickly cleared, and EBV, which causes a latent infection, the expression of CD127 is significantly reduced in CD8$^+$ T cells in chronic HIV infection (Boettler, et al., 2006; Wherry, et al., 2006). Furthermore, the emergence of CD127 expression on antigen-specific CD8$^+$ memory cells correlated with enhanced proliferative capacity, decreased disease markers and viral clearance in acute HBV infection. IL-7R mediated signaling is mainly a homeostatic mechanism to support the survival of memory T cells and prevent them from undergoing apoptosis. This has been convincingly demonstrated in a murine model of LCMV infection in which the kinetics of IL-7R expression inversely correlated with the level of apoptosis in antigen-specific cells (Kaech, et al., 2003).

Another interesting correlation seen in acute LCMV infection in mice and acute HBV infection in humans is the expression of CD127 and PD-1, the receptor for programmed death ligand -1 (PD-L1). PD-1, a member of the CD28 co-stimulatory receptor family, negatively regulates T cell activation, proliferation, and cytokine production by inhibiting TCR signaling, and sensitizes T cells to apoptosis (reviewed by Zha, et al., 2004). Inhibition of the PD-1 signal restores functions in persistently stimulated 'stunned' or exhausted T cells as has been demonstrated *in vivo* in a chronic LCMV infection model (Barber, et al., 2006). Very recently, three different studies of PD-1 expression on $CD8^+$ T cells in chronic HIV infection in humans showed that a higher frequency of PD-1^+ cells were found within the HIV-specific population as compared to populations specific for acute viruses or well-controlled persistent viruses within the same donor, and the cells with the highest levels of PD-1 expression were the most sensitive to apoptosis (Day, et al., 2006; Trautmann, et al., 2006; Petrovas, et al., 2006). Additionally, PD-1 expression correlated with decreased production of TNFα, and IL-2 but not IFNγ, suggesting that PD-1 contributes to HIV disease progression not only by impeding the survival of the virus-specific memory T cells but also altering their anti-viral functions.

We and others have observed that dengue-specific T cells have an impaired functional phenotype early during acute infection which is characterized by the inability of these cells to produce IFNγ after *in vitro* antigenic stimulation and to proliferate in culture (Mathew, et al., 1999; Mongkolsapaya, et al., 2003). We therefore included PD-1 in our phenotypic analysis of tetramer-specific T cells from dengue patient samples.

I. THESIS OBJECTIVES

Early and enhanced immune activation are two important hallmarks of disease severity seen in DHF patients. Serotype cross-reactive memory T cells found in dengue-immune PBMC are likely to play a role in DHF immunopathology by getting re-activated during heterologous dengue infection and undergoing preferential expansion as compared to cells newly primed by the second serotype. The incomplete sequence homology between the 4 dengue serotypes means that differences in the sequences of cross-reactive T cell epitopes can result in altered activation profiles among virus-specific memory T cells.

Hence, our hypothesis was that (a) the stimulation of serotype cross-reactive T cells in dengue-immune PBMC with natural epitope variants would result in a qualitatively and qualitatively altered *in vitro* response, and (b) distinct phenotypic and functional subsets may contribute to DHF immunopathology. This work is presented in three parts:

Chapter III: **a.** Identification of HLA-A*02-restricted T cell epitopes

b. Frequencies of epitope-specific cross-reactive T cells in 4 donors

c. IFNγ, TNFα, and MIP-1β co-expression and analysis of quantitative and qualitative changes following stimulation with variant epitopes.

d. Sequential stimulation of dengue immune PBMC with variant epitopes to directly examine functional cross-reactivity

Chapter IV: **a.** Comparison of epitope-specific frequencies in patient PBMC during acute illness and convalescence

b. Phenotypic comparisons of tetramer$^+$ cells between DF and DHF patients

c. Serotype cross-reactivity in the tetramer$^+$ T cell population

Chapter V: Analysis of patterns of cross-reactivity in T cell clones following APL stimulation:

a. Changes in cytokine production

b. Changes in sensitivity in cytolysis without alterations of other functions

c. Alterations in sensitivity to tetramer binding.

CHAPTER II

MATERIALS AND METHODS

A. Experimental vaccine strains and human subjects of vaccination.

The 4 donors included in the post-vaccine immune response studies were subcutaneously immunized with undiluted live attenuated monovalent vaccines of the various serotypes as shown in Table I. Infection with dengue was confirmed by antibody titers and virus isolation. A flavivirus naïve Massachusetts resident was also included in the study. The D1 and D3 vaccinee developed symptoms compatible with dengue fever (Innis, et al., 1988; Gagnon, et al., 1996). PBMC were isolated by Ficoll-Hypaque and density gradient centrifugation. They were resuspended at 10^7/ml in RPMI 1640 containing 20% FBS and 10% DMSO and cryopreserved until use. HLA typing of these donors done at the tissue typing center at the University of Massachusetts Medical Center (Worcester, MA) identified all the donors as HLA-A*02^+.

B. Peptides

The amino acid sequence of serotype 3 H87 strain, (GenBank accession no. NC 001475) was initially chosen to generate a list of nonamer epitopes based on the peptide binding motif for HLA-A*0201 using the epitope prediction algorithm (available online at ⟨http://bimas.dcrt.nih.gov/molbio/hla_bind⟩). Twenty-five sequences with the highest binding scores were chosen, and the peptides were synthesized at the University of Massachusetts Medical School Peptide Core Facility. The four identified epitopes were identical in D3 CH53489 (GenBank accession no. AF017733, nonstructural; and

Table I. *Vaccination history and class I HLA alleles of study subjects*

Donors	Serotype and Vaccine Strain	Months after Vaccination PBMC Obtained	Class I HLA Type		
			A	B	C
1	D1 45AZ5	4, 8	2,11	27,60	1,3
2	D3 CH5348	168, 192	2,24	7,62	3,7
3	D2 16681	12	2,29	44	5
4	D2 16681	12	2,3	7,44	5,7

GenBank accession no. M86733, structural). Variant peptides were synthesized based on the D1 45AZ5 (GenBank accession no. NC_001477), D2 NGC (GenBank accession no. M29095), and D4 814669 (GenBank accession no. AF326573).

C. IFNγ ELIspot assay.

Multiscreen-IP filter plates (Millipore, Billerica, MA) were coated with primary anti-IFNγ mAb (1D1K) at 10μg/ml in sterile PBS and incubated overnight at 4^0C. The plates were then washed in PBS and blocked with 10% FCS/RPMI for 2 hr at 37^0C. PBMC were thawed and washed with RPMI 1640 containing 10% heat-inactivated human AB serum. 3 x 10^5 cells were added to each well at 50μl/well. Peptides were added at 10μg/ml in a volume of 50μl/well. Each stimulation was done in triplicate. The plates were incubated for 16-20 hr at 37^0C. Cells were then removed and the plates were washed thrice with PBS and 0.05% Tween-PBS. 100 μl of the secondary biotinylated anti-IFNγ mAb (7-B6-1) at 4μg/ml was added to the plates which were then left at room temperature for 2 hr. Plates were then washed and incubated with 1:400 dilution of horseradish streptavidin peroxidase at 100μl/well at room temperature for 45 min. Plates were washed and incubated with 200μl/well of the filtered substrate (1AEC tablet was dissolved in 2ml of DMF; the solution was diluted at 1:100 in NaAc solution) for 10 min at room temperature. Plates were then washed and left to air-dry before being read manually. The number of spots in negative control wells were averaged and subtracted from the average number of spots in the other wells.

D. Bulk culture of PBMC.

PBMC were thawed, and washed in 10% FCS/RPMI. 3 x 10^6 cells were stimulated with either homologous virus (m.o.i = 1) or peptide 10μg/ml and cultured in 24 well plates in 10% FCS/AIM-V. The bulk cultures were tested in CTL assays between days 7-10 after stimulation. If required, the cells were re-stimulated on day 14 with gamma irradiated autologous PBMC in 1ml of fresh medium containing 10U/ml IL-2, 0.1 μg/ml anti-CD3 mAb 12F6, and either virus or peptide at m.o.i=1 or 10μg/ml respectively. Bulk cultures were re-stimulated every two weeks after that with allogeneic feeders, IL-2 and anti-CD3.

E. Cloning of PBMC and maintenance of T cell lines

PBMC which had been stimulated in bulk culture for 14 days were plated at concentrations of 1, 3, 10, and 30 cell/well in 96-well round bottom plates in 50μl of 10%FCS/AIM-V media containing 10U/ml IL-2, 0.1 μg/ml anti-CD3 mAb 12F6, and 10^5 allogeneic irradiated feeder cells. The wells were fed with fresh media containing IL-2 every 3 days for 2 weeks before being screened in CTL assays against target cells pulsed with the peptide used in the initial stimulation of the bulk culture. The cells from the positive wells were collected and transferred into 48 well plates and ultimately expanded into 24 and 6 well plates with regular 2 week re-stimulations.

In some cases, peptide-stimulated bulk culture cells were first stained with tetramers for 20min at room temperature, washed and stained with surface mAbs for CD3 and CD8 before being sorted using FACSAria. Tetramer$^+$ cells were collected in T cell

media and sorted into 96 well plates at 1 and 3 cells/well and maintained as previously described.

F. CTL assays.

Autologous BLCLs were used as the antigen-presenting target cells. They were either infected overnight with recombinant vaccinia viruses expressing dengue virus proteins or were pulsed with peptide at the start of the CTL assay. Cells were then washed and labeled with 0.25mCi of ^{51}Cr (Dupont NEN, Boston, MA) for 1 hr at 37^0C. Cells were washed thrice to remove unincorporated ^{51}Cr, counted, and distributed into 96 well plates at 1500 cells/well and at an effector:target ratio of 100:1 for bulk culture CTL and 10:1 for CTL of cloned T cells. Uninfected targets were incubated with 10μg/ml of peptide for 30min at 37^0C before the addition of effector T cells. Plates were then incubated for 4 hr at 37^0C. The plates were then harvested for supernatant using the Skatron collection system and ^{51}Cr content was measured in a gamma counter. The percent specific ^{51}Cr was calculated as follows: (cpm experimental release – cpm spontaneous release)/(cpm maximum release – cpm spontaneous release) x 100. Maximum values were obtained by incubating target cells with Renex detergent and minimum values were obtained by incubating target cells with media. All assays were performed in triplicate and the values were averaged.

G. Intracellular staining and flow cytometry.

PBMC were thawed and washed with RPMI 1640 containing 10% heat-inactivated human AB serum. Except in the peptide titration experiments where the

concentration varied, cells (10^6) were stimulated with 10 μg/ml peptide and incubated at 37°C for a total of 6 h with Golgi Plug (Brefeldin A; BD Pharmingen) added during the last 5 h. PBMC were washed with FACS buffer (2% FBS and 0.1% sodium azide in PBS) and incubated with 50 μg/ml mouse IgG for 15 min at 4°C to block nonspecific binding to IgG Fc receptors. Cells were then stained for surface markers such as CD3 (PE-Cy7 Ab; Caltag Laboratories) and CD8 (PerCP Ab; BD Biosciences) at 4°C for 30 min. After washing with FACS buffer, cells were fixed and permeabilized with the Cytofix/Cytoperm reagent (BD Pharmingen) for 20 min at 4°C. This was followed by a wash in PermWash buffer (BD Pharmingen) and intracellular staining with Abs for CD69 (PE), IFN-γ (FITC), TNF-α (allophycocyanin), MIP-1β (PE) at 4°C for 30 min. All Abs were obtained from BD Pharmingen. Data were acquired on a FACSAria machine (BD Biosciences) and analyzed using versions 4.2, 4.5, or 6.1 of FlowJo software (Tree Star). In the experiments where the PBMC were co-stained with more than one cytokine antibody, fluorescence compensation was attained by using single-color control samples. The specificity of the co-stain was checked during data acquisition in order to ensure that it was not due the bleeding out of one fluorochrome into another channel. The lymphocyte gate included side-scatter and forward-scatter low populations that were selected for $CD3^+$ and $CD8^+$ cells. The number of events collected for each sample varied between 150,000 and 250,000, depending on the donor. The number of $CD3^+CD8^+$ cells analyzed in each of the donors are as follows: donor 1, 20,000 ± 2,000; donor 2, 27,000 ± 2,000; donor 3, 44,000 ± 3,000; donor 4, 60,000 ± 3,000. Each experiment included isotype controls for each cytokine-Ab conjugate. Based on these, gates were first set on

the medium-stimulated panel, and the same gating was then applied to all other samples within each donor.

H. CFSE staining and sequential stimulation of PBMC

PBMC were thawed and resuspended in RPMI 1640 medium at 10 x 10^6/ml. Cells were stained with CFSE at 1 μg/ml for 25 min at 37°C in the dark. Cells were washed three times with cold RPMI 1640 medium containing 10% FBS, stimulated with 10 μg/ml homologous peptide and 25ng/ml IL-7, and incubated in the dark. IL-2 (50 U/ml) was added on day 3. A total of 3 x 10^5 bulk culture cells were mixed with 7 x 10^5 autologous BLCLs. They were restimulated with 10 μg/ml of either homologous or heterologous peptides on day 5 and day 14, or left unstimulated for 6 hrs. Cells were then washed and stained for surface markers (CD3 and CD8) and intracellular cytokines (IFN--allophycocyanin, TNF--AlexaFluor 610, MIP-1-PE) as previously described.

I. Study design and samples from patients in Thailand.

The study design has been reported in detail elsewhere (Mathew, et al., 1998). Briefly, the subjects enrolled in the study were Thai children 6 mo-14 of age with acute febrile illness and were diagnosed with DF or DHF according to WHO guidelines. Serology and virus isolation from acute phase plasma were used to confirm acute DV infections, and primary and secondary infections were distinguished based on serologic response. Blood samples were obtained during acute illness (0 -8 days after study entry), early convalescence (8-11 days after study entry), and late convalescence (6 mo – 3 years after study entry). PBMC were isolated by density gradient centrifugation, cryopreserved,

and stored at -70°C. 10 HLA-B*07^{+} subjects were identified based on serologic HLA class I typing on fresh blood obtained 6 mo or more after acute illness (22). Frozen PBMC were shipped on dry ice to the University of Massachusetts Medical School (Worcester, MA) for analysis.

J. Generation of HLA-B*07 tetramer

The HLA class I tetramer with HLA-B*07 restricted D2/3/4 NS3 222-231 peptide (APTRVVAAEM) was made as previously described at the Peptide Core Facility of the University of Massachusetts Medical School (25). Refolded monomer was mixed with streptavidin-phycoerythrin conjugate (BD Pharmingen, San Diego, CA) at a molar ratio of 4:1 to form tetramers.

K. HLA-peptide tetramer staining and phenotypic characterization of patient PBMC

Cryopreserved patient PBMC were thawed and washed in RPMI medium containing 50% FCS. 3 x 10^5 cells were incubated with 0.5 μg tetramer for 20 min at room temperature. Cells were washed twice, left on ice for 5 min, and then stained with mAbs for cell surface antigens using PerCp-conjugated anti-CD8 (BD Biosciences), APC-conjugated anti-CD3, and FITC-conjugated anti-CD69, anti-CD38, anti-HLA-DR, anti-CCR7, and anti-CD62L (BD Pharmingen) for 30min on ice. Some samples were also stained with anti-CD27, anti-CD28, anti-CD45RA, anti-CD45RO, and anti-PD-1 depending on the number of cells recovered after the thawing procedure. Isotope control IgG-FITC was included for each patient at each time point. Cells were washed twice and fixed with 1%

Paraformaldehyde before being analyzed by FACS. Data were acquired on a BD FacsCalibur Flow Cytometer. 150,000 – 200,000 events were collected for each sample. The data were analysed using FlowJo version 6.1.1 software (Tree Star). The cells were gated through CD3 and CD8.

L. Tetramer and intracellular staining of T cell clones.

T cell clones were removed from cultures between 7 – 10 days after re-stimulation and washed with 10% FCS/RPMI. 3 x 10^5 T cells were co-cultured with 7 x 10^5 autologous BLCL and 10µg/ml peptide in 1ml media for 6 hr with GolgiStop or GolgiPlug added for the last 5 hr. When staining for CD107a, 20ul of PE-anti-CD107a mAb was added at the beginning of the assay. Cells were washed and stained with tetramer for 30 min at 4^0C and then washed. The remainder of the protocol for staining for intracellular cytokines is as described previously.

CHAPTER III

IDENTIFICATION OF HLA-A*02-RESTRICTED CD8$^+$ T CELL EPITOPES AND THE QUANTITATIVE AND QUALITATIVE ALTERATION OF CROSS-REACTIVE T CELL RESPONSES BY THEIR HETEROLOGOUS VARIANTS

The selective expansion of cross-reactive CD8$^+$ T cells present in the antigen-specific memory repertoire during immune response to a secondary dengue viral infection has been previously demonstrated (Mathew et al., 1998; Mongkolsapaya et al., 2003; Mongkolsapaya, et al., 2006). While dengue-specific CD8$^+$ T cell epitopes have been identified in both structural and non-structural viral proteins, cross-reactivity between serotypes is more common among the latter, since they are more highly conserved (Kurane et al., 1991 and 1998; Lobigs et al., 1994; Zivny et al., 1995). With the exception of two recently isolated class I-restricted T cell epitopes, none of the others are presented by HLA alleles which are predominantly found among humans living in high-risk areas. In order to identify CD8$^+$ T cell epitopes presented by common HLA alleles and to study the effect of variant epitope sequences on T cell cross-reactivity, we used an epitope prediction algorithm to generate two lists of the 25 9-mer peptides which had the highest binding scores for the HLA-A*02 allele, based on the D2 and D3 serotypes. Twelve of these peptides appeared on both lists, albeit with different binding scores, suggesting that these could be cross-reactive T cell epitopes (Table II). We tested these peptides in IFNγ ELISPOT assays using PBMC from donors who had received a single experimental mono-valent dengue vaccine corresponding to the D1, 2, and 3 serotypes (Table I; Chapter II).

Table II: Predicted HLA-A*0201-restricted 9-mer epitopes common to D3 and D2 serotypes

D3 sequences

Peptide[a]	Sequence	Score[b]
2815	KLLTKPWDV	6755
851	ILWENDIKL	1235
1161	VLFTFVLLL	1174
2201	IVLEFFMMV	1133
2424	LLMRTSWAL	1007
2413	VMLLVLCAV	726
2965	YMWLGARYL	647
49	LVMAFIAFLL	565
2148[c]	**LLLGLMILL**	**309**
1907	ILTDGPERV	237
2353	**VLLVTHYA**	**171**
2423	**LLLMRTSWA**	**171**

D2 sequences

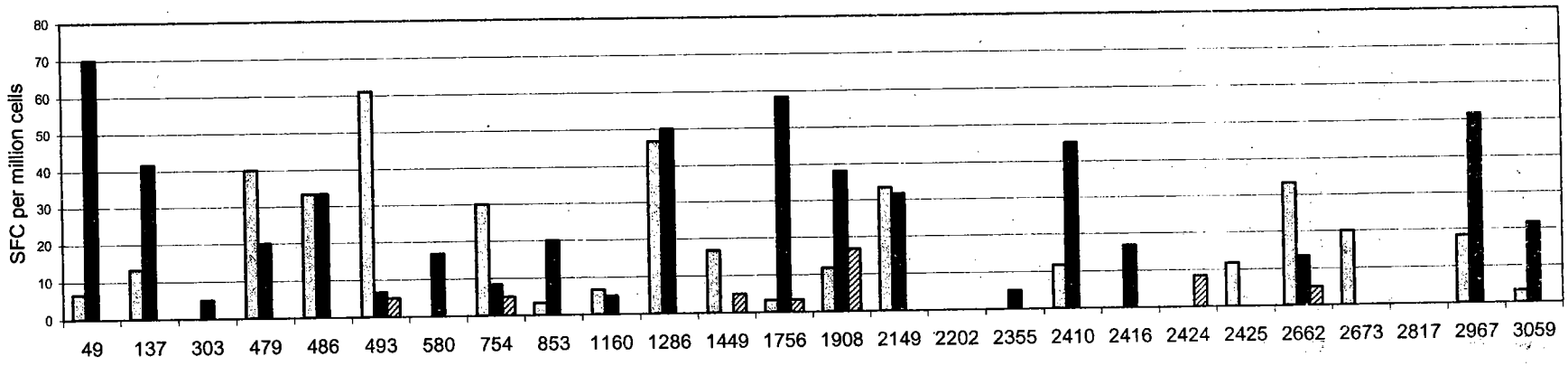
Peptide	Sequence	Score
2817	RLLTKPWDV	1930
853	ILSENEVKL	148
1161	ILLVAVSFV	5534
2202	IILEFFLIV	730
2425	LMMRTTWAL	727
2416	LLVLCLTQV	271
2967	YMWLGARFL	647
49	ALVAFLRFL	205
2149	**LLLTLLATV**	**1006**
1908	ILTGDEERV	237
2355	**FLLVAHYAI**	**177**
2424	**VLMMRTTWA**	**171**

[a] The peptide numbers refer to the starting amino acid position of the epitope in the dengue genome

[b] The binding score refers to the half-time dissociation of peptide-HLA complexes and are arranged in the decreasing order for the D3 sequences

[c] The sequences in bold show the 9-mers that were recognized by dengue-immune PBMC in ICS assays.

A.



B.

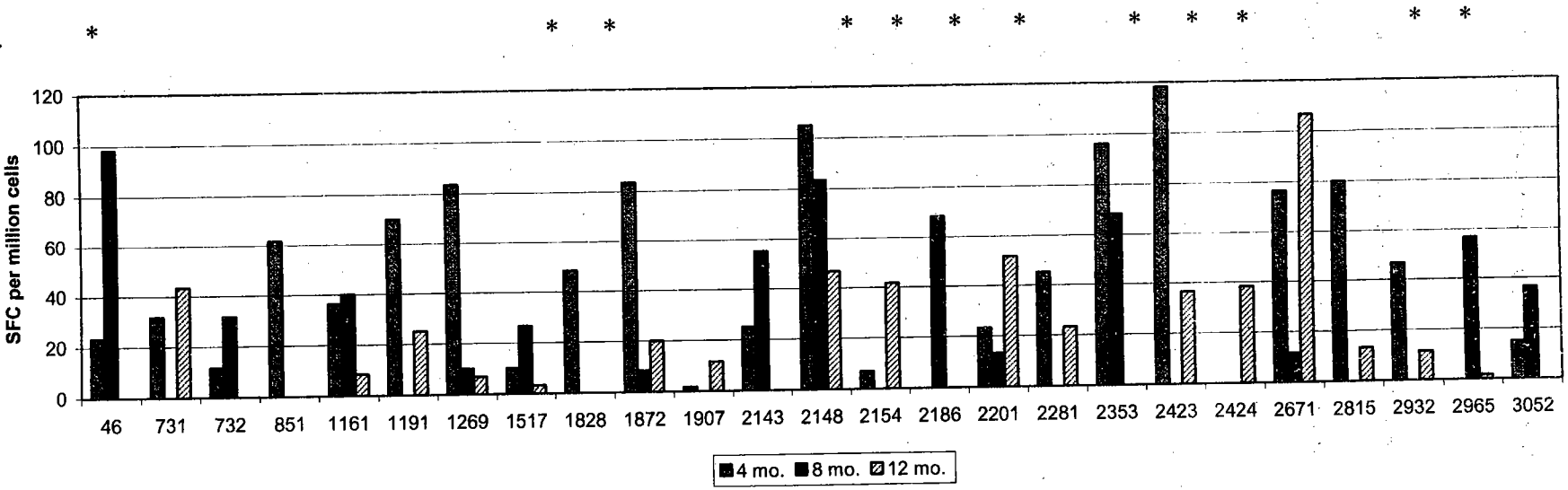


Figure 2: Screening of responses to predicted HLA-A*02 epitopes by ELISPOT. Cryopreserved PBMC taken from a D1 vaccinee at 4, 8, and 12 months post-vaccination were thawed and stimulated with 4 ug/ml of (A), D2-sequence based peptides, or (B), D3-sequence peptides for16 hrs in triplicate. The number of spots counted in the media control was then subtracted from the averages. Peptides indicated by (*) in the D2 panel are present in the D3 panel with altered sequences. SFC = Spot forming cells

A. IFNγ ELISPOT and CTL responses to D2 and D3 peptides in D1 vaccinated donor.

The D2 and the D3 peptides were first tested against donors vaccinated with D2 and D3 serotype vaccines. No IFNγ producing cells were detected in either of the D2 donors against any of the peptides and only the D3 NS4b 2423 peptide induced a precursor frequency of 17 $IFN\gamma^+$ spots/million cells in the D3 vaccinated donor. Since the PBMC from the D2 donors and D3 donor were taken 2 years and 16 years post-vaccination respectively, we next examined cross-reactive responses against the D2 and D3 peptides in a D1 vaccinee from whom PBMC were available as early as 4 months post-vaccination. The IFNγ responses against the D3 peptides were in general higher than those against the D2 peptides (Figure 2A, 2B). This may be due to the greater homology in sequence between D1 and D3 serotypes. There were no responses detected against any of the D2 peptides in PBMC obtained at 1 year, suggesting a loss of cross-reactive cells over time. The specificity of the response also changed over time with different peptides dominating the response at 4 months as compared to the other time points. Of the 4 D2 peptides and 10 D3 peptides that induced more than 40 $IFN\gamma^+$ spots/million cells in the 4 month bleed, CTL responses against only the D2 493 peptide (34%) and the D3 2353 peptide (26%) were detectable in 7 day old bulk cultures stimulated with the homologous D1 virus (Figure 3). The recognition of these two sequences at the bulk culture level might be attributable to the fact that the amino acid sequences between the D1 (homologous) serotype and the heterologous D2 or D3 variants differ by one (493) or two (2353) residues.

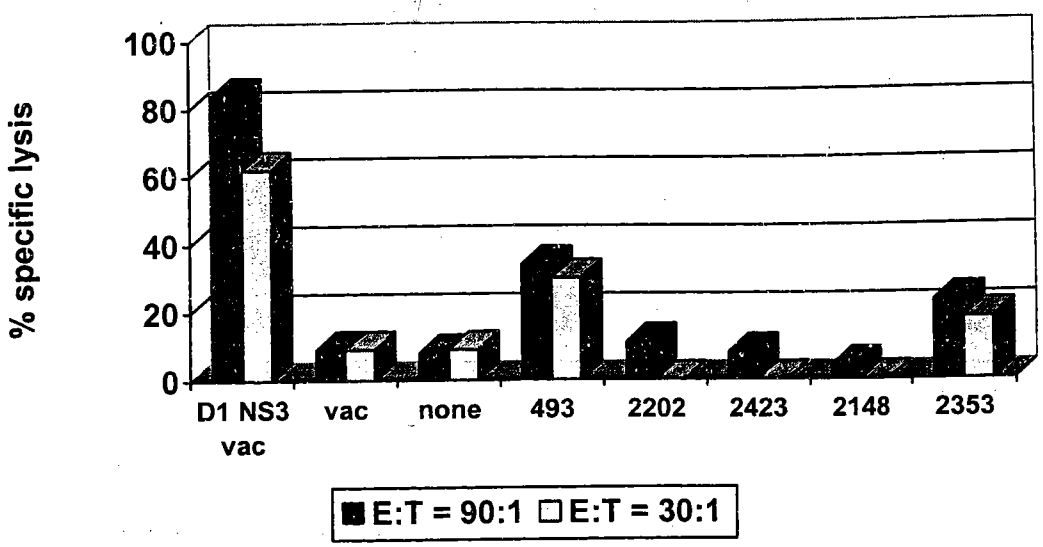


Figure 3: Specific lysis of peptide-pulsed autologous targets. D1 immune PBMC were stimulated with D1V at m.o.i of 1. After 7 days, the bulk culture (effectors) was tested for virus-specfic CTL activity against autologous BLCL (targets) which were uninfected, or infected overnight with recombinant vaccinia expressing D1 NS3, or which were pulsed with 25μg/ml peptide for 30 min before the assay. % specific lysis was calculated as described in Chapter II, Materials and methods.

Table III: Frequencies of IFNγ-secreting epitope-specific $CD8^+$ T cells in PBMC of dengue-immune subjects

Start position	Protein	Serotype	Sequence	Score[a]	% IFNγ producing $CD69^+$ cells[b]			
					1[c]	2	3	4
2353	NS4b	D3	VLLLVTHYA	171	0.64±0.05[d]	0.45±0.03	0.15[e]	0.58
2423	NS4b	D3	LLLMRTSWA	171	0.47±0.02	0.34±0.05	0.18	0.19
2148	NS4a	D3	LLLGLMILL	309	0.35±0.04	0.24±0.04	0.17	0.12
493	E	D2	FLDLPLPWL	497	0.19±0.03	0.20±0.03	0.10	0.13

[a] Scores indicate the predicted half-time of dissociation from class I HLA molecules
[b] PBMC were first gated on $CD3^+CD8^+$ lymphocytes
[c] Donors (Table 1)
[d] Data is representative of one of several independent experiments
[e] Donors 3 and 4 were only tested once due to limiting cell numbers

B. Responses to predicted epitopes by intracellular staining for IFNγ and TNFα:

Since the responses detected using IFNγ ELISPOT were low in magnitude, we continued our analyses using a flow cytometry-based approach to simultaneously detect the secretion of other cytokines and the expression of the activation marker CD69. We first studied PBMC from the D3-immune HLA-A*02$^+$ donor. Cells were stimulated with 10ug/ml of peptide. Frequencies of more than 0.1% of CD69$^+$ IFNγ producing cells were detected in the CD3$^+$ CD8$^+$ T cell population in response to stimulation by 4 of the 25 peptides (Figure4A). The highest response was to the NS4b 2353 peptide (0.45% CD69$^+$ IFNγ$^+$ cells), which had also stimulated high responses in the D1 donor. The frequency of cells specific for NS4b 2353, NS4b 2423, and NS4a 2148 were higher than the frequency of cells specific for two previously identified HLA-B*62 epitopes in this donor (Zivny et al., 1999). Three of the 4 peptides stimulated responses of more than 0.1% even at 0.1 ug/ml (Figure 4C). Responses to these four peptides were also detected in the 3 other donors who were vaccinated with other dengue serotypes (Table III). No responses were detected in a HLA-A*02 negative dengue-immune donor or in a HLA-A*02 positive flavi-naïve donor (data not shown). In the D3-immune donor, all 4 peptides induced a TNFα response which was higher than the corresponding IFNγ response, suggesting that the epitope-specific population was comprised of sub-populations of cells which secreted TNFα but not IFNγ. In addition to IFNγ, TNFα and CD69 up-regulation, 3 of the 4 peptides also induced proliferation responses as measured by CFSE dilution (Figure 4B). Cells that underwent more than 6 divisions over 5 days ranged from 5% to 16% of CD3$^+$ CD8$^+$ T cells remaining in the culture with the highest proliferation being in response to the D3 2423 peptide.

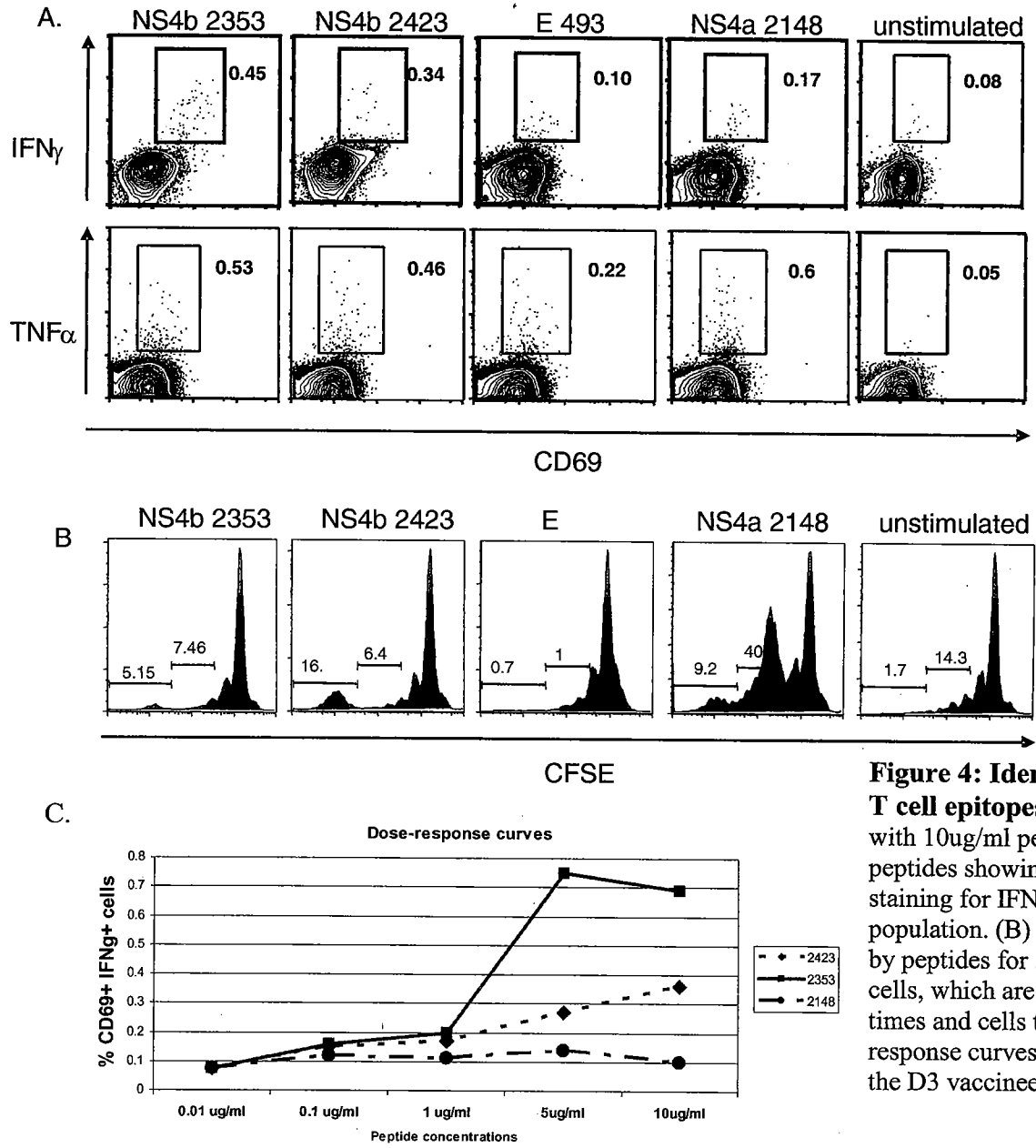


Figure 4: Identification of four novel HLA-A*0201-restricted T cell epitopes. PBMC from D3 vaccinee were stimulated for 6 h with 10ug/ml peptide and analysed by FACS. 4 of the 25 tested peptides showing positive responses are shown in (A) intracellular staining for IFN-γ, TNF-α, and CD69 in the $CD3^+CD8^+$ lymphocyte population. (B) PBMC were stained with 1μM CFSE and stimulated by peptides for 5 days. Panels represent cell division in $CD3^+CD8^+$ T cells, which are further gated into cells that have divided two to five times and cells that have undergone more than five divisions. (C) Dose response curves for 3 of the peptides were measured in PBMC from the D3 vaccinee (donor 2).

C. Analysis of IFNγ, TNFα, and MIP-1β co-expression following heterologous stimulation by variant sequences of the NS4b 2353 epitope

Because of variation in amino acid sequences between the four dengue serotypes (Table IV) and because the evidence from initial studies that showed that vaccinated individuals responded to heterologous serotypes, we next investigated the range of cytokine production within each epitope-specific T cell population and determined whether these expression profiles were skewed following stimulation with variant sequences of these epitopes from heterologous serotypes. Of the four epitopes, the E493 and the NS4b 2423 epitopes are highly conserved among all four serotypes whereas the NS4a 2148 and NS4b 2353 epitopes are less conserved (Table IV).

We were interested in examining TNFα and MIP-1β responses to the epitopes since high levels of both these inflammatory factors are produced during dengue infection (Cardier, et al., 2005; Spain-Santana, et al., 2001). We hypothesized that variant peptides might skew the response to a TNFα/MIP-1β profile. Responses to NS4b 2353, which was the most immunogenic epitope in all four donors, are shown in figure 4. PBMC were stimulated with each of the peptide variants and double stained for IFNγ/TNFα (Figure 5A), and IFNγ/MIP-1β (Figure 5B). In 3 of the 4 donors, the highest total cytokine responses were stimulated by the heterologous variants of this epitope. The highest response to the homologous peptide was seen only in the donor who had been immunized 16 years prior to obtaining the PBMC, suggesting that the pattern in this donor could be due to the attrition of cross-reactive T cells over time. The cytokine response in each donor was composed of $IFN\gamma^+$, $TNF\alpha^+$, $IFN\gamma^+TNF\alpha^+$, and $IFN\gamma^+MIP\text{-}1\beta^+$ cells.

Table IV: Sequences of epitopes and their heterologous variants

Start position	Protein (residues)	Serotype	Sequence
2353	NS4b (111[a]-119)	D1	V L M L V A H Y A
		D2	F L L V A H Y A I
		D3	V L L L V T H Y A
		D4	L V M L L V H Y A
2423	NS4b (181-189)	D1	I L L M R T T W A
		D2	V L L M R T T W A
		D3	L L L M R T S W A
		D4	L L L M R T T W A
2148	NS4a (56-64)	D1	M L L A L I A V L
		D2	L L L T L L A T V
		D3	L L L G L M I L L
		D4	M L V A L L G A M
493	E (211-219)	D1	F L D L P L P W T
		D2	F L D L P L P W L
		D3	F F D L P L P W T
		D4	F F D L P L P W L

[a]Numbers indicate the position of the epitope within each viral protein based on the dengue 3 serotype

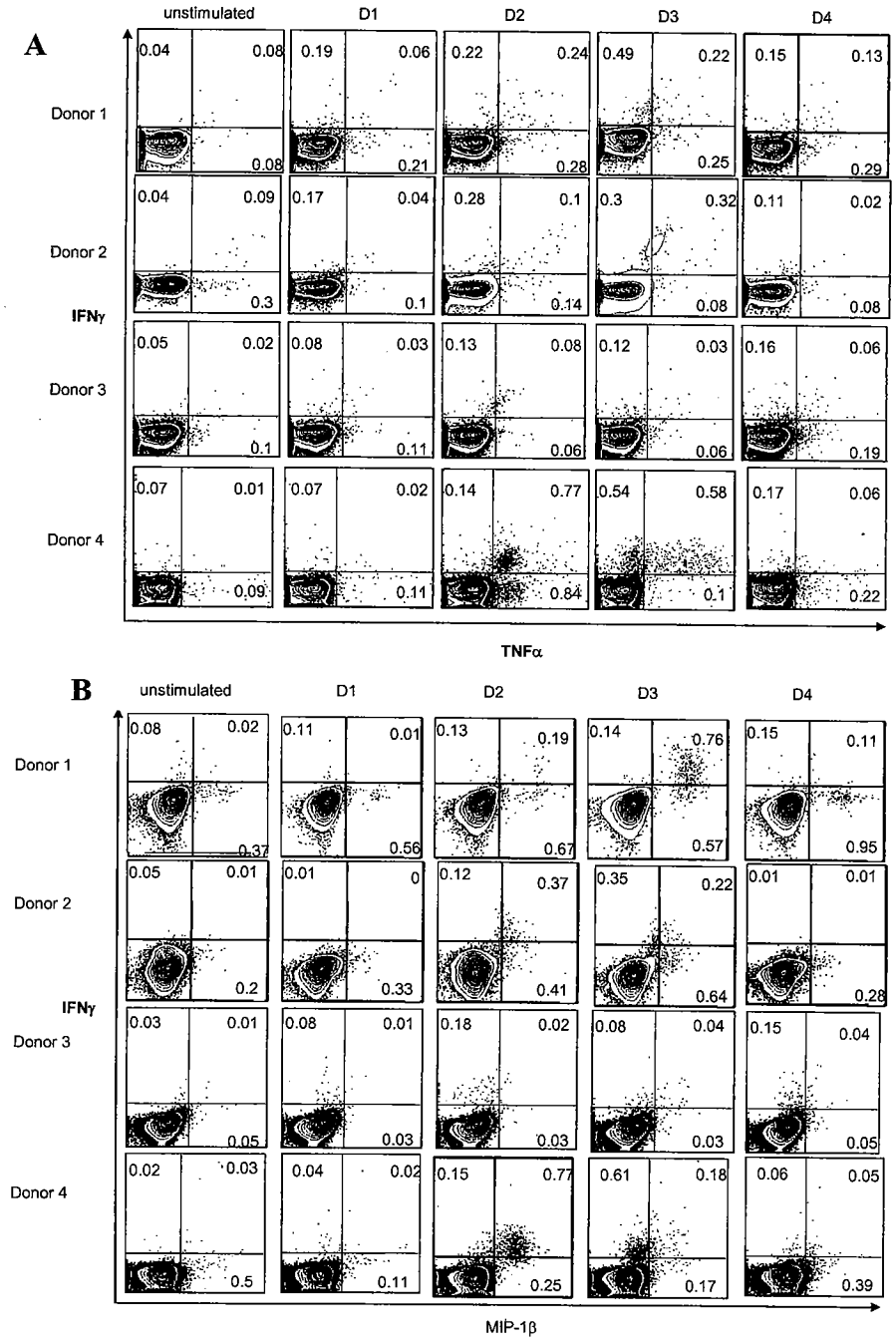


Figure 5: Heterogeneous cytokine expression following stimulation with variant sequences of NS4b 2353. A. PBMC from all four donors were stimulated with all four variants of the NS4b 2353 epitope for 6 h. Contour plots show simultaneous intracellular staining for IFNγ and TNFα in the $CD3^{+}CD8^{+}$ population. B. Double intracellular staining for IFNγ and MIP-1β.

The relative sizes of these subsets varied depending on both the donor as well as the serotype of the stimulating peptide. In 3 of the 4 donors, the frequencies of MIP-1β^+ cells were higher than the corresponding frequencies of IFNγ^+ and TNFα^+ cells. In the same 3 donors, the D2 and D3 variants induced the highest frequencies of IFNγ/TNFα and IFNγ/MIP-1β cells. Other changes were seen in the hierarchy of the various subsets, with different variants skewing the response towards different cytokine producing cells. For example, in donor 1, the D3 variant induced 0.76% IFNγ^+/MIP-1β^+ and 0.57% MIP-1β^+ cells whereas the D2 and D4 variants stimulated cells to produce MIP-1β^+ alone. The skewing of the cytokine profile was also apparent in donor 4 where the prevalent populations in response to D2 peptide stimulation were TNFα^+, IFNγ^+TNFα^+, and IFNγ^+/MIP-1β^+ cells. In contrast, the prevalent populations in response to D3 peptide stimulation were IFNγ^+ and IFNγ^+TNFα^+ cells.

D. Analysis of quantitative and qualitative changes in response to the variant sequences of all 4 epitopes by triple cytokine staining

Since the initial double staining experiments revealed the existence of distinct subsets of epitope-specific cells that were either single positive for each cytokine or double positive for IFNγ/TNFα and IFNγ/MIP-1β, we wanted to further define the heterogeneity of these populations by analyzing for the co-expression of all 3 cytokines. An example of the gating analysis of SEB-stimulated samples stained simultaneously for IFNγ, TNFα, and MIP-1β is shown in Figure 6. Seven different populations of cytokine producing cells (IFNγ^+, TNFα^+, MIP-1β^+, IFNγ^+TNFα^+, IFNγ^+MIP-1β^+, TNFα^+MIP-1β^+, IFNγ^+TNFα^+MIP-1β^+) were identified (Figure 6D-6I).

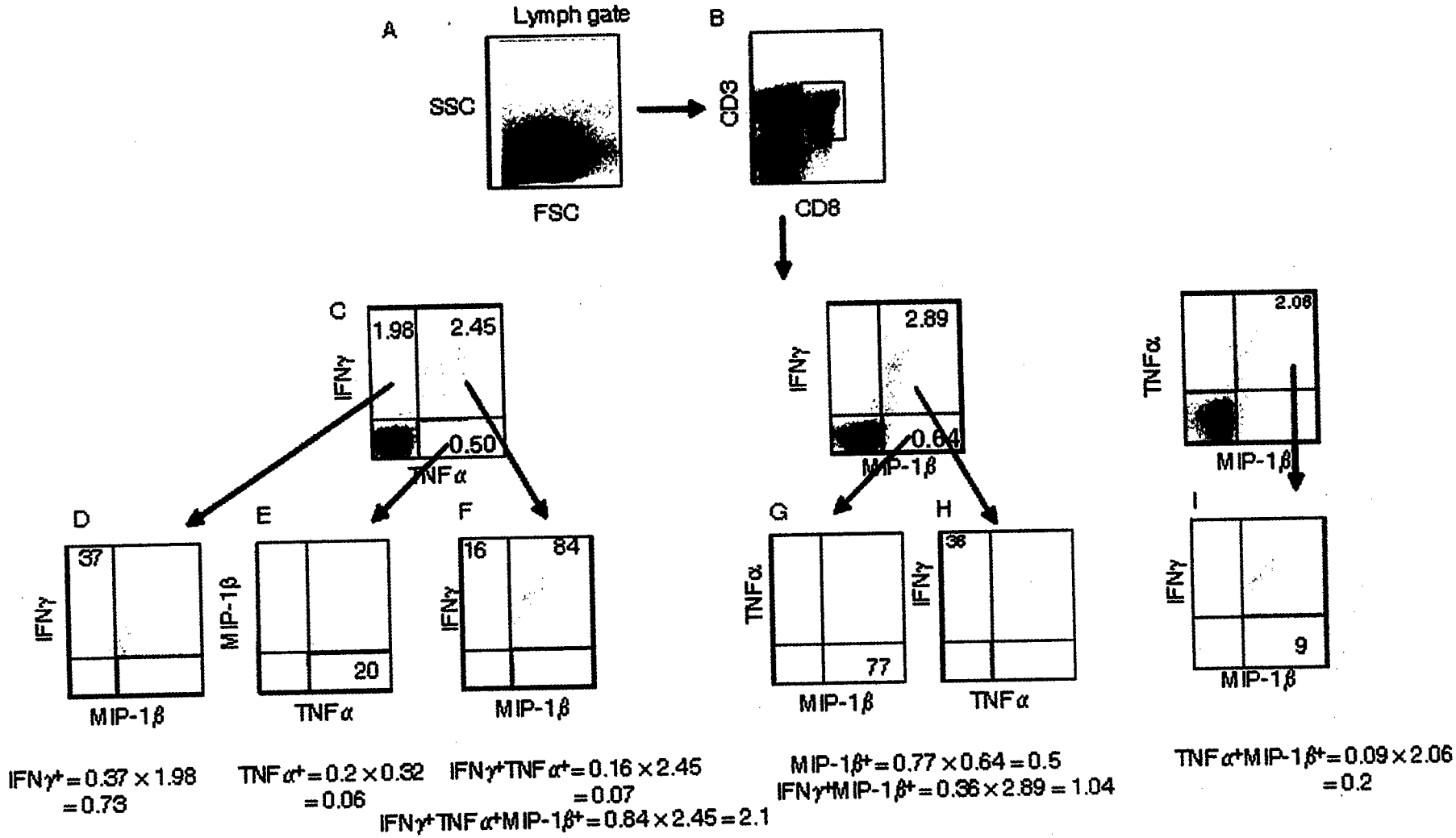


Figure 6: Example gating for the analysis of single, double, and triple cytokine subsets of SEB-stimulated donor 4 PBMC. A PBMC were gated for lymphocyte population, followed by $CD3^+CD8^+$ selection (B). C, $CD8^+$ T cells were then gated for each of the three cytokine combinations. D, E, and G, Single cytokine populations. F, Frequencies in the left quadrant were used for calculation of $IFN\gamma^+$ $TNF\alpha^+$, and frequencies in the right quadrant for $IFN\gamma^+TNF\alpha^+MIP\text{-}1\beta^+$. H and I, Double cytokine populations. Example calculations of subset frequencies are shown at the bottom of each set of panels.

Results of such analyses were highly reproducible. In the D3-immune subject, (donor 2) from whom large numbers of PBMC were available, the analysis was repeated in 3 separate experiments; in almost all cases, the standard deviation was less than or equal to 0.03% (Table V) and the pattern of responses across serotypes was consistent in all repeated experiments.

The total frequency of $CD8^+$ T cells responding functionally to all 4 epitopes ranged from 0.65% - 4%, with cells responding to the variants of the NS4b 2353 epitope dominating the overall response in all 4 donors (Figure 7A). When the response to each epitope variant was compared across donors, the same epitope variants usually induced the highest quantitative response in all donors regardless of the serotype that they were immunized with, as seen with D3 NS4b 2343 and D2 NS4a 2148 (Figure 7B, 7C). Higher responses to heterologous variants were more common than the responses to homologous peptides.

Heterologous stimulation also altered the qualitative nature of the response, since all the epitopes and their variants either did not stimulate all 7 cytokine subsets or altered the relative sizes of these subsets. These changes were both donor-dependent as well as epitope variant-specific. Thus, although the D3 NS4b 2423 and D2 NS4a 2148 variants induced the highest responses in all 4 donors, the profile of the response to each variant differed in each donor. Thus the heterologous response to D3 NS4b 2423 in donors 1, 3, and 4 were dominated by MIP-1β or TNFα while the homologous response to this peptide in donor 2 was dominated by MIP-1β and IFNγ. While the heterologous response to D2 NS4a 2148 in donors 1 and 2 were dominated by IFNγ producing cells, the

Table V: Summary of triple cytokine analysis of D3 vaccinee

		I[a]	T	M	IT	IM	TM	ITM	Total[b]
	D1[c]	0.14±0.02[d]	0	0.13±0.02	0	0.02±0.02	0	0	0.29±0.03
Ns4b	D2	0.12±0.02	0	0.19±0.03	0.04±0.03	0.33±0.03	0.01±0.00	0.01±0.02	0.69±0.08
2353	D3	0.25±0.05	0	0.42±0.02	0.23±0.01	0.18±0.03	0.17±0.05	0.13±0.03	1.37±0.1
	D4	0.09±0.02	0	0.11±0.08	0	0.01±0.01	0	0	0.21±0.08
	D1	0.06±0.02	0.02±0.03	0.17±0.02	0	0.02±0.01	0	0	0.27±0.02
Ns4b	D2	0.13±0.03	0	0.10±0.03	0	0.04±0.02	0	0	0.27±0.03
2423	D3	0.23±0.02	0	0.21±0.02	0.09±0.01	0.07±0.02	0.07±0.02	0.05±0.02	0.71±0.07
	D4	0.09±0.04	0	0.08±0.00	0	0.01±0.02	0	0.01±0.01	0.19±0.04
	D1	0.06±0.02	0	0.14±0.06	0	0.03±0.03	0	0	0.23±0.05
Ns4a	D2	0.19±0.03	0	0.07±0.04	0.01±0.01	0.06±0.02	0	0	0.33±0.03
2148	D3	0.12±0.02	0	0.01±0.02	0.02±0.02	0.02±0.02	0	0	0.15±0.03
	D4	0.09±0.01	0	0.01±0.02	0.01±0.02	0.01±0.02	0	0	0.12±0.02
	D1	0.02±0.03	0±0.01	0	0	0.01±0.02	0	0	0.04±0.01
E 493	D2	0.15±0.04	0.01±0.01	0.01±0.01	0	0.02±0.03	0	0	0.18±0.03
	D3	0.12±0.02	0	0	0	0.01±0.02	0	0	0.13±0.03
	D4	0.15±0.02	0	0.12±0.03	0	0.03±0.03	0	0	0.3±0.03

[a] I = $IFN\gamma^+$; T = $TNF\alpha^+$; M = $MIP\text{-}1\beta^+$; IT = $IFN\gamma^+TNF\alpha^+$; IM = $IFN\gamma^+MIP\text{-}1\beta^+$; TM = $TNF\alpha^+MIP\text{-}1\beta^+$; ITM = $IFN\gamma^+TNF\alpha^+MIP\text{-}1\beta^+$

[b] Indicates the total frequency of cytokine producing cells responding to each peptide

[c] Indicates dengue serotype

[d] Mean value of three independent experiments ± standard deviation

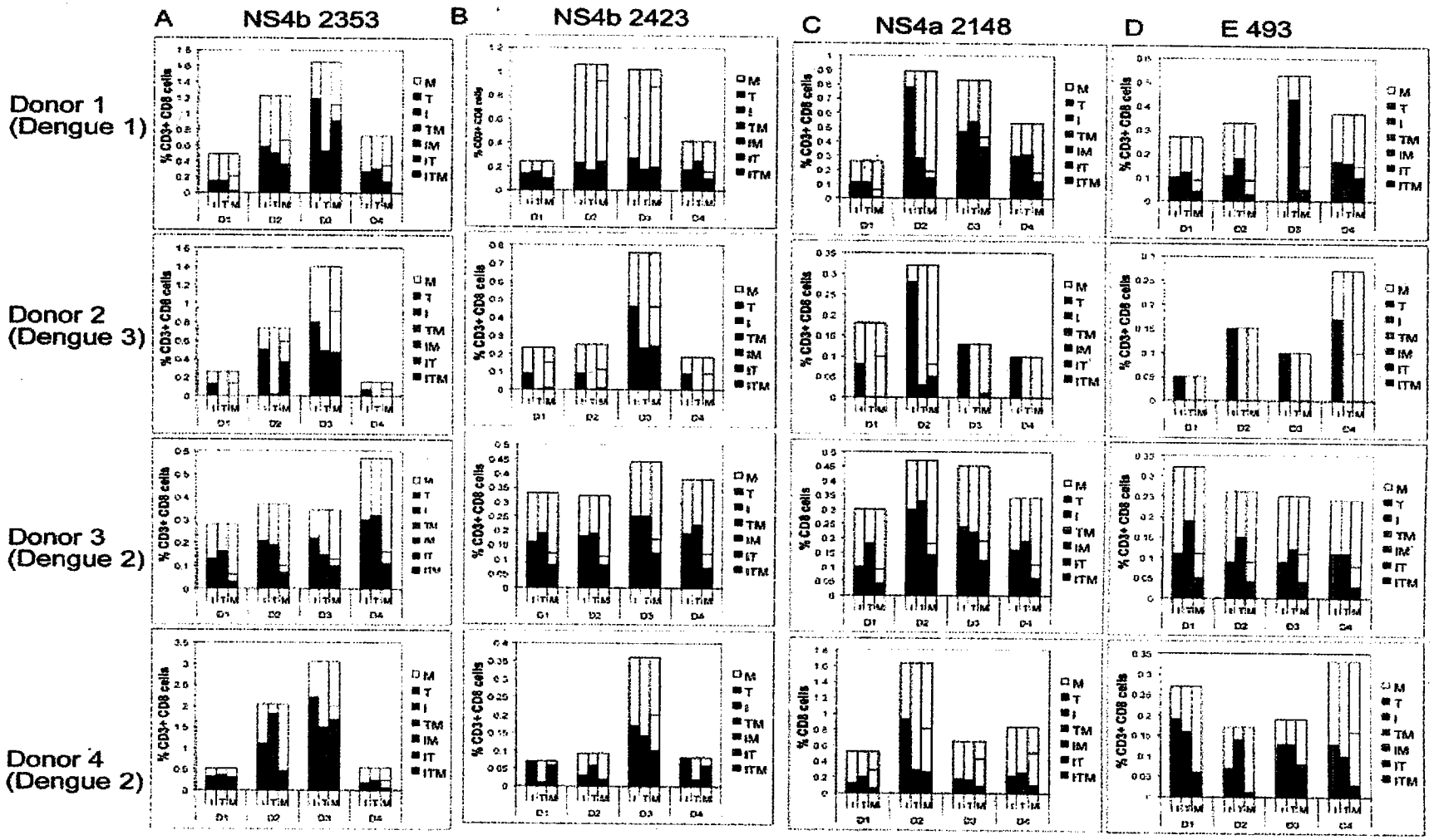


Figure 6. Quantitative and qualitative alterations in functional response following heterologous peptide stimulation. Triple cytokine stainings was performed on PBMC from all four donors stimulated with variants of all four epitopes. Each graph displays the responses to the four variants of the peptide indicated at the top of the column of the PBMC from the donor listed on the left. The height of each bar shows the total frequency of all $CD3^+CD8^+$ T cells responding to that peptide. Each bar also is divided into three bars that show the combined frequencies of cells that produce IFNγ (I), TNFα (T), or MIP-1β (M). The frequencies of cells in unstimulated cultures were subtracted separately for each population. Subpopulations of cells producing different combinations of these three cytokines are displayed with a different color as shown in the graph legends. For example, IFN-secreting cells include $IFN\gamma^+$, $IFN\gamma^+TNF\alpha^+$, $IFN\gamma^+MIP\text{-}1\beta^+$, and $IFN\gamma^+TNF\alpha^+MIP\text{-}1\beta^+$ cells.

homologous response in donors 3 and 4 was represented equally by IFNγ/TNFα and IFNγ/MIP-1β populations, respectively.

In other examples of donor-specific changes, stimulation of D1 immune PBMC (donor 1) with D3 NS4b 2353 resulted in all 7 functional populations with the $IFN\gamma^+MIP\text{-}1\beta^+$ subset being the largest. In contrast, the D1 NS4b 2353 peptide induced $IFN\gamma^+$, $TNF\alpha^+$, $MIP\text{-}1\beta^+$, and a very small population of $TNF\alpha^+MIP\text{-}1\beta^+$ cells (Figure 7A). Similarly, in donor 2 (D3 immune), although the homologous D3 NS4b 2353 and NS4b 2423 peptides induced 6 different sub-sets of cytokine-producing cells, stimulation with the other variants of these epitopes only gave rise to mainly $IFN\gamma^+$ and $MIP\text{-}1\beta^+$ cells (Figure 7A, 7B). In contrast, in donor 3, although the responses to all variants of all the epitopes were mediated by all 7 subsets, the frequency of cells producing MIP-1β was lower than the frequencies of cells producing IFNγ and TNFα.

This extensive functional heterogeneity of the population of cells re-activated *in vitro* during a secondary stimulation suggests that the quality of the recall response during heterologous dengue infection *in vivo* will depend on which of these subsets undergo selective expansion and dominate the secondary response.

E. Sequential stimulation of dengue immune PBMC by different variant peptide sequences directly reveals cross-reactive T cells

In the experiments described above, PBMC immune to one serotype responded by producing cytokines when stimulated with peptides from other serotypes, showing that these epitopes could stimulate cross-reactive responses. However, the results of these experiments did not indicate if the same population of epitope-specific cells was

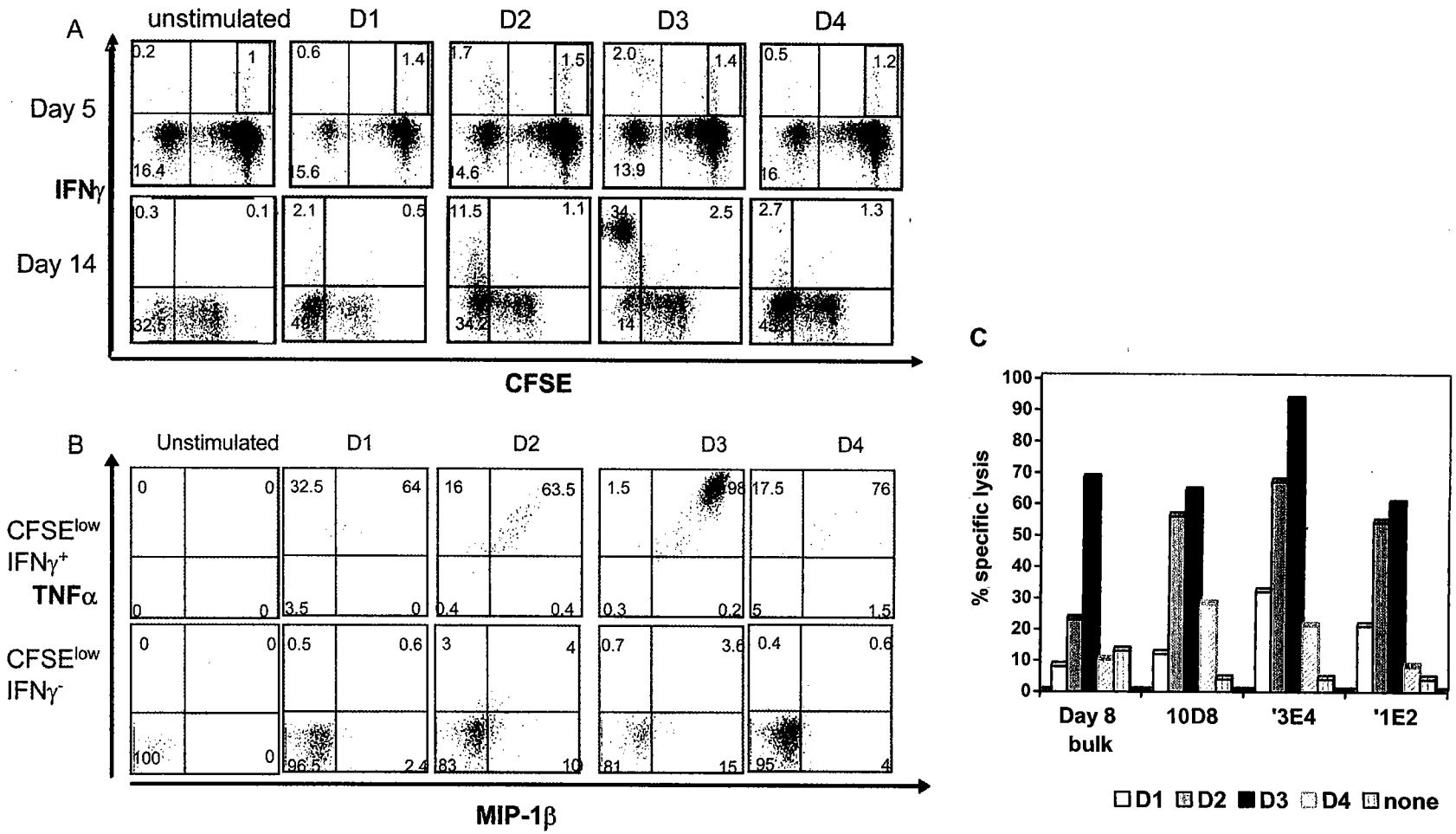


Figure 8: Sequential stimulation of dengue-immune PBMC with heterologous peptides directly reveals serotype cross-reactive T cells. PBMC from dengue 3-immune donor 2 were stained with 1μM CFSE and stimulated with D3 NS4b 2423. (A) On days 5 or 14 of culture, cells were stimulated with all variants of this epitope and stained intracellularly for IFNγ, TNFα, and MIP-1β. In the dot plots shown, $CD3^+CD8^+$ T cells that have divided six or more times are in the left quadrants. For day 5 samples, undivided $IFN\gamma^+$ cells were separately gated, and the frequencies are shown within the rectangle gate. (B) Proliferated cells that secrete IFNγ ($CFSE^{low}IFN\gamma^+$) or were $IFN\gamma^-$ ($CFSE^{low}IFN\gamma^-$) were examined for secretion of TNFα and MIP-1β. (C) Specific lysis of peptide-pulsed autologous BLCL by CFSE-stained Day 8 bulk culture cells or cell lines derived from this bulk culture by limiting dilution.

responding to the variant peptides or if each variant peptide activated a different population of epitope-specific T cells. In order to understand this, we developed an assay which would measure functional responses of the same cell population to two different peptide stimuli.

PBMC were stained with CFSE at the time of stimulation with the homologous peptide (first stimulus). After 5 or 14 days, the cells were washed and re-stimulated for 6 hrs with the same or heterologous peptides and stained for cytokine production (second stimulus). The cross-reactive population would be represented by the $CFSE^{low}cytokine^{+}$ group, the population specific for the homologous peptide would be represented by the $CFSE^{low}cytokine^{-}$ sub-set and the population specific only for the second heterologous peptide would be represented by the $CFSE^{high}cytokine^{+}$ sub-set.

Stimulation of Dengue 3 immune PBMC with the homologous D3 NS4b 2423 peptide induced proliferation of epitope-specific $CD8^{+}$ T cells. By day 5, fully divided $CD3^{+}CD8^{+}$ cells constituted ~16% of the culture and increased to ~48% by day 14 (Figure 8A). Re-stimulation with homologous peptide on day 14 induced $IFN\gamma^{+}$ production by 67% of the divided cells; 0.25% - 0.52% of the undivided cells that remained undivided at day 5 of culture also produced IFNγ after restimulation. However, by day 14 of culture, essentially none of remaining undivided or partially divided cells were capable of producing $IFN\gamma^{+}$ after re-stimulation.

In contrast, only 22% of the day 14 proliferated cells were $IFN\gamma^{+}$ following D2 peptide stimulation. The number of cells which secreted IFNγ in response to D1 and D4 peptide stimulation was even lower. This suggests that a third of the expanded D3 NS4b 2423-specific T cell population was cross-reactive for D2 NS4b 2423 but had very little

cross-reactivity for the D1 and D4 variants. Since direct ex-vivo stimulation of this donor's PBMC with D1, D2, and D4 peptides induced comparable levels of cytokine production (Figure 7B), these results suggest that under long-term culture conditions, clones that are cross-reactive for D2 and D3 sequences alone have a proliferative advantage. This pattern of cross-reactivity was consistent with that seen in NS4b 2423-specific T cell clones obtained from limiting dilution. Most of the clones derived from D3 peptide stimulated bulk lines as well as the bulk culture itself were highly cross-reactive for D2 peptide and less so for D1 and D4 variants in cytolysis assays (Figure 8C).

The pattern of cytokine staining by $CFSE^{low}$ cells in response to re-stimulation with homologous D3 or heterologous peptides also differed. The intensity of IFNγ staining of $CFSE^{low}$ cells was lower after stimulation with heterologous D2 peptide than after stimulation with homologous D3 peptide. All of the $CFSE^{low}IFN\gamma^{+}$ cells in cultures stimulated by homologous peptides co-expressed TNFα and MIP-1β (Figure 8B). In contrast, there was a substantial population of cells (16% – 32% of $CFSE^{low}IFN\gamma^{+}$ population) which did not express MIP-1β in response to heterologous stimulation. The majority of the $CFSE^{low}IFN\gamma^{-}$ population did not express TNFα. But 10% - 15% of these cells expressed MIP-1β following re-stimulation with D2 and D3 peptides revealing an unexpected pattern of functional heterogeneity represented by this population of $CFSE^{low}IFN\gamma^{-}TNF\alpha^{-}MIP\text{-}1\beta^{+}$ cells.

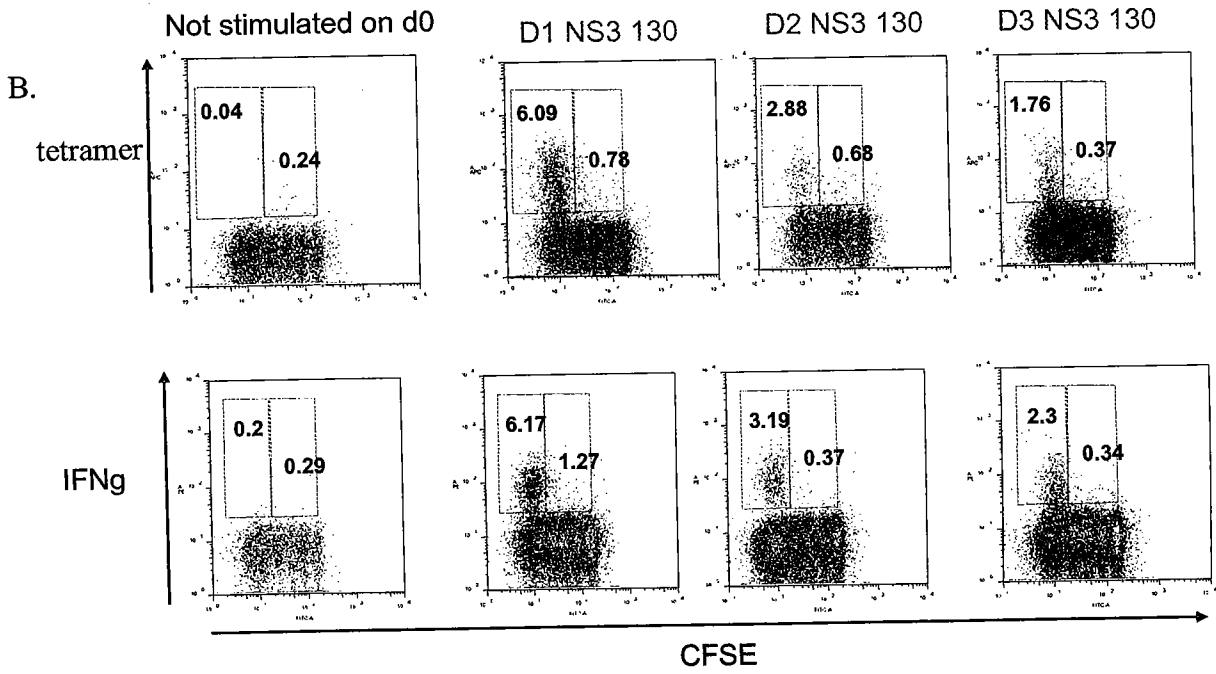
In order to determine if these patterns of cross-reactivity was epitope-specific, we examined responses to variants of other epitopes. The response to a HLA-A11*01 restricted epitope (D1 NS3 130) and its variants was studied in D1 immune PBMC by staining with tetramer and anti-IFNγ. The D2 and D3 variants of this epitope differ by

one and two amino acids, respectively, from the D1 sequence (Figure 9A), and T cells in D1 peptide-stimulated bulk cultures are cross-reactive for both variants since they efficiently lyse both D2 and D3-pulsed autologous targets (Figure 9C). The cross-reactivity works in the other direction with D3 peptide stimulated bulk cultures recognizing D1 and D2-pulsed targets equally well in CTL assays. However, the D2 peptide-stimulated bulk cultures showed low frequencies of epitope-specific CTLs and poor cross-reactive recognition of D1 and D3 pulsed targets.

D1 immune PBMC were stimulated on Day 0 with the homologous D1 peptide, re-stimulated on Day 8 with the three variants for 6 hr, and stained with the corresponding tetramers or for intracellular IFNγ. D1-tetramer staining and IFNγ intracellular staining both showed epitope-specificity in only 6% of the total cells for homologous peptide re-stimulation while staining with the variant tetramers detected 3% of cells that were cross-reactive for D2 and 2% of cells that were cross-reactive for D3 (Figure 9B). This hierarchy of cross-reactivity was similar to that observed by staining for IFNγ and unlike that observed by CTL lysis in the D1 bulk cultures (Figure 9C) suggesting that cross-reactivity is segregated by effector function

However, tetramer staining may not accurately measure the size of the antigen-specific population. Activated T cells may have decreased avidity for tetramer due to changes in the reorganization of the TCR on the cell surface (Drake et al., 2005; Blohm et al., 2002). We observed such a result when we examined the sequential stimulation response of D3-immune PBMC to a HLA-B*07 restricted epitope NS3 222-231 and its D1 variant. The difference of a single aa (D1:Ser → Glu) (Figure 10A) between the two sequences had little effect on the ability of bulk cultures stimulated with the D2/3/4

A. D1 NS3 130 = G T S G S P I V N R E
D2 NS3 130 = G T S G S P I V D R K
D3 NS3 130 = G T S G S P I I N R E



C.

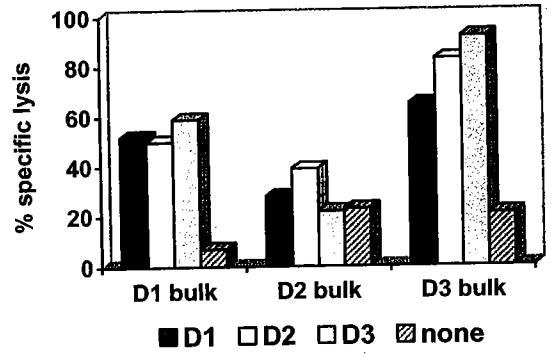


Figure 9: Tetramer staining of PBMC sequentially stimulated with heterologous peptides. PBMC from D1 immune donor were CFSE stained and stimulated with homologous D1 NS3 130 on day 0. An unstimulated control was also included. A. Amino acid sequences of the HLA-A11*01 restricted epitope. B. PBMC were either left unstimulated on Day 8 or re-stimulated with homologous and heterologous peptides and were stained tetramer specific for the D1, D2, or D3 sequences or for intracellular IFNg. C. Specific lysis of autologous BLCL pulsed with 10ug/ml of peptide by Day 8 bulk cultures at E:T = 50: 1.

A. D1 = A P T R V V A A S M
D2/3/4= A P T R V V A A E M

B.

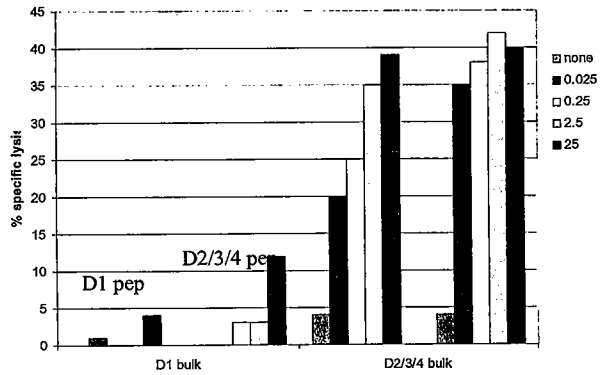
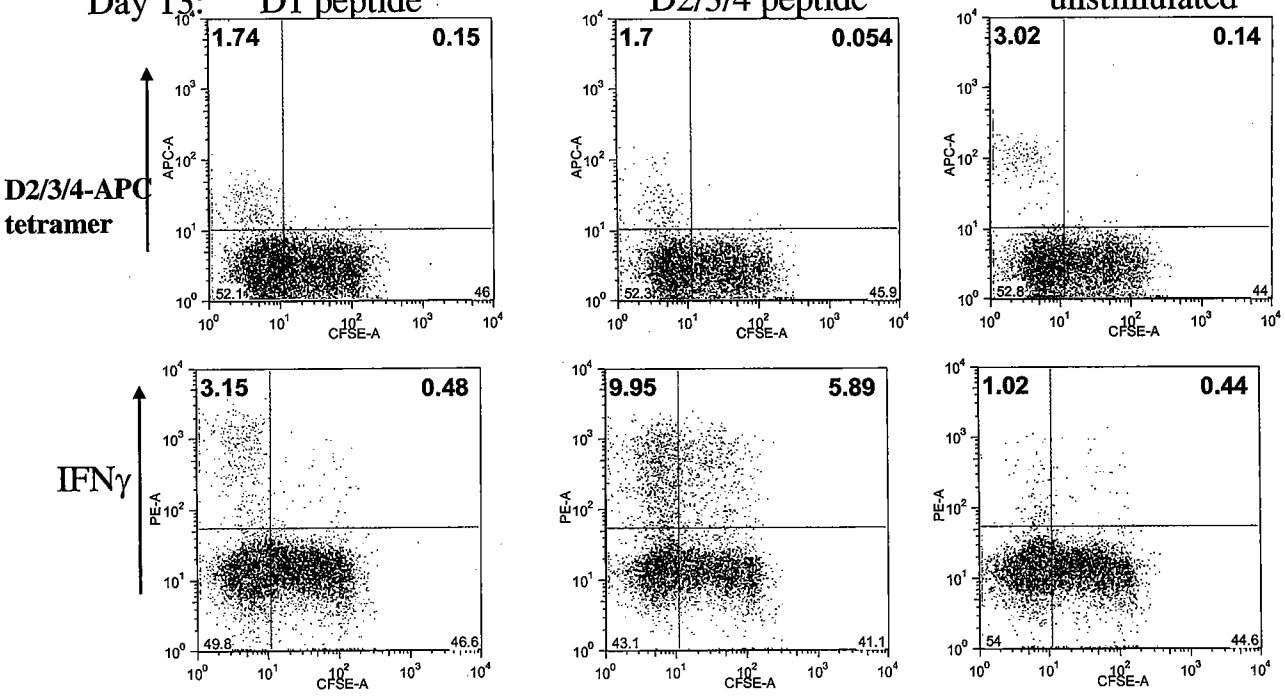


Figure 10: Tetramer staining of PBMC sequentially stimulated with heterologous peptides. PBMC from D13immune donor were CFSE stained and stimulated with homologous D2/3/4 NS3 222 on day 0. A. Amino acid sequences of the HLA-B*07 restricted epitope. B. Specific lysis of autologous BLCL pulsed with D1 or D2/3/4 peptide by Day 13 bulk cultures at E:T = 50: 1 and T cell line 3C3 at E:T = 10:1. C. PBMC were either left unstimulated on Day 13 or re-stimulated with homologous and heterologous peptides and stained with IFNγ and tetramer specific for the D2/3/4 sequence.

C Day 13: D1 peptide D2/3/4 peptide unstimulated



peptide to cross-reactively recognize and lyse D1-pulsed target cells (Figure 10B). However, the reverse pattern of cross-reactivity was not seen since the D1-peptide stimulated bulk cultures were able to minimally recognize the D2/3/4 variant at very high concentrations.

This pattern in the cross-reactivity of response between cells specific for the two sequences was seen in the IFNγ response of the CFSE-stained cells and in their ability to bind tetramer. The bulk culture stimulated with the heterologous D1 peptide on day 0 did not produce IFNγ upon stimulation with either peptide on day 13 nor show affinity for the tetramer, suggesting that the D3-specific memory cells in this donor's PBMC had low avidity for the variant peptide and did not proliferate (data not shown).

In contrast, in the bulk culture stimulated on day 0 with the homologous D2/3/4 peptide, 10% of the total culture produced IFNγ upon re-activation with the same peptide and a third of these cells (~3%) were cross-reactive for the variant peptide (Figure 10C, bottom panel). Homologous re-stimulation also induced IFNγ production among 6% of the incompletely proliferated $CFSE^{mid\text{-}high}$ population, which was a pattern not observed with the other peptides studied (Figures 8, 9). The pattern of tetramer staining, however, was surprisingly different from that of cytokine production. Only 3% of cells stimulated with the D2/3/4 peptide on day 0 were $tetramer^{+}$ on day 13 and were all found among the $CFSE^{low}$ population. In the cultures that were re-stimulated on day 13, only 1.7% of the $CFSE^{low}$ cells were $tetramer^{+}$ suggesting that the remaining 1.3% had down-regulated their TCR upon re-activation. These $tetramer^{+}$ cells were also mostly $IFN\gamma^{+}$ but there was a significant fraction of $tetramer^{+}$ cells which did not secrete IFNγ (Figure 11A).

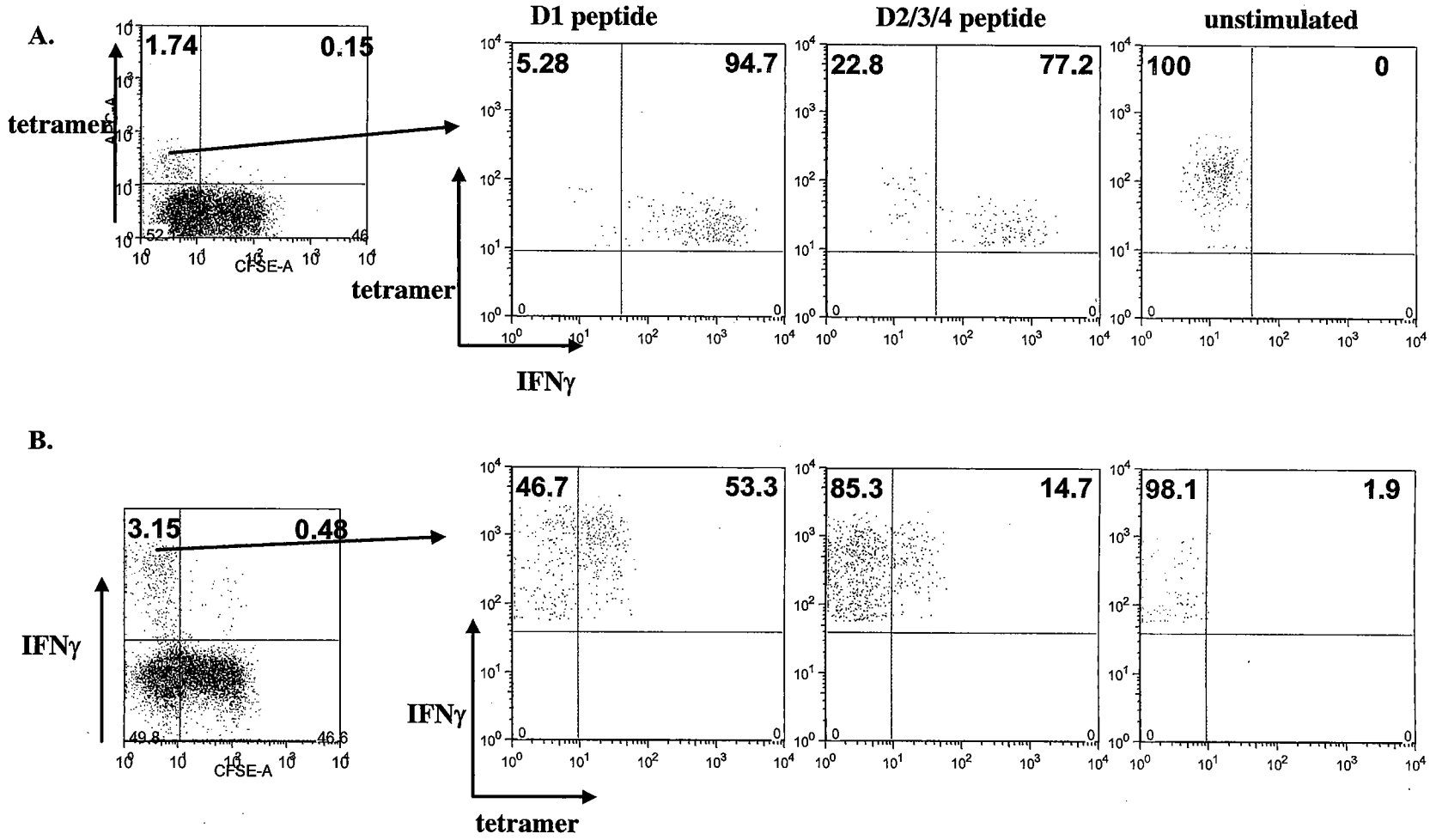


Figure 11. Heterogeneity of function within epitope-specific population of cross-reactive CD8$^+$ T cells. (A). Tetramer$^+$ cells in the CFSE^{low} population from the experiment described in Figure 10 were selected and analyzed for the secretion of IFNγ. (B) IFNγ$^+$ cells from the same experiment were selected and analyzed for the ability to bind tetramer.

We also examined the pattern of tetramer staining among the $CFSE^{mid\text{-}high}IFN\gamma^{+}$ population and found that the $tetramer^{+}$ subset was included within this population (Figure 11B). We interpret these results to mean that there is a hierarchy in the functional avidity of these antigen-specific cells with the cells of the highest avidity retaining the ability to bind tetramer and produce IFNγ after having fully proliferated, the proliferated cytokine-producing but non-tetramer-binding cells being of intermediate avidity, and those that produced cytokine without fully proliferating or binding tetramer to be of low avidity. In this sense, the 3% of the D2/3/4-specific bulk culture cells (Figure 10B) which produced IFNγ in response to the heterologous peptide would have the same high avidity to the variant as to the homologous peptide.

F. Chapter Summary

Our objective was to study the patterns of cross-reactive responses to immunodominant T cell epitopes and their variants in dengue-immune PBMC in order to understand the complexities of the secondary immune response to heterologous dengue serotypes. We identified 4 novel HLA-A*02 restricted epitopes which were recognized in the 4 available vaccinated donors. The frequencies of the cells specific for these epitopes as measured by intracellular IFNγ production were higher than those measured during convalescence in infected subjects (Loke et al., 2001; Mongkolsapaya et al., 2003 and 2005). Stimulation of dengue-immune PBMC with the variant sequences of these epitopes altered both the size and the functional profile of the secondary response as compared to homologous peptide stimulation. The quantitative changes seen in response to the altered peptides were:

- In each donor, the peptide that induced the highest response was frequently a heterologous sequence
- The same variant of some epitopes (D2 2148 and D3 2423) induced the highest response in all donors regardless of their primary serotype
- Of the 4 epitopes, each donor showed the highest response to one of the variants of NS4b 2353.

Stimulation with variant sequences also induced qualitative changes in response:

- The numbers and relative frequencies of the various cytokine-producing subsets varied by donor
- Within a donor, variant peptide stimulation altered both the types of subsets that responded, as well as their frequencies.

We also developed a novel assay which allowed the direct demonstration of serotype cross-reactive responses to variant peptide stimulation. The sequential stimulation of CFSE-stained PBMC with homologous peptide followed by heterologous peptide (after a proliferation interval) revealed cross-reactivity patterns and hierarchies of functional avidity. Responses to the secondary (heterologous) stimulation were mediated by cells that had divided after primary stimulation with the homologous sequence, i.e cross-reactive cells.

Thus, the presence of a diverse functional epitope-specific population and the changes in the frequencies of functional subsets following heterologous secondary stimulation suggest that the nature of the memory response (protective vs pathologic) could be determined by the dominant functional subset. These changes in functional profiles were both donor-dependent (i.e., primary serotype) and epitope-specific.

Additionally, the higher immunogenicity and avidity of the variants of D2 and D3 serotypes suggest a role for the serotype of the secondary infection in determining disease severity.

CHAPTER IV

ANALYSIS OF FREQUENCY, KINETICS OF RESPONSE AND PHENOTYPE OF EPITOPE-SPECIFIC CD8$^+$ T CELLS DURING ACUTE DENGUE DISEASE AND CONVALESCENCE IN THAI PATIENTS

Studies which examined T cell responses during acute dengue illness have reported a correlation between disease severity and increased T cell activation. In these studies, patients with DHF had elevated levels of cytokines and soluble cytokine receptors early in the illness, and increased expression of CD69 on CD8$^+$ T cells (Green et al., 1999). Quantitation of epitope-specific T cells via IFNγ ELISPOT and tetramer staining showed increased epitope-specific T cell frequencies in DHF patients as compared to those with mild dengue disease (Zivna et., 2002; Mongkolsapaya et al., 2003; Mongkolsapaya et al., 2006). However, little has been published on the phenotype of responding T cell sub-sets during dengue infection. In studies performed by Mongkolsapaya et al, the frequencies of tetramer$^+$ cells were found to be low during early illness with peak frequencies occurring at 2 weeks after the resolution of fever. Since the characteristic symptoms of DHF, namely plasma leakage and shock, appear at the time of defervescence, this finding is at odds with the notion that activated CD8$^+$ T cells contribute to DHF immunopathology. In order to learn more about the nature of dengue-specific T cells in terms of their changing frequencies and phenotypes through the course of secondary immune response, we studied CD8$^+$ T cells specific for a HLA-B*07 restricted NS3 epitope in PBMC obtained during acute illness and convalescence from 10 dengue-infected Thai subjects.

A. Identification of HLA-B*07 restricted NS3-222-231 epitope and construction of tetramer:

The HLA-B*07-restricted $CD8^+$ T cell epitope NS3 221-232 was initially identified as a 12mer epitope LAPTRVVAAEME recognized by convalescent-stage PBMC of a D4 virus-infected patient and T cell clones established by limiting dilution (Mathew et al., 1998; Zivna et al., 2001). This sequence is conserved among D2V, D3V, and D4V and differs by 2 amino acids – serine for alanine at position 9 and alanine for glutamic acid at position 12 in the D1V sequence. This epitope was recognized in IFNγ ELISPOT assays in several dengue-infected HLA-B*07 donors during acute infection and convalescence. T cell clones specific for the 12mer epitope were able to recognize the 10mer NS3 222-231 epitope in CTL assays but not the 2 predicted 9mers, 221-229 and 222-230, present within this region. Tetramers folded with the 9mer 221-229 did not stain these cells (data not shown). We therefore constructed a HLA-B*07 tetramer with the peptide corresponding to the 10mer 222-231 for further studies. This tetramer efficiently stained an HLA-B*07-restricted patient PBMC-derived cell line (Figure 12A). The specificity of the tetramer was confirmed by staining an HLA-B*07-restricted cell line specific for a different NS3 epitope (Figure 12B) as well as PBMC taken from a dengue-immune HLA-B*07^- donor (Figure 12C) and from a dengue-naïve HLA-B*07^+ donor (Figure 12D). The tetramer was also recognized by around 4% of $CD8^+$ T cells from a dengue-immune HLA-B*07^+ patient (Figure 12E).

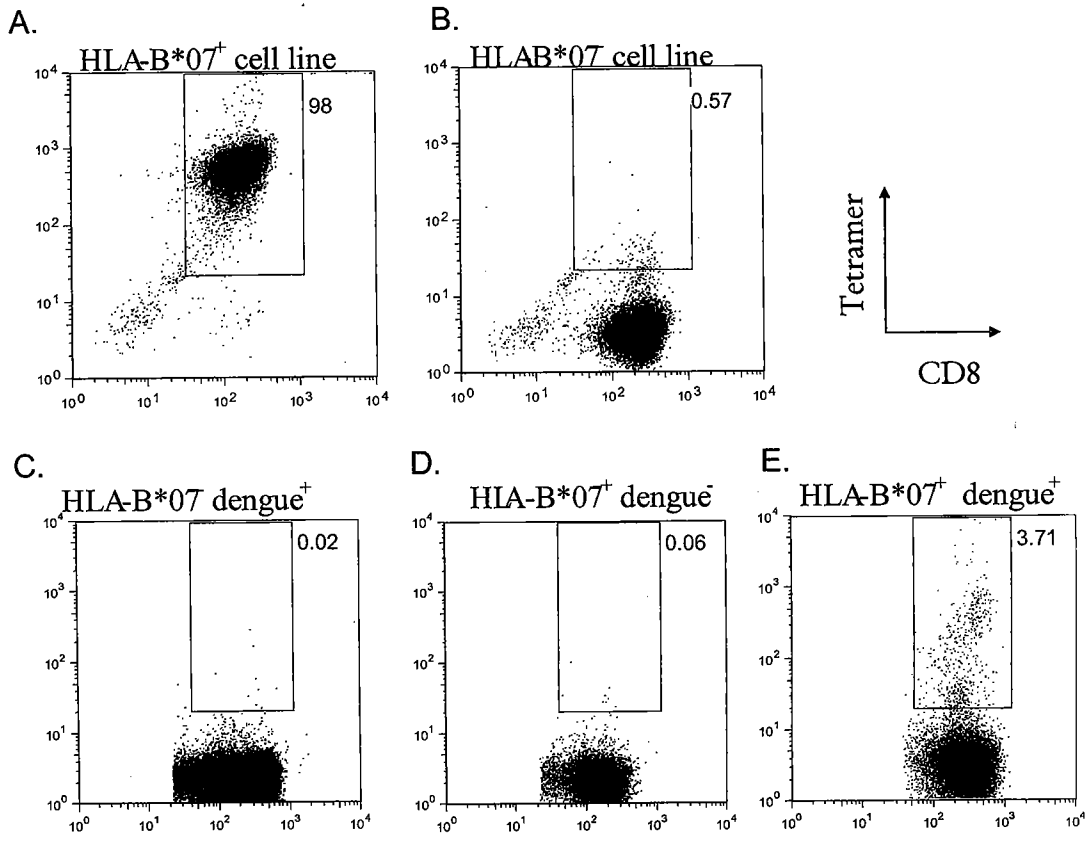


Figure 12: Specificity of HLA-B*07 tetramer staining: The tetramer constructed with the 10mer NS3 222-231 based on the D2/3/4 sequence and conjugated to APC was used to stain (A) NS3 222-231 specific CD8+ T cell line. (B). NS3 527-536 specific cell line. PBMC from (C) HLA-B*07- dengue infected patient, (D) HLA-B*07+ dengue naïve donor and (E) HLA-B*07+ dengue infected patient. Cells were incubated with tetramer for 20 min. at room temperature and 30 min. with surface mAbs for CD3 and CD8 on ice.

B. Frequencies of epitope-specific cells in patient PBMC during acute illness and convalescence:

The 10 patients enrolled in the study were all under 15 years of age. There were 5 DF and 5 DHF cases, and all but 1 subject were serologically defined as having secondary infections with dengue virus (Table VI). Samples were obtained as early as 5 days before the resolution of fever and as late as 9 days after defervescence. Convalescence samples were obtained during follow-up visits at 6 months – 1 year after the resolution of illness.

NS3 222-231-specific T cells were detected in all donors, with frequencies ranging between 0.23% - 8.12% of $CD8^+$ $CD3^+$ T cells in acute stage samples stained with the tetramer corresponding to the D2/3/4 sequence (Figure 13A, 13B). These frequencies are higher than those detected by IFNγ ELISPOT assay in some of these patients in a previous study (Zivna et al., 2001) and also higher than those detected during acute illness by other groups. Also, $tetramer^+$ cells were observed even in those PBMC in which no IFNγ responses had been detected.

Serial bleeds were available in 5 donors during acute illness, starting as early as 5 days before defervescence. In 2 of these donors, the kinetics of the response was seen to change rapidly. In subject C95-098 with secondary grade II DHF, the frequency peaked at 7.4% of $CD8^+$ T cells 3 days before defervescence and underwent a 3- fold decrease by day 5 after fever resolution. In subject C94-094 with secondary grade I DHF, $tetramer^+$ T cells underwent a massive 6-fold expansion from 1.5% to 7% of $CD8^+$ T cells within a day and underwent a 3-fold decrease a day later (Figure 13C). In the remaining three donors, 2 of whom had DF, the frequencies of $tetramer^+$ cells were lower than in the DHF

Table VI: Characteristics of patients in the study.

Subject[a]	Diagnosis	Serology	Serotype	HLA Class I Alleles A	B	C
C95-029	DF	Primary	D3	24, 24	7, 75	1, 6
C96-089	DF	Secondary	D1	24, 11	7, 60	7
C94-115	DF	Secondary	D3	2, 29	7, 60	3
K97-093	DF	Secondary	D3	24, 33	7, 44	7, 7
C99-014	DF	Secondary	D3	11, 33	7, 44	4, 6
C94-094	DHF grade 1	Secondary	D4	2, 11	7, 13	3, 7
C94-132	DHF grade 2	Secondary	D4	11, 29	7, 75	8
C95-098	DHF grade 2	Secondary	D1	29	7, 61	3
C97-036	DHF grade 2	Secondary	D3	11, 33	7, 75	6
C97-014	DHF grade 3	Secondary	D3	3, 11	7, 13	4, 6

[a]Age of subjects: 6 mo – 14 yrs

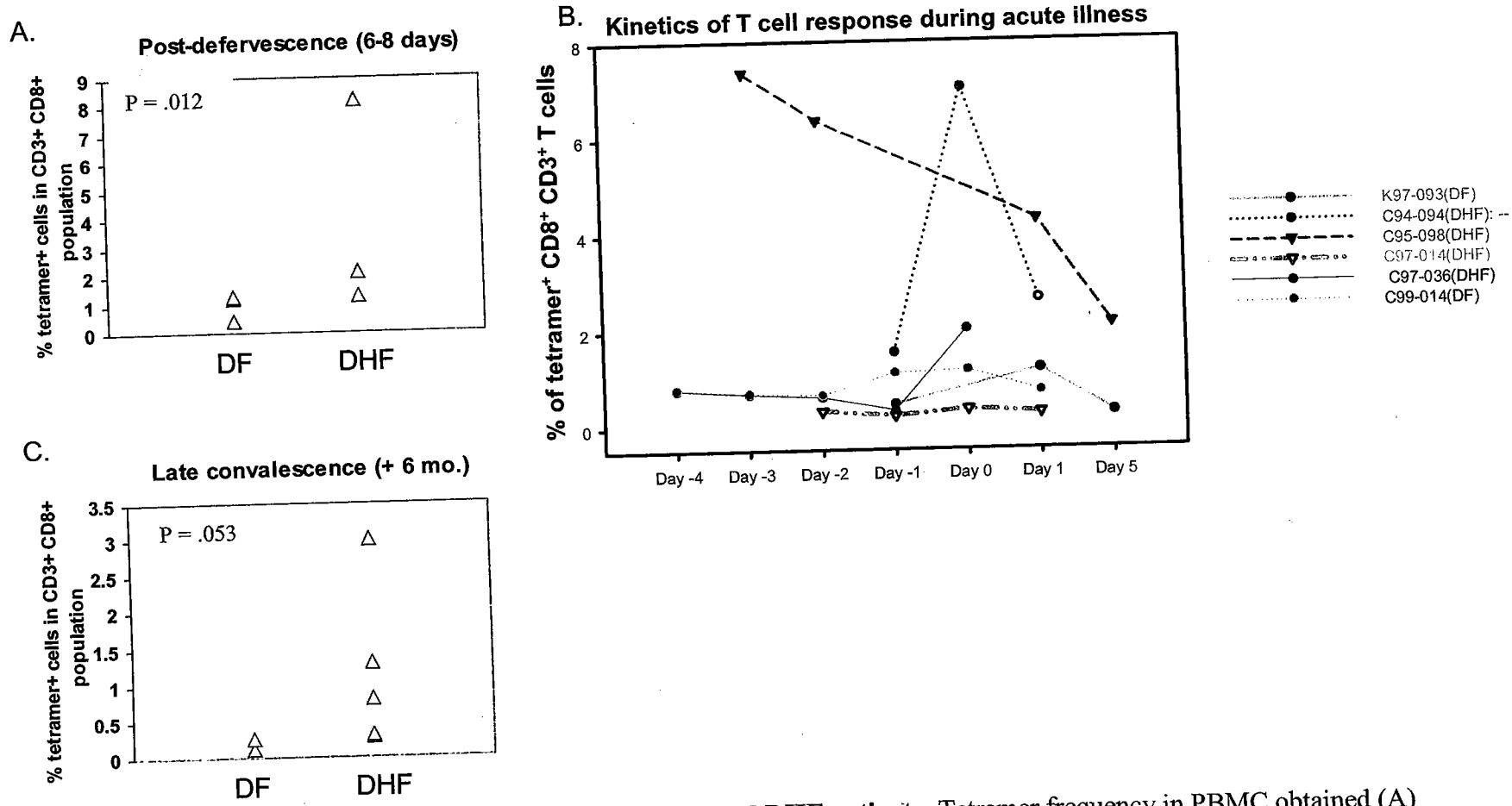


Figure 13. Frequencies of tetramer+ T cells in DF and DHF patients: Tetramer frequency in PBMC obtained (A) 6-8 days after the resolution of fever during acute illness, (B) between 6 months to 1 year following hospitalization. (C) Kinetics of response in 5 patients from whom serial bleeds were obtained during acute illness. Day 0 is the day of defervescence. Cells were first gated on CD3+ CD8+ lymphocytes before analysis of tetramer staining

donors on all the study days. The peak of the response again occurred on the day of defervescence with tetramer$^+$ cell numbers decreasing thereafter.

The magnitude of response was also seen to correlate with disease severity. Tetramer$^+$ T cell frequencies were higher in DHF patients both during acute illness as well as in convalescence. While the frequencies of tetramer$^+$ cells were in general lower during convalescence than those observed during acute illness, 2 of the DHF patients had frequencies as high as 1.8% and 3% tetramer$^+$ T cells even at 6 months following recovery (Figure 13C).

C. Phenotypic analysis of tetramer$^+$ CD8$^+$ T cells:

The changes in phenotype which occured during the activation and expansion of antigen-specific cells during dengue infection were studied by staining patient PBMC with antibodies for activation markers CD69, CD38, and HLA-DR, homing receptors CCR7 and CD62L, and when cells were available, for markers such as CD45RA and CD45RO that would help in the differentiation of memory subsets as well.

An example of the staining for activation markers is shown in Figure 14. The expression of CD69 in both DF and DHF patients decreased following the clearance of virus by day 9 after being admitted into the study (Figure 15). While fewer than 15% of the tetramer$^+$ cells were CD69$^+$ in DF patients, this number was as high as 80% in the 3 of the 4 DHF patients. There were no CD69$^+$ cells found during late convalescence (6 months post-infection) in DF donors whereas ~10% of tetramer$^+$ cells in the DHF patients expressed CD69 at this stage. All donors also showed a

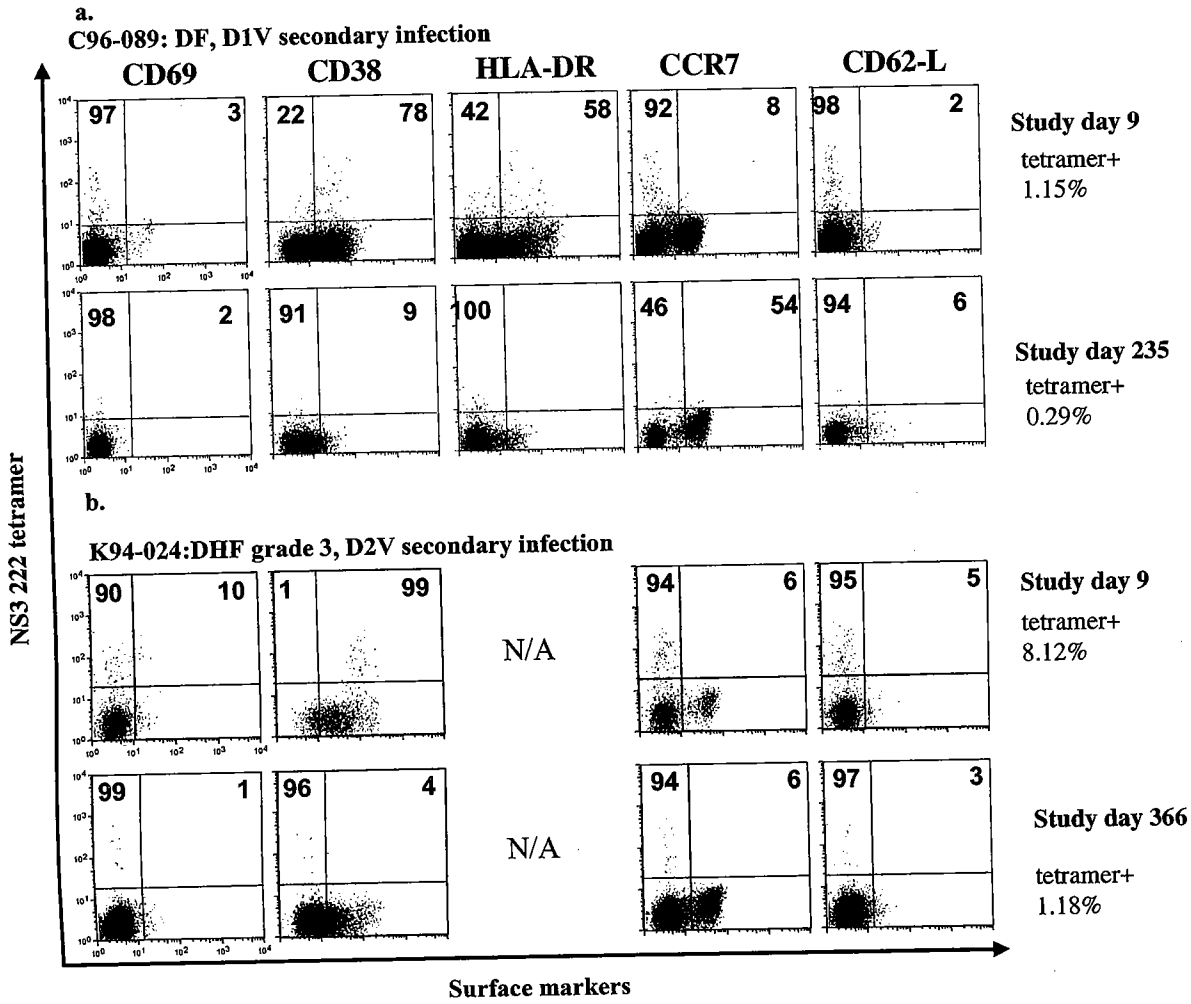


Figure 14. Phenotype of tetramer+ CD8+ T cells during acute illness vs convalescence: Tetramer and activation/differentiation phenotype staining of PBMC gated on CD3+CD8+ population from (a) DF patient and (b) DHF patient. The numbers within the plots indicate the percentage of surface expression of individual markers within the tetramer+ population.

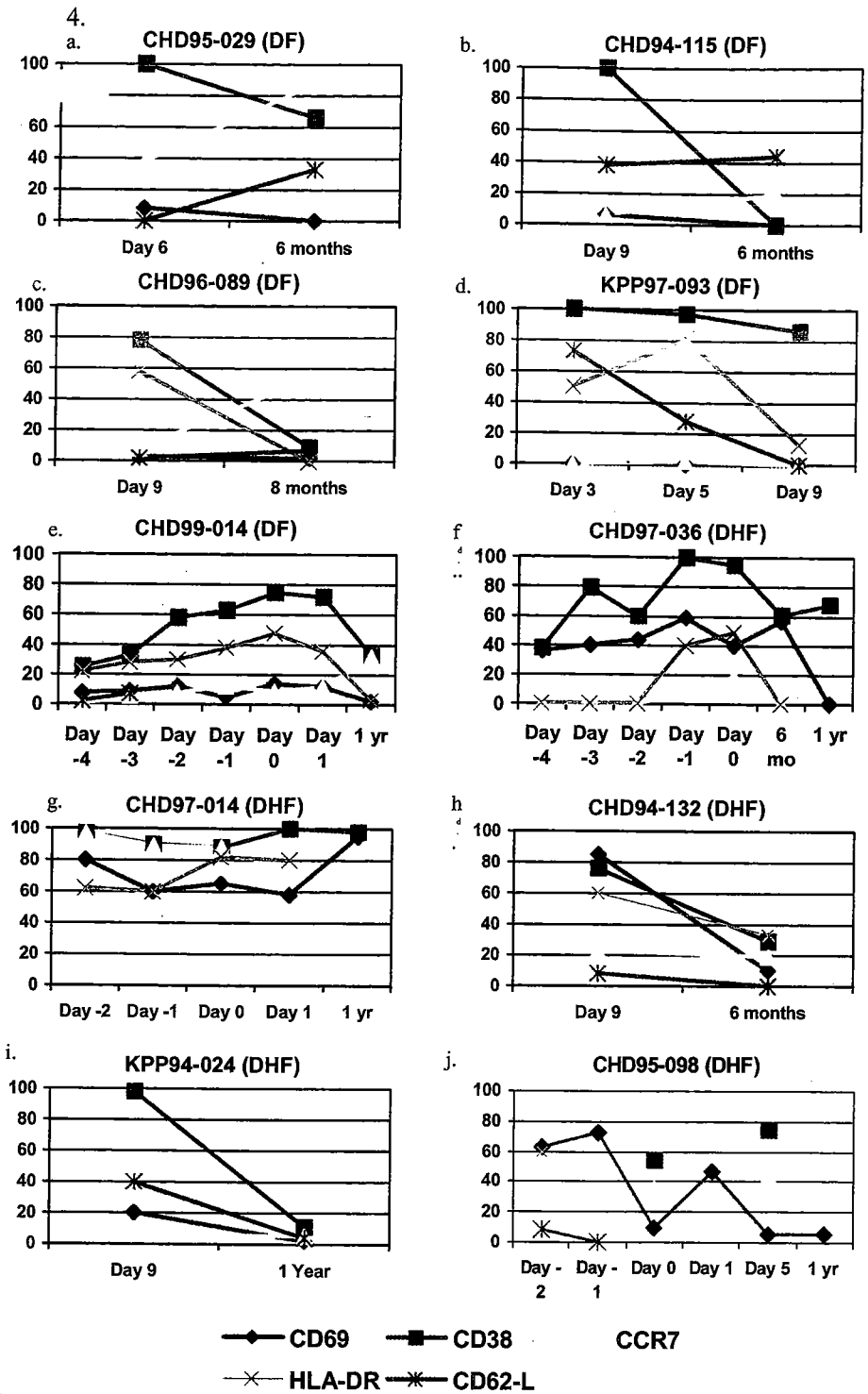


Figure 15. Kinetics of expression of activation and differentiation markers on tetramer+ T cells: Patient PBMC obtained from both acute phase of illness (Days 1-8) and convalescence were co-stained with tetramer and CD69, CD38, CCR7, and HLA-DR. Populations gated for CD3+ CD8+ staining were further analyzed for staining by tetramer and surface mAbs. The percentages shown in the graphs indicate the percentage of marker+ cells within the tetramer+ population.

the presence of other activated T cells.

In contrast to the CD69 pattern of expression, ~ 80% or more of the tetramer$^+$ cells were CD38$^+$ by day 5 in all the donors in acute illness, and remained high in some donors even during convalescence (Figure 15A,D,F,G). There was donor-to-donor variability in the expression of HLA-DR on tetramer$^+$ cells. In general, HLA-DR expression was higher in the acute stage than during convalescence. However, one patient showed sustained high expression of HLA-DR into convalescence (Figure 15C), and one patient showed higher HLA-DR expression during convalescence as compared to the acute stage (Figure 15G).

The tetramer$^+$ cells in all 4 DF donors and 2 of the DHF donors studied were predominantly CCR7$^-$ with ~ 20% of tetramer$^+$ cells expressing CCR7 in the post-defervescence stage. This suggests that most of the epitope-specific T cells in the periphery are effector T cells at the time of viral clearance. CCR7 expression was restored in DF subjects at the convalescent visit, with up to 80% of tetramer$^+$ cells being CCR7$^+$. In contrast, in one DHF subject from whom multiple bleeds were available during the febrile stage, almost none of the tetramer$^+$ cells expressed CCR7 during acute disease and were still CCR7$^-$ in convalescence. This low expression of CCR7 during convalescence was also seen in the other DHF subjects suggesting that the majority of the epitope-specific T cells that are present in the post-DHF repertoire have retained an effector memory phenotype.

Several recently published studies of CD8$^+$ T cell responses during chronic infection have shown the expression of programmed death receptor -1 (PD-1) on antigen-specific memory T cells to correlate with impaired cellular functions and increased

specific memory T cells to correlate with impaired cellular functions and increased sensitivity to apoptosis (Day, et a., 2006; Petrovas, et al., 2006; Trautmann, et al., 2006). Since we and others have observed that dengue-specific $CD8^+$ T cells show decreased ability to proliferate and secrete IFNγ in the early stages of acute infection, we examined the dengue $tetramer^+$ cells for PD-1 expression. In both the DF and DHF donors tested, PD-1 was expressed on more than 90% of the $tetramer^+$ population at the peak of the acute response while being completely absent in the convalescent samples (Figure 16A, 16B). Interestingly, PD-1 was also upregulated on a significantly large portion of the $tetramer^-$ $CD8^+$ T cell population, which may explain the profound immunosuppression of T cell responses seen immediately following viral infection.

D. Serotype cross-reactivity of $tetramer^+$ cells:

We were next interested in further analyzing the epitope-specific response for serotype cross-reactivity since the patients included in the study had secondary dengue infections with known viral serotypes. PBMC taken around the time of defervescence from 2 DHF patients with secondary D3 infections were stained simultaneously with tetramers containing the D1 variant as well as the D2/3/4 sequence. In one patient, the $tetramer^+$ response on fever day -1 was represented by a population of cells which were either cross-reactive (0.94%), or specific for the D1 sequence (~ 2%) with fewer cells being specific for the secondary serotype (0.41%) alone (Figure 17A). However, 2 days later, the epitope-specific response had altered such that fewer cross-reactive cells were seen and most of the $tetramer^+$ cells were specific for the D1 variant sequence alone. The opposite trend was seen in another patient with regard to the changing avidities of the

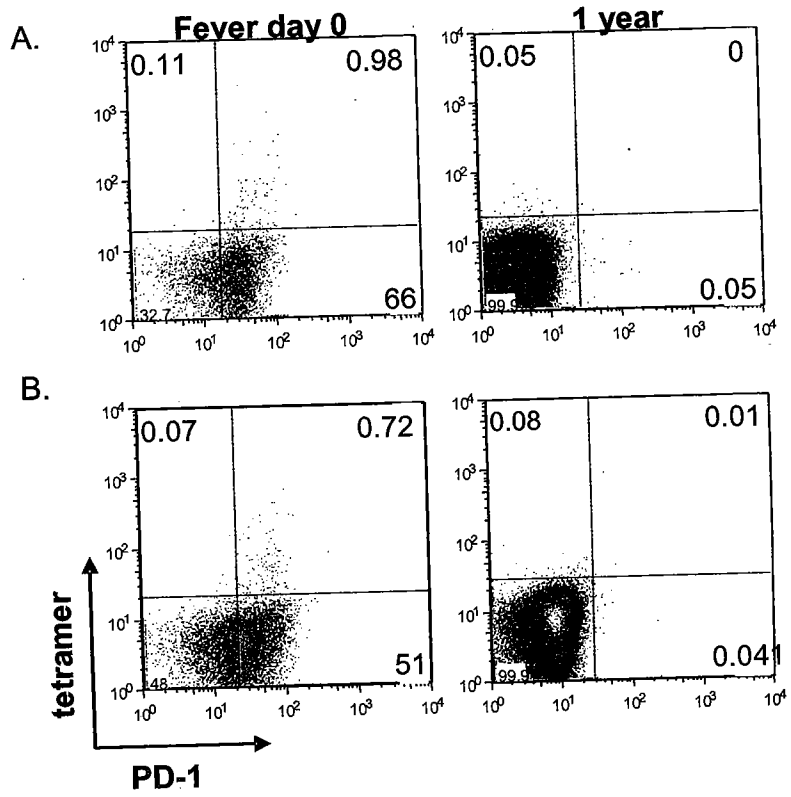


Figure 16. Expression of PD-1 during acute illness and convalescence: PBMC obtained on fever day 0 (day of defervescence) and from 1 year after the resolution of illness from (A) patient 97-036 (DHF) and (B) patient 99-014 (DF). Cells were co-stained with APC-conjugated D1-tetramer and FITC-conjugated anti-PD-1. Populations were gated on CD3+CD8+ cells and then analyzed for PD-1 expression. The percentages shown in the graphs indicate the percentage of marker+ cells within the tetramer+ population.

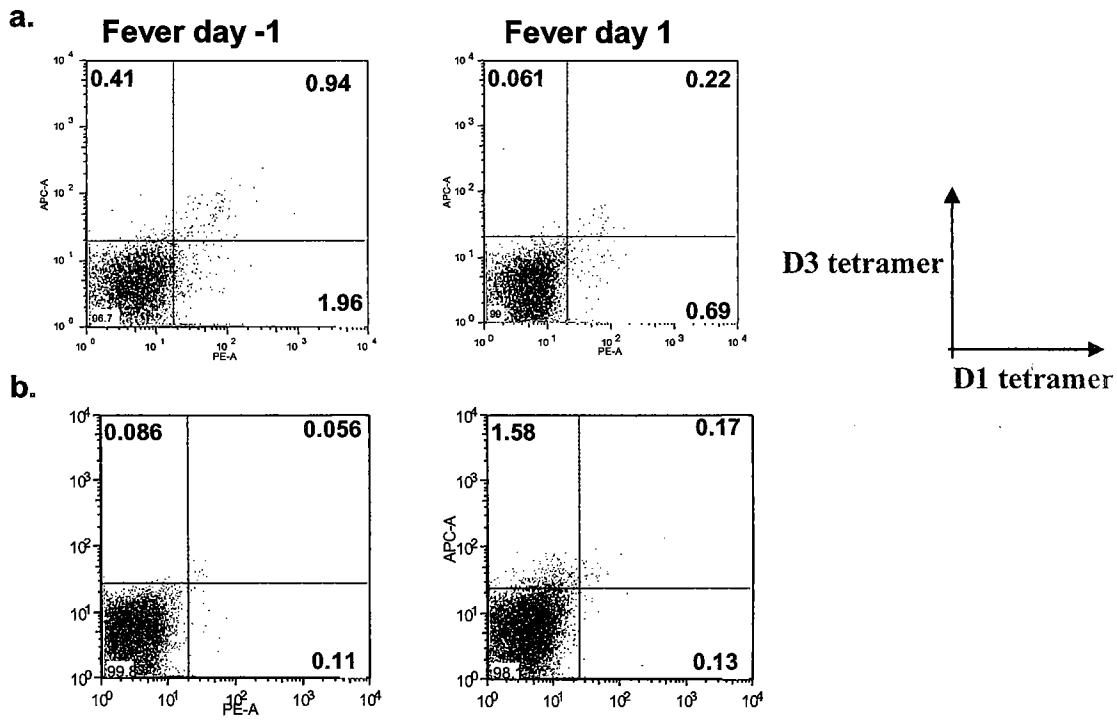


Figure 17. Serotype cross-reactivity within the tetramer+ T cell population: PBMC were obtained on fever day -1 (day before defervescence) and fever day +1 (day after deferevescence) from (A) patient 97-104 and (B) patient 97-036, both of whom had secondary D3 infection. Cells were simultaneously stained with PE-conjugated D1 tetramer and APC-conjugated D2/3/4 tetramer. The numbers within the quadrants represent the percentage of tetramer+ cells within the CD3+CD8+ gate.

epitope-specific response over time. The total tetramer$^+$ CD8$^+$ frequencies in this patient was less than 0.3% on fever day -1 with few cells staining with both tetramers and most of the cells with higher avidity for the variant D1 tetramer (Figure 17B). On fever day +1, the total tetramer$^+$ population had expanded 6-fold to 1.88% and a majority of these cells now showed higher avidity for the tetramer containing the homologous D2/3/4 sequence. These data suggest that the response to a secondary serotype can vary in overall avidity over the time course of the response with T cells with higher avidities for the infecting serotype disappearing over time or vice-versa.

E. Chapter summary:

Our objective was to study the kinetics of the antigen-specific T cell response in dengue-infected subjects in order to determine if the frequency and phenotypic profile of tetramer$^+$ T cells in DHF patients is different from that found in patients with DF and thereby understand the basis for DHF immunopathology in humans. We studied the NS3 222-231 specific responses in 10 patients (5 DF; 5 DHF) and found that:

- The magnitude of the response correlated with disease severity both during acute illness as well as during convalescence.
- Tetramer$^+$ cells were found as early as 5 days before defervescence and the peak of the response occurred around the day of defervescence.
- Tetramer$^+$ cells detected early during acute illness had a phenotype consistent with recent activation as seen by CD69 staining, with DHF patients showing higher percentages of tetramer$^+$CD69$^+$ cells than DF patients.

- Tetramer$^+$ cells from DHF patients showed low CCR7 expression even during convalescence indicating an altered funcational capacity.
- A majority of the tetramer$^+$ expressed PD-1 during acute illness. PD-1 was also expressed on about 50% of tetramer$^-$ CD8$^+$ T cells during this time.
- Simultaneous staining of PBMC from 2 DHF patients with homologous and heterologous tetramers showed serotype cross-reactive responses in both with the avidities of T cell for both tetramers changing over the time course of infection.

Our results show that the T cell response to secondary dengue infection is mediated by a heterogeneous population of memory CD8 T cells that undergo a robust program of expansion during acute infection. These virus-specific T cells persist in high numbers even after the viremic phase has ended. This study strengthens the view that DHF immunopathology is a consequence of an early and enhanced immune response

CHAPTER V

NATURAL EPITOPE VARIANTS MAY MODULATE IMMUNE RESPONSE TO SECONDARY DENGUE INFECTION BY INDUCING FUNCTIONAL DICHOTOMY IN MEMORY $CD8^+$ T CELL CLONES

Our studies on post-vaccination responses in individuals immunized with a single dengue serotype showed a functional heterogeneity which appeared to have a unique signature in each individual (Chapter III; Bashyam, et al., 2006). Antigen-specific $CD8^+$ T cells responded to natural variant peptides from heterologous serotypes in a manner which was both subject-specific as well as peptide/variant-specific. For example, one patient showed strong TNFα responses to stimulation by all peptide variants while the single variant of some peptides induced the strongest overall response in all vaccinees regardless of their primary serotype.

Other aspects of functional heterogeneity were revealed in peptide/variant stimulated short-term bulk cultures. The sequences which induced the highest cytokine responses in direct *ex vivo* assays did not always induce the strongest proliferative response in a CFSE-based assay which tracked both the expansion and cytokine production of antigen-specific cells in response to sequential stimulation by homologous and heterologous peptides. While the parameters of this study precluded us from making connections between a functional profile and the corresponding clinical outcome, our results suggested that the variation in the responses could reflect the emergence of a specific set of antigen-specific clones with different functional avidities for cognate vs. altered ligands during secondary infection.

Our third aim, therefore, was to examine the patterns of heterogeneity that exist at the clonal level in response to varying ligand concentrations and the difference in functional profiles between different clones which have the same specificity.

A. Isolation of serotype cross-reactive $CD8^+$ T cell lines and their specificities.

All the $CD8^+$ T cell clones described in this section recognize different epitopes on the NS3 protein (Table VII). The HLA-B*62 restricted clones were previously isolated from the PBMC of the D3 immune donor after stimulation with the D2 peptides from PBMC obtained 13 years post-vaccination (Zivny , et al., 1999). The HLA-B*07-restricted clone 11E was isolated from the convalescent PBMC of a D1 infected donor after stimulation with a D1 peptide (Zivna, et al, 2002). These clones were expanded in media containing IL-2, anti-CD3 mAb, and allogeneic irradiated feeder cells and allowed to rest for 10 days after re-stimulation before being used in experiments.

The HLA-A*1101 epitope was originally described by Mongkolsapaya, et al., (2003) in Thai donors. We found that this epitope and its variants were recognized by our D1 vaccinee as well, with the D3 peptide stimulating the strongest IFNγ response but the D1 tetramer showing the highest staining (Figure 18A). Tetramer staining and cytokine secretion seemed to be functionally segregated since there were no $IFN\gamma^+tetramer^+$ observed, and the hierarchy of the frequency $IFN\gamma^+$ response (D2>D3>D1) did not match the hierarchy of tetramer staining (D1>D2>D3).

We used PBMC taken 4 months after D1 vaccination to isolate cell lines. Two HLA-A*1101-restricted cell lines which were cross-reactive for all variants were isolated directly from limiting dilutions of peptide-stimulated bulk cultures (Figure 18B).

Table VII: Specificity of serotype cross-reactive dengue-specific $CD8^+$ T cell clones:

Donor (Primary serotype)	Epitope	Sequences	HLA restriction	Secondary serotype (*In vitro* stimulation)	T cell clone[a]
D3 donor	NS3 235-243	D1:ALKGMPIRY D2:ALRGLPIRY D3:AMKGLPIRY D4:ALRGLPIRY	HLA-B*62	D2	JK 1F8 JK62
	NS3 71-79	D1:DVKKDLISY D2:SVKKDLISY D3:DVKKDLISY D4:DVRNDMISY	HLA-B*62	D2	JK41
D1 donor	NS3 527-536	D1:EARKTFVEL D2:EQRKTFVEL D3:ESRKTFVEL D4:EQRKTFVEL	HLA-B*07	D3	11E
	NS3 130-140	D1:GTSGSPIVNRE D2:GTSGSPIVDR D3:GTSGSPIINR	HLA-A*1101	D1 D2	3C2 10C11

[a] All the T cell clones except 3C2 were isolated following limiting dilution of bulk cultures stimulated with peptides and screening for cytolytic activity against autologous BLCLs pulsed with the same peptide used for stimulation.

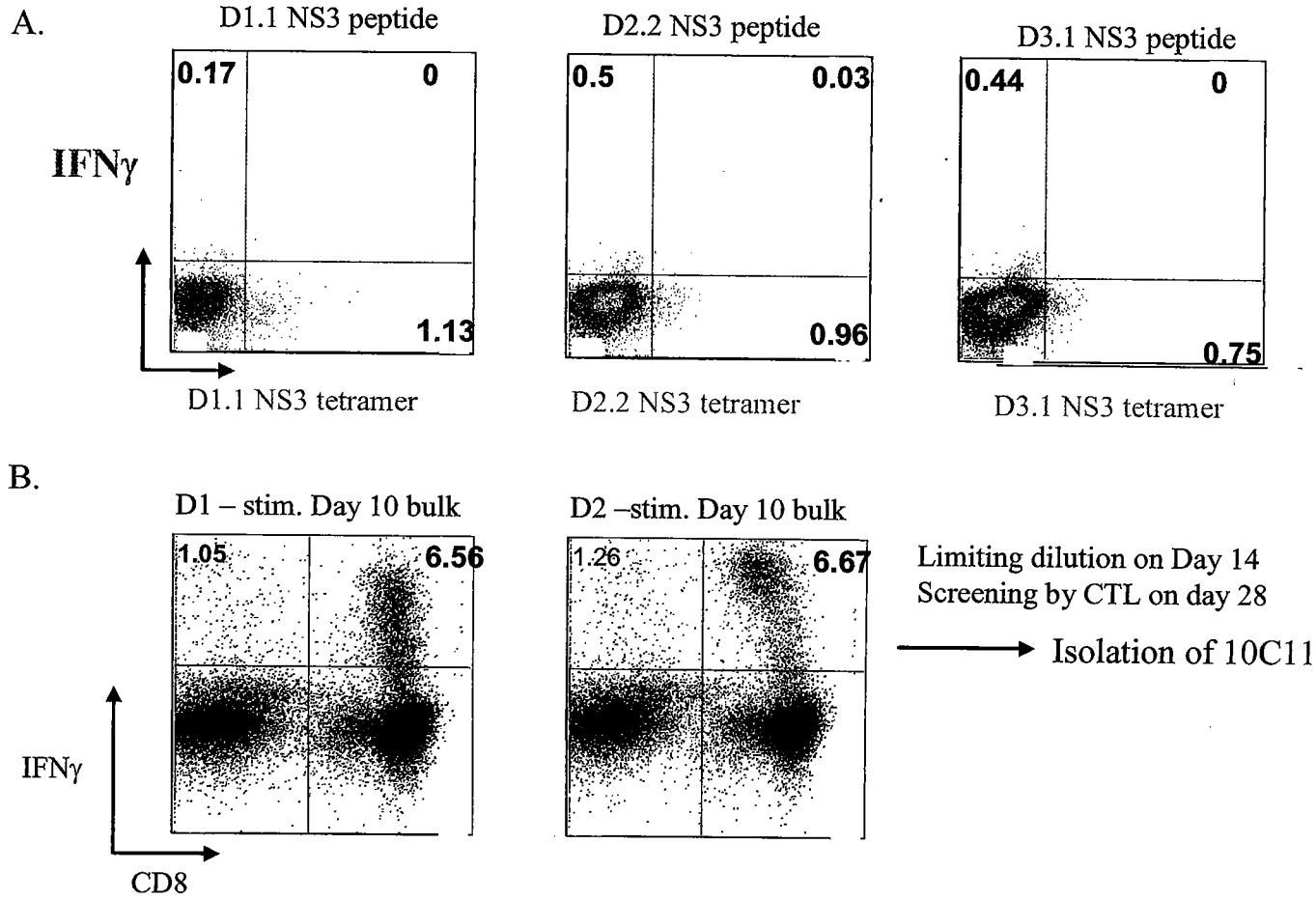


Figure 18: Responses to HLA-A*1101 restricted NS3 130epitope in D1 immune PBMC. (A) PBMC from a 4 mo. post-vaccination bleed were stimulated with 10μg/ml peptide for 6 hr and stained for tetramer and intracellularIFNγ. (B) PBMC were stimulated with10 μg/ml peptide and cultured in media containing IL-7 and IL-2 for 10 days. Bulk cultures were co-cultured on day 10 with peptide-pulsed autologous BLCLs for 6 hr and stained for IFNγ.

Simultaneous staining for IFNγ and tetramer binding of the bulk cultures that were re-stimulated for 6 hrs showed a heterogeneous population where there were twice as many $IFN\gamma^+tetramer^+$ cells as those that only produced IFNγ or only bound tetramer (Figure 19A). We attempted to isolate clones which could have such divergent effector profiles by sorting for tetramer staining (Figure 19B). The limiting dilution of cells recovered from the tetramer sort yielded one viable cell line 3C2 which was cross-reactive for all variants by CTL but did not stain with the D2 tetramer (Figure 19C).

B. Selective induction of effector functions

One mechanism by which functional heterogeneity can be established within a clonal population with a single specificity is the selective activation of some effector functions. Clone JK62 was previously found to be cross-reactive for D2 and D3 (homologous) sequences but not the D4 variant of NS3 71 peptide (Zivny, et al., 1999). When we examined the cytokine responses by intracellular staining, we found that the clone was not as efficient at producing IFNγ, TNFα, or MIP-1β as compared to its ability to lyse cells (Figure 20). In response to D3 stimulation, 14% of the responding cells were $IFN\gamma^+TNF\alpha^+$, only 4% were $IFN\gamma^+MIP\text{-}1\beta^+$, and around 2% of the total responding cells produced a single cytokine. In contrast, heterologous stimulation with D2 sequence induced poor IFNγ, TNFα, and MIP-1β despite activating robust cytotoxicity in this clone indicating a segregation in the activation of various effector functions.

This selectivity in the induction of cytolytic vs cytokine functions was also seen in clone 11E specific for the NS3 527 peptide (Figure 21A, 21B). While the heterologous D3 variant induced >60% lysis of autologous targets even at low peptide concentrations,

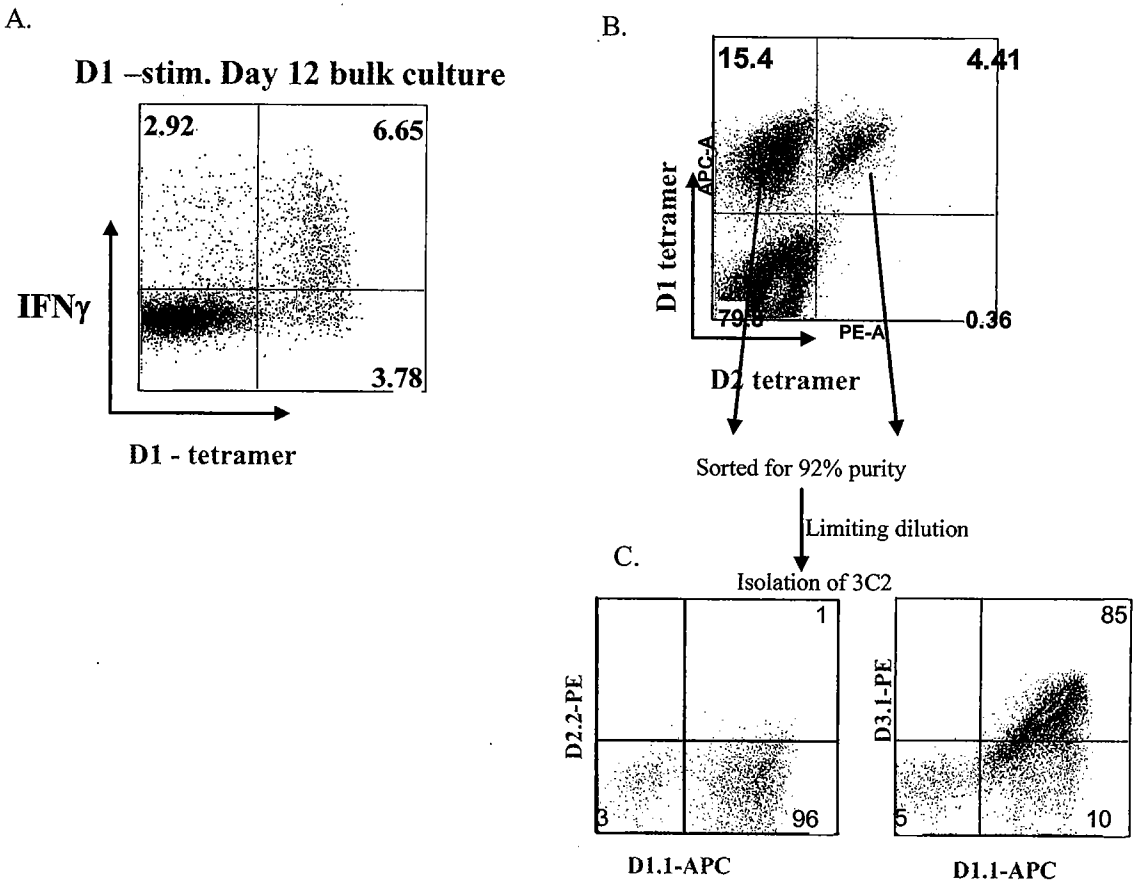


Figure 19: Functional heterogeneity in bulk T cell responses and isolation of clone by tetramer sort. D1 immune PBMC were stimulated with D1 NS3 130 and cultured for 12 days. (A) On day 12, bulk culture cells were stimulated with 10ug/ml peptide for 6 hrs and analyzed for tetramer binding and IFNγ production. (B)Bulk culture cells were co-stained with variant tetramers in order to identify cross-reactive and serotype-specific populations and sorted. (C) Limiting dilution of sorted cells yeilded one long-term clone which was co-stained with variant tetramers.

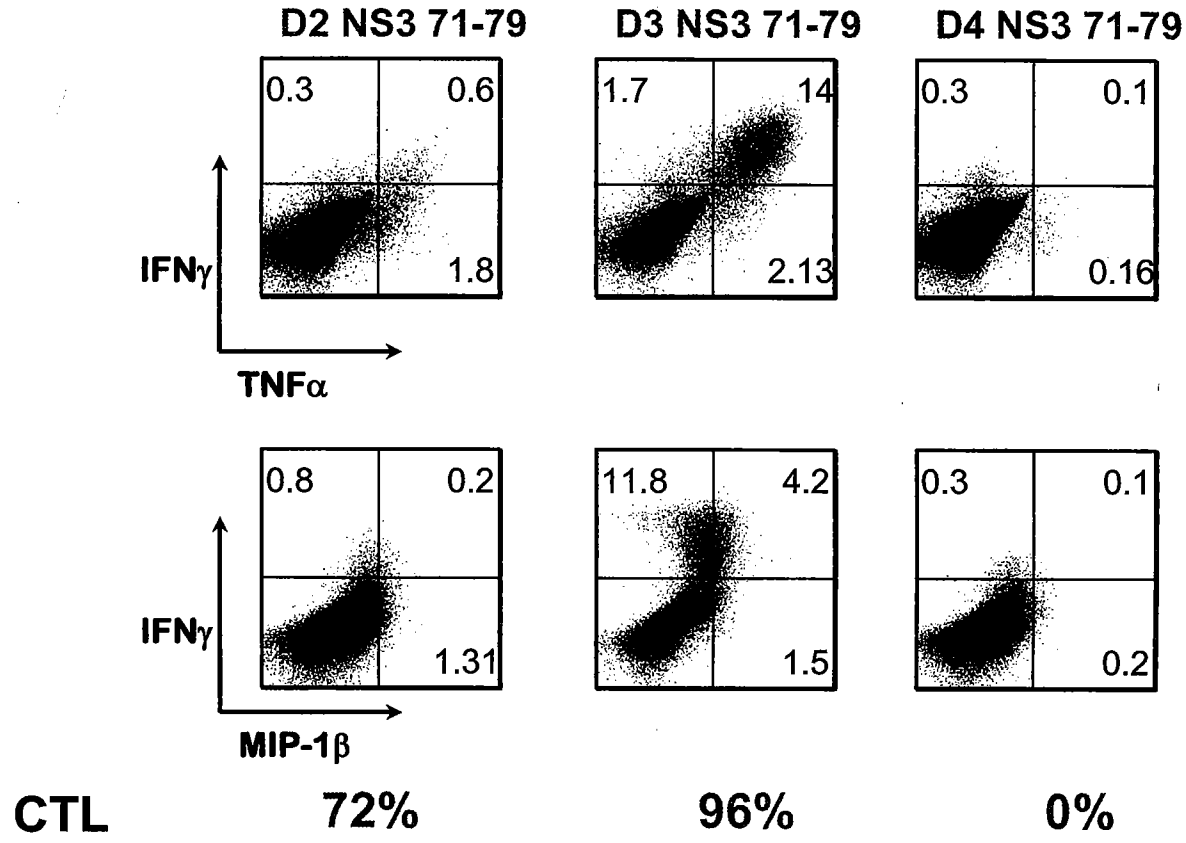


Figure 20: Segregation between cytokine production and cytolysis in effector response to variant ligand stimulation: Clone JK62 isolated from a D3-immune donor was tested for its response against all the variants in 6 hr ICS assays where cells were co-stained for IFNγ, TNFα, and MIP-1β. At the same time, they were also tested in CTL assays against peptide-pulsed autologous targets. Peptides were used at 10μg/ml concentrations in all assays. Both assays were performed at least twice with this clone.

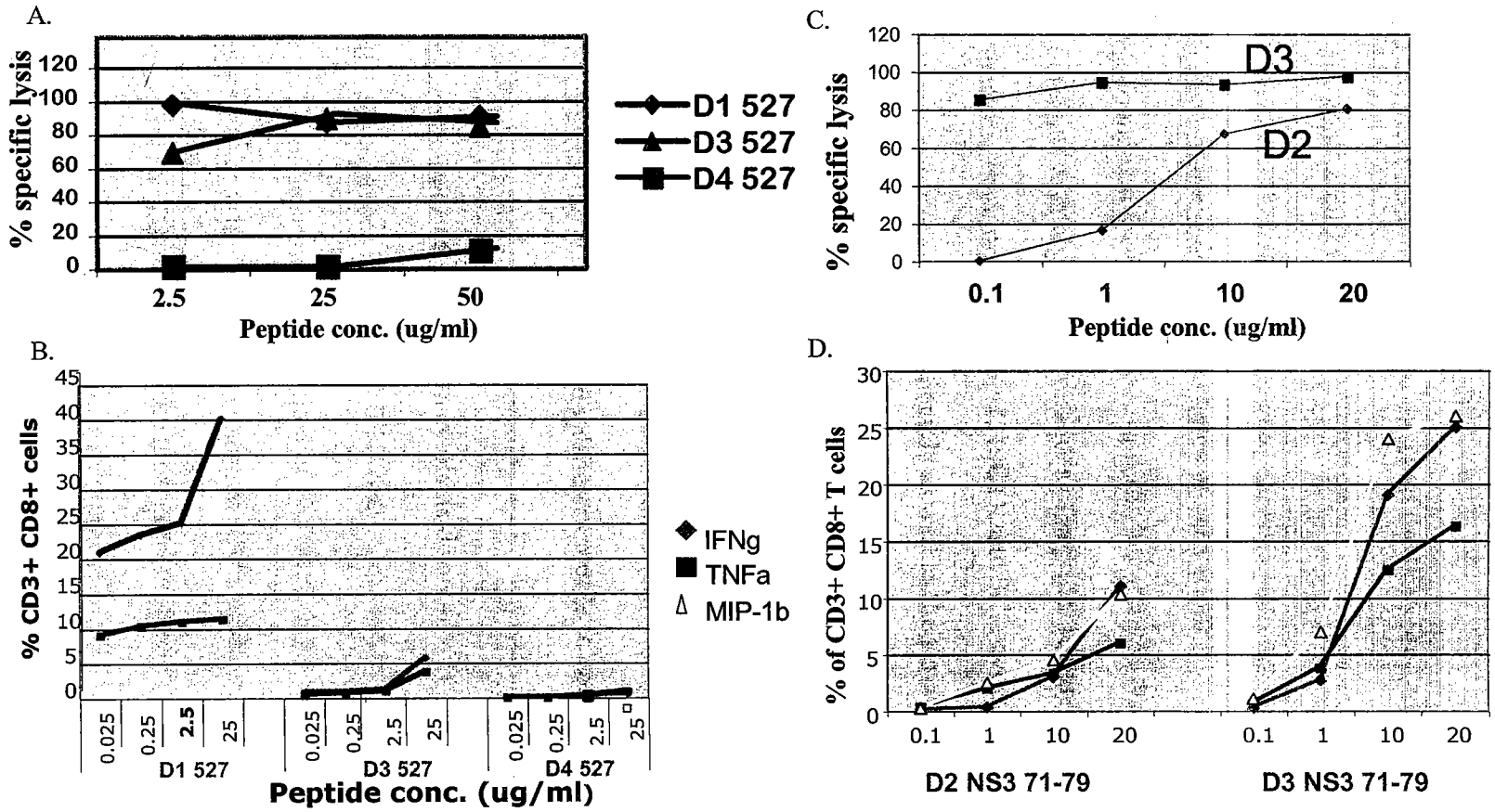


Figure 21. Alteration of functional profile in response to partial agonists: The cytolytic and cytokine responses of HLA-B*07 restricted 11E clone (a, b) and HLA-B*62 restricted JK41 clone (c, d) were measured to varying concentrations of peptides. The responses to the homologous sequences (primary serotype) are shown in blue.

<5% of cells were activated to produce IFNγ or TNFα even at high peptide concentrations. Stimulation with the homologous D1 peptide also induced diversity within the cytokine response with less than half the IFNγ producing cells also producing TNFα at any peptide concentration.

C. Hierarchy of functions and thresholds of activation:

We found that there was a clone-specific hierarchy in the activation of different effector functions with higher concentrations of peptides required to elicit more than one effector function. For clones 11E, JK41, 10C11, and 3C2, cytolysis was induced at lower concentrations of both homologous peptide as well as the variant ligands as compared to cytokine production (Figure 21, 22). The 10C11 clone had the same response profile to all 3 sequences with cytolysis being elicited first, higher concentrations being required for the production of IFNγ and MIP-1β, and even the highest concentrations of peptide failing to induce the production of TNFα (Figure 22A).

In contrast, the 3C2 clone had a different profile to each of the three variants (Figure 22B). Stimulation with the homologous D1 sequence induced robust responses only at concentrations of 1μg/ml with cytolysis/degranulation responses in 80% of the cells, MIP-1β responses in 25%, and IFNγ responses seen in <10% of the cells. In contrast, D2 peptide stimulation resulted in decreased degranulation but not cytolysis, and both IFNγ and MIP-1β production seen in <10% of the cells at the highest peptide concentrations. The D3 variant was the most potent ligand for this clone since the threshold for the activation of effector function was 3 logs lower as compared to the other two sequences. While the induction of cytolysis required the mimimum concentration of

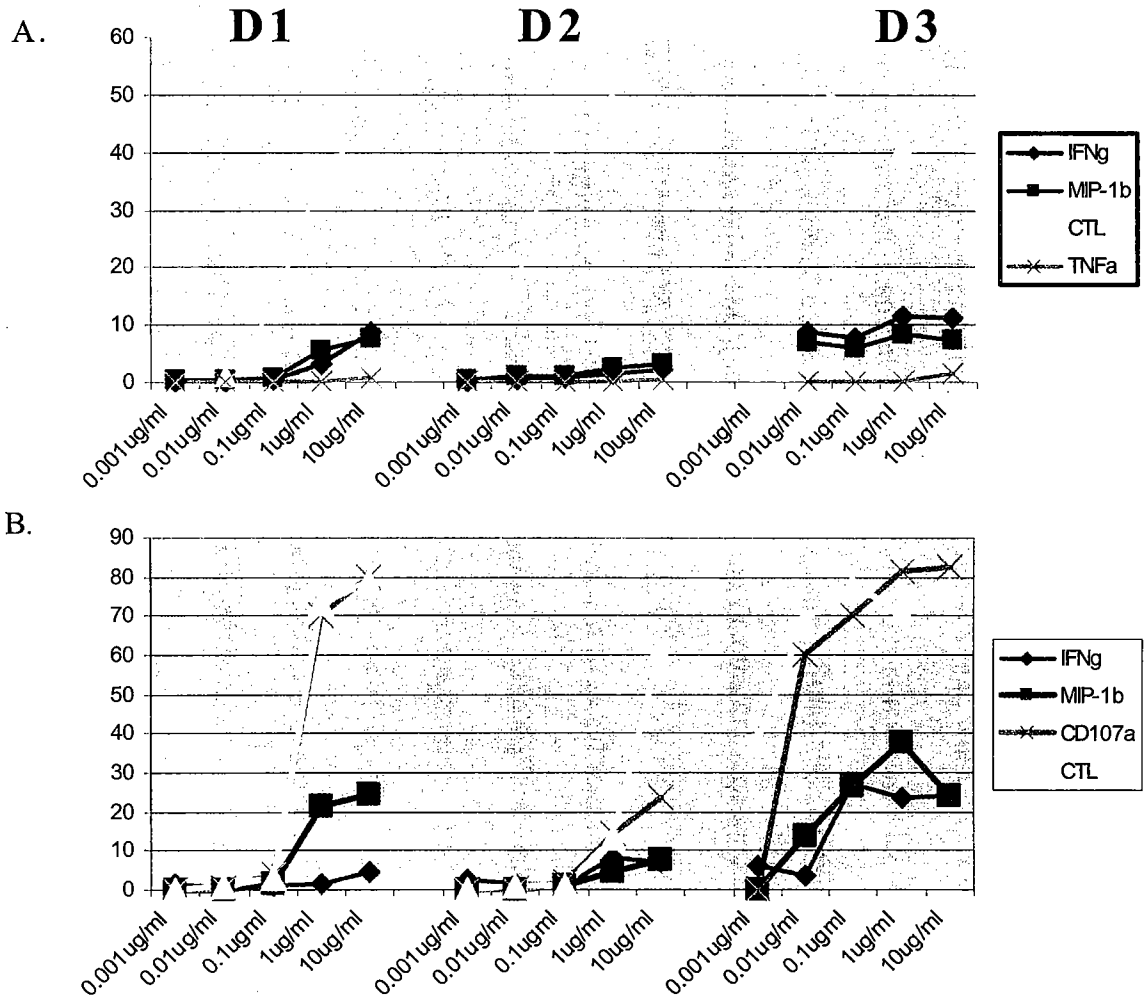


Figure 22. Alteration of functional profile in HLA-A11*01 restricted clones specific for NS3 130: The cytolytic and cytokine responses of 10C11 (a) and 3C2 (b) to varying concentrations of peptides presented on autologous BLCL. While both clones were derived from the same D1 immunized donor, 10C11 and 3C2 were isolated from D2 and D1 stimulated bulk cultures respectively.

peptide at 0.001μg/ml, degranulation was detected at 0.01 μg/ml, and IFNγ and MIP-1β had a similar dose response curves with the peak responses seen in 25- 40% of the cells at >0.1μg/ml.

D. Avidity differences as measured by tetramer staining:

We also examined the ability of peptide-stimulated T cells to bind tetramer in order to analyze avidity of the cross-reactive T cells for their variant ligands as measured by TCR down-regulation. Clone 1F8 showed robust and equal cytolysis of cells pulsed with 10μg of all three variants but a hierarchy in IFNγ responses with the highest response of 82% seen for the D3 peptide, followed by D1 in 75% and then D2 in 54% of the cells (Figure 23).However, tetramer staining showed that, while 100% of the cells bound tetramer when left unstimulated, about 8% of cells stimulated with the D3 peptide and producing IFNγ showed decreased tetramer staining. Since such a down-regulation was not seen with the other two peptides suggesting that the D3 sequence is a more potent ligand than D1 and D2.

In another example, the 10C11 cell line isolated from D1 immune PBMC after *in vitro* D2 stimulation showed strong cytolytic responses against all variants of the HLA-A*1101 restricted NS3 130 epitope even at very low concentrations of peptide and very low cytokine responses even at the highest concentrations of peptide (Figure 22A). However, only ~15% of the cell line bound tetramer when unstimulated, and activation with various peptides caused a dose-dependent loss of tetramer staining at different rates depending on the sequence (Figure 24). A higher percentage of cells secreted IFNγ at lower concentrations of D2 peptide than D1 peptide. In spite of this higher sensitivity to

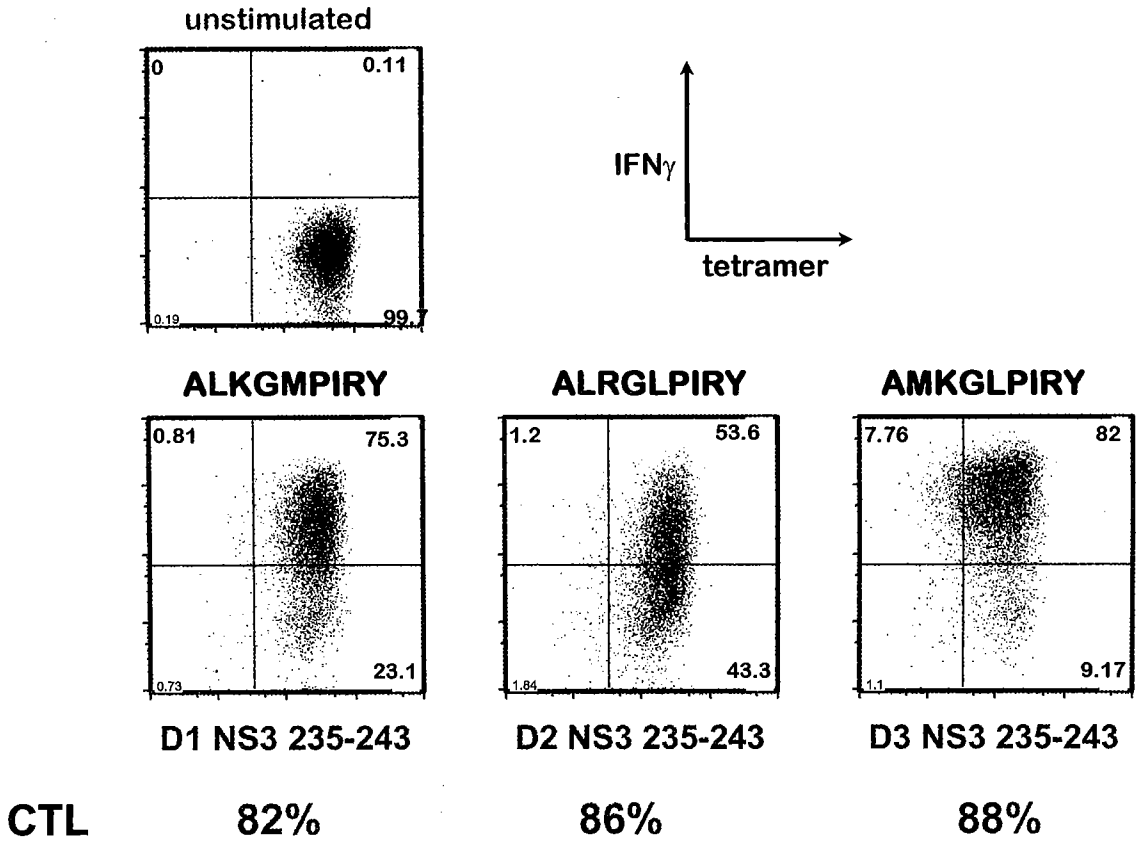


Figure 23: Tetramer down-regulation in IFNγ producing peptide-stimulated T cells: Clone1F8 isolated from a D3 immune donor was tested for its response against the D2 and D4 variants in 6 hr ICS assays where cells were co-stained for IFNγ and D3-tetramer. At the same time, they were also tested in CTL assays against peptide-pulsed autologous targets. Peptides were used at 10μg/ml concentrations in all assays. Both assays were performed at least twice with this clone.

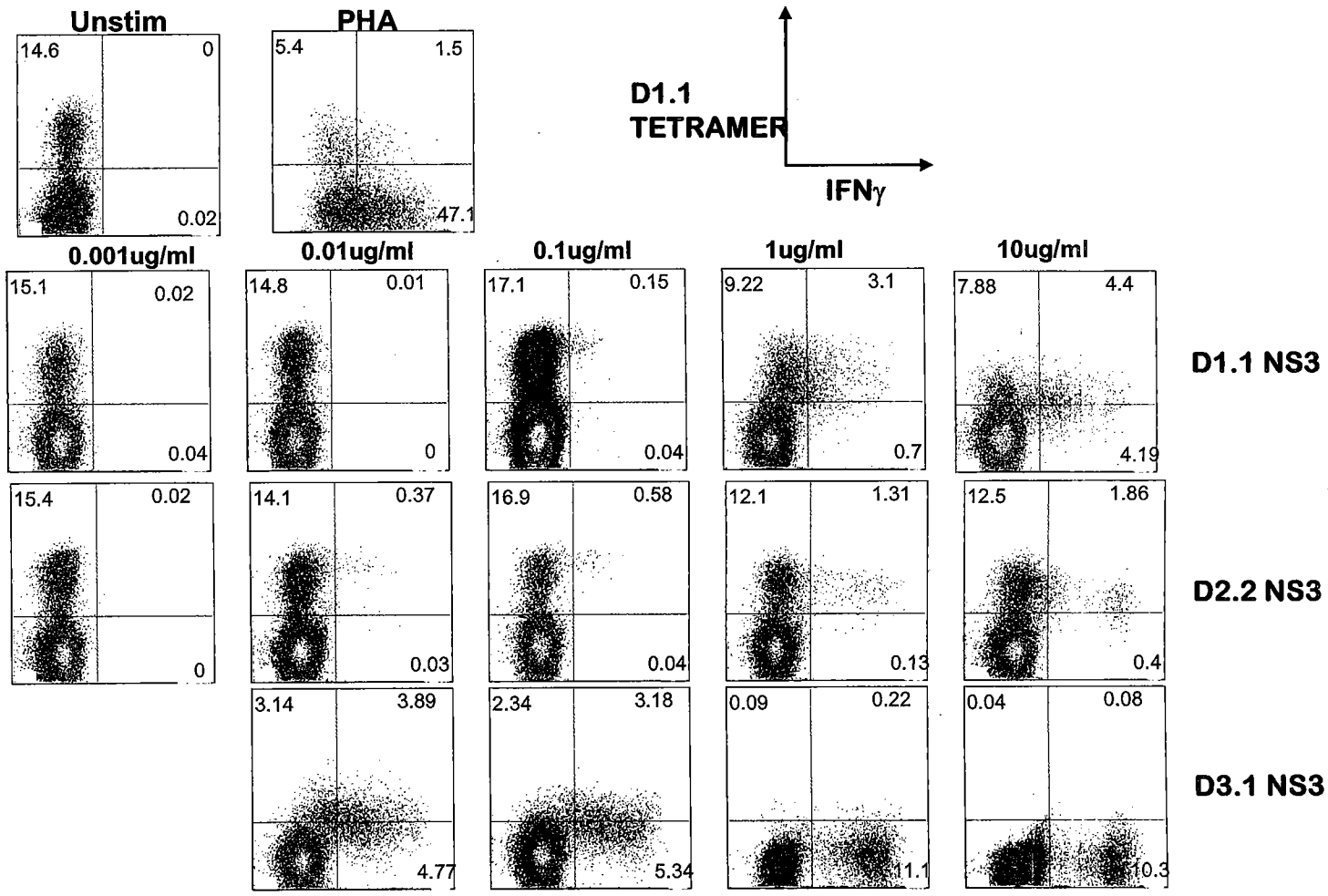
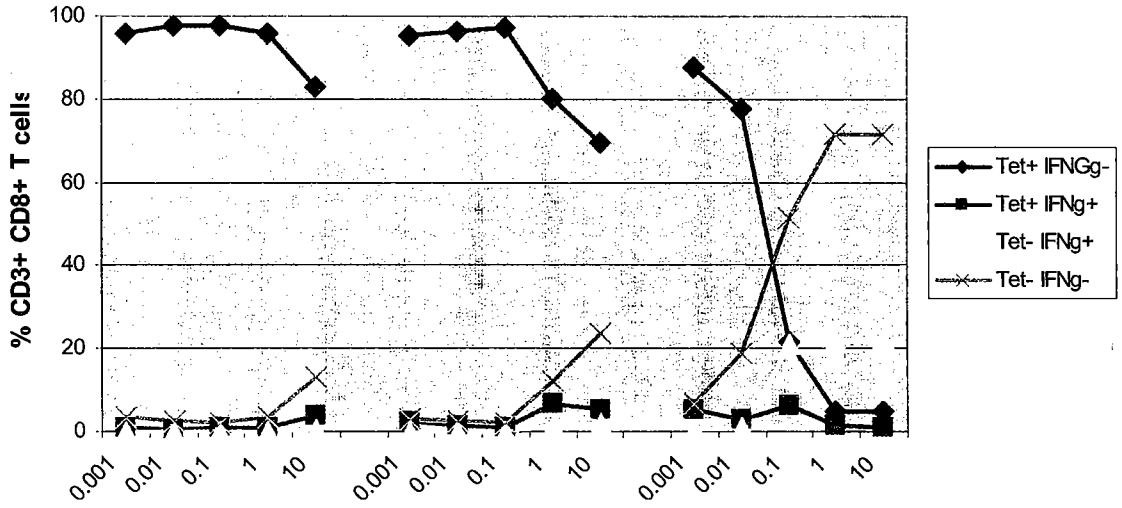


Figure 24: Peptide dose-dependent down-modulation of tetramer in activated cells: The 10C11 line was isolated from D1 immune PBMC after *in vitro* D2 peptide stimulation and tested in dose-response ICS 6 hr assays against peptide-pulsed autologous BLCL. Cells were stained with tetramer, permeabilized and fixed, and stained with anti-IFNγ antibody. Plots show $CD3^+$ $CD8^+$ gates cells.

D2 in terms of IFNγ production, D1 was the more potent ligand since D1 activated cells down-regulated their TCR-bound tetramer at 1μg/ml while very little down-regulation was seen in D2 activated cells even at the highest concentration. In comparision with D1 and D2, the heterologous D3 peptide was the ligand to which the cell line had the strongest avidity since even the lowest concentration of D3 not only induced IFNγ secretion in half the responding population but also resulted in loss of tetramer staining in all of these cells.

The D3 sequence proved to be a strong agonist in clone 3C2, which also has the same specificity. As seen in Figure 22B, this clone was proficient in degranulation and cytolytic activity at the lowest peptide concentration of 0.001μg/ml with higher concentrations of 1μg/ml of the D3 peptide required to elicit cytokine function in a third of the responding population. Co-staining of functional markers with tetramer shows that although ~100% of the clone bound tetramer when left unstimulated or stimulated with less than 1μg/ml of D1 or D2 peptide, cells started to down-regulate tetramer-bound TCR when stimulated with as little as 0.001μg/ml (Figure 25A, 25B). The dose response to the D3 sequence also showed that, although very few cells retained the ability to bind tetramer at high peptide concentrations, not all tetramer$^+$ cells which down-regulated tetramer were able to degranulate or produce IFNγ, and this population was in fact larger than the numbers of cells in which the loss of tetramer staining was accompanied by one or more effector function. This suggests that functional heterogeneity is even found within a clonal population where all the cells have the same specificity but different functional avidity for the ligand.

A.



B.

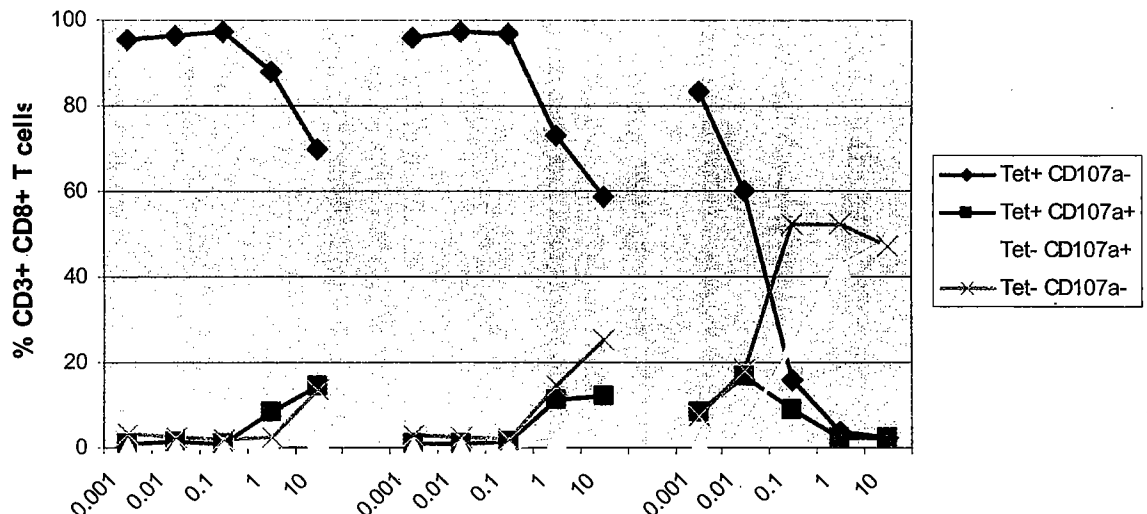


Figure 25: Comparison of functional avidities to variant ligands: The 3C2 clone isolated from D1 immunized donor following *in vitro* stimulation with D1 peptide was stimulated in 6 hr ICS assays to measure degranulation, IFN? production, and ability to bind tetramer. The responses are graphed on $CD3^+$ $CD8^+$ lymphocytes which bound tetramer but were not functionally active ($Tet^+IFN\gamma^-$ and $Tet^+CD107a^-$), functionally active while still able to bind tetramer ($Tet^+IFN\gamma^+$ and $Tet^+CD107a^+$), activated cells which lost tetramer staining ($Tet^-IFN\gamma^+$ and $Tet^-CD107a^+$), and cells which down-regulated tetramer but did not show function ($Tet^-IFN\gamma^-$ and $Tet^-CD107a^-$).

E . Chapter Summary.

We examined the functional profiles of 6 different clones isolated from 3 different donors and specific for 4 different epitopes by stimulating them with varying concentrations of variant ligands and assaying for cytolysis and cytokine production. We found that:

- There was a segregation of effector function in response to some variants with only cytolysis but low or absent cytokine production seen in some cases. There was a further divergence of cytokine production within a clonal population with some cells producing IFNγ but not TNFα.
- There were distinct thresholds of activation for different effector functions with degranulation and cytolysis requiring the lowest doses of antigen. There was a further hierarchy within cytokine producing cells with increasing doses of peptide required to elicit cytokines in the following order: MIP-1β < IFNγ < TNFα.
- Down-regulation of tetramer in peptide dose response assays further defined functional hierarchy.
    - Not all tetramer$^+$ cells were functional
    - Cells responded functionally to variants despite being unable to bind that variant tetramer.
    - Cells which were activated to undergo degranulation and cytokine production down-modulated bound tetramer. A greater degree of down-regulation was observed in cytokine-producing tetramer bound cells than in degranulating tetramer-bound counterparts.
    - Cells lost their sensitivity to tetramer at high peptide concentrations

Some cells which down-regulated tetramer appeared to be anergic

The diverse functional profiles and the existence of distinct activation thresholds for different functions among these dengue-specific serotype cross-reactive clones have significant implications for the mix of cytokines, chemokines and cytotoxic T cells that are generated during secondary immune response to heterologous infection. The absence or reduction in the numbers of cells capable of a certain function such as IFNγ production could have a detrimental effect while contributing to protective immunity if the affected response happens to be TNFα or MIP-1β. Antigen levels are also likely to dictate the nature of the immune response since high peptide doses can elicit enhanced function or result in functional anergy while low peptide doses skew the profile into a predominantly cytolytic response.

CHAPTER VI

DISCUSSION

The selective expansion of cross-reactive $CD8^+$ T cells present in the antigen-specific memory repertoire during the immune response to a heterologous viral infection has been demonstrated in several models of viral infection. The response mediated by this reamplified population is not necessarily protective in nature and, in fact, has been shown in many situations to be immunopathologic (Selin, et al., 1998; Chen, et al., 2001; Mon, et al., 2003; Haanen, et al., 1999; Klenerman and Zinkernagel, 1998). In these studies, immunopathology was associated with attrition of response, an alteration in the nature of the effector functions of memory $CD8^+$ T cells, or impaired viral clearance. In chronic infections such as those caused by HIV and HBV, viral persistence has been attributed to the emergence of viral escape mutations and T cell epitope variants which act as antagonists of anti-viral CTL (Klenerman, et al., 1994; Bertoletti, et al., 1994). T cell antagonism has also been shown to result in protective memory response and survival in mice that would normally succumb to lethal immunopathology during co-infections with an antagonist-APL expressing variant virus (Hunziker, et al., 2002).

Thus, differences in amino acid sequence between an original T cell epitope and its variants can impact the functional outcome of a secondary infection. The four serotypes of dengue virus do not share complete homology, leading to the possibility that $CD8^+$ T cell epitopes not conserved between serotypes will function as altered peptide ligands during secondary infection and skew the memory response towards an immunopathological outcome.

This laboratory previously showed that a variant dengue peptide functioned as a partial agonist ligand by stimulating robust cytolysis but weak proliferation and IFNγ production (Zivny, et al., 1999). The goal of this thesis was to analyze the breadth and complexity of cross-reactive T cell responses to dengue virus. We studied uncloned T cell populations by stimulating with natural variants from heterologous serotypes in order to determine if stimulation with variant ligands would lead to altered profiles of effector function. We also examined dengue-specific serotype cross-reactive T cell clones in order to determine whether a polyfunctional response could exist within a clonal population, to understand how effector profiles changed in response to peptide dose, and to examine the changes in the functional hierarchy caused by dengue epitope variants which acted as partial agonists.

A. Epitope identification strategy

Previously identified $CD8^+$ T cell dengue epitopes were found to be restricted by infrequently found HLA alleles such as HLA-B*62 and HLA-B*07, and the frequencies of cells specific for some of these epitopes were also very low. Our strategy therefore was to identify new epitopes restricted by the HLA-A*0201 allele which is commonly found in SE Asian populations. We used a computer algorithm to predict 9-mer peptides from the sequences of D2 and D3 serotypes that would best fit the HLA-A*02 binding motif based on the half-life of dissociation of peptide-HLA complexes. The lists for each serotype had several sequences in common, with the binding scores being identical in a few cases and vastly different for most variants. However, the results from our screening assays did not reflect these differences.

The screening of these peptides in IFNγ ELISPOT assays showed very weak responses. Two possibilities that explain this result are that either the predicted peptides with high scores were not actually produced by the processing machinery of the cells, or that the primary immunization of the donor with the experimental vaccine induced cells with low affinity to the epitopes. However, we were able to detect peptide-specific responses in intracellular staining assays. It is possible that the two techniques differed in their results because of the different peptide concentrations used (4μg/ml peptide in ELISPOT vs. 10ug/ml in ICS), the negative effect of cryopreservation on the ability of IFNγ producing cells to form discernable spots in ELISPOT assays as reported by some groups (Weinberg, et al., 2002), or due to the activation of peptide-specific cells that are able to produce IFNγ but not secrete it. Of the 25 peptides tested, 4 were eventually identified as the strongest inducers of IFNγ and TNFα response in intracellular staining assays.

B. Quantitative and qualitative aspects of functional heterogeneity in uncloned PBMC.

We observed high frequencies of $CD8^+$ T cells to all four HLA-A*02 restricted epitopes in four vaccinated individuals. The total numbers of functionally active $CD8^+$ T cells specific for all four epitopes in each donor ranged from 0.7% to 4%, and the frequencies of cells specific for each epitope as measured by intracellular IFNγ secretion ranged from 0.12% to 0.64% of $CD8^+$ T cells. This is comparable to frequencies of 0.02% to 2.5% of $CD8^+$ cells specific for a HLA-A*11-restricted dengue NS3 epitope during the acute phase of illness and is higher than frequencies of 50 $IFN\gamma^+$ cells/10^6 PBMC seen

within 4 months of dengue infection in patients in Thailand and Vietnam (Mongkolsapaya, et al., 2003; Mongkolsapaya, et al., 2005; Loke, et al., 2001).

Staining for IFNγ, TNFα, and MIP-1β simultaneously revealed that very few or none of the cells produced all three cytokines, and that the majority of the responding population produced only one of the three cytokines. Although this heterogeneity of cytokine response in human PBMC to HIV and CMV epitopes has been reported elsewhere (Appay, et al., 2000; Sandberg, et al., 2001; Betts, et al., 2006), none of these studies examined the effect of variant peptide stimulation. We found that some variant sequences induced higher frequencies of $TNF\alpha^+$ cells and MIP-$1\beta^+$ than $IFN\gamma^+$ cells. One explanation for this finding is that these cells contain preformed TNF-α mRNA (Slifka, et al., 1999) and store MIP-1β in cytolytic granules that can be released even by low avidity T cell interactions with pMHC (Wagner, et al., 1998; Zaitsova, et al., 2001), which would lead to faster secretion kinetics for these two cytokines.

The detection of higher numbers of cells producing TNFα and MIP-1β than IFN-γ after peptide stimulation suggests that these cytokines might be a better indicator of the size of the epitope-specific population. It also would be interesting to analyze the cytokine secretion patterns of these epitope-specific T cells in the context of tetramer staining. This also could help answer the question of which cytokine accurately reflects the actual numbers of epitope-specific cells.

The heterogeneous cytokine expression we observed could be significant in determining the clinical outcome of the immune response. In addition to having antiviral properties, TNFα and MIP-1β also are inflammatory cytokines (Dayton, et al., 1985; Cocchi, et al., 1995). MIP-1β is a chemoattractant for both monocytes/macrophages and

$CD8^+$ T cells. The secretion of MIP-1β may result in immunopathology by two mechanisms: the recruitment of monocytes to the site of infection might result in higher viral load due to Ab-enhanced infection of these cells by virus, and the recruitment of activated T cells during secondary infection might result in higher levels of inflammatory cytokines. Thus, a situation where the secondary response in a D1 immune donor is dominated by cells specific for epitopes (NS4b 2423 and E493) whose heterologous variants (D2 and D3) not only enhance the size of the response but also preferentially activate MIP-1β or TNFα production, could result in increased disease severity (Figure 7; top panel).

C. Potential influence of primary and secondary serotype on disease severity.

The size and pattern of responses to each epitope and its variant were unique to each donor. However, within a donor, there were some similarities in the functional profile to different peptides. The cytokine response in the D3 immunized donor was characterized by low TNFα responses while in D2 immunized donor 3, the response to all stimulations was represented by all 7 sub-sets of cytokine producing cells (Figure 7; panels in 2nd and 3rd row). If these functional profiles are found to be consistent for a broader set of epitopes within each donor, the secondary response in these subjects would likely be dominated by T cells with subdued inflammatory and enhanced anti-viral properties.

Previous studies from our laboratory showed that dengue-specific $CD4^+$ T cells from immunized subjects showed the highest IFNγ response to inactivated lysates of Vero cells infected with the homologous serotype of dengue virus and to the homologous

sequence of known CD4 epitopes identified so far (Mangada, et al., 2004). The $CD8^+$ T cell response to individual epitopes did not follow the same trend. A single variant of two of the four epitopes studied induced the highest total cytokine response in all four donors. Also, the D3 sequence of the NS4b 2353 peptide induced cytokine responses in >1.5% of $CD8^+$ T cells in three of the four donors. The immunogenicity of peptides D2 NS4a 2148, D3 NS4b 2423, and D3 NS4b 2353 in these *in vitro* assays did not correlate with their algorithm-based predicted binding scores, which were low. However, it is possible that these three sequences have the highest immunogenicity *in vivo* since the binding scores do not take into account the half-life of dissociation between pMHC and the TCR complex.

Some epidemiological studies have associated dengue 2 and dengue 3 infections with greater disease severity (Sangkawibha, et al., 1984; Endy, et al., 2002; Leitmeyer, et al., 1999). In our studies, when the total cytokine responses to all four epitopes of each serotype were compared (Fig. 6), peptides of the D2 and D3 serotypes induced the highest responses in all 4 donors. A majority of our long-term T cell clones also showed a higher avidity to the D3 sequence of their epitopes. It is not known whether there are any molecular structures or other viral factors that determine D3 prevalence and support its association with severe outbreaks as compared to other serotypes (Nisalak, et al., 2003). A recent study which modeled the effects of ADE on the ability of co-circulating serotypes to survive and evolve showed that serotypes that undergo enhancement have a competitive advantage and are able to persist within an endemic population (Cummings, et al., 2005). It is possible that the D3 serotype, which is known to undergo ADE and co-circulate, has escaped this selection pressure by mutating antigenic epitopes. Although

responses to a broader group of epitopes would need to be examined in more subjects in order to make definitive conclusions about the immunodominance of D3 epitopes, these preliminary findings are consistent with the hypothesis that secondary infection with these two serotypes might result in enhanced cytokine response and result in immunopathology in HLA-A*02^{+} individuals.

D. Cross-reactivity and functional heterogeneity in short-term T cell cultures.

We developed a novel method to directly demonstrate serotype cross-reactivity among epitope-specific cells in CFSE-stained bulk cultures. These were set up by *in vitro* stimulation with the homologous peptide and sequentially stimulated after expansion by variant peptides. Proliferating cells underwent 8 divisions in about 10 days and the serotype-cross-reactive cells were identified by their CFSE^{low}IFNγ^{+} profile when re-stimulated with heterologous peptide (Figure 8). These cross-reactive cells were functionally diverse since there a significant fraction (16% - 32%) of these proliferated cells secreted TNFα but not MIP-1β, in contrast to the secretion of all three cytokines by ~100% of homologous peptide-restimulated cells.

There was also a substantial portion of CFSE^{low} cells which did not produce any of the three cytokines following either homologous or variant peptide stimulation or show tetramer staining (Figures 8, 10, 11). It is possible that these cells represent non-epitope specific cells that have undergone cytokine-stimulated, antigen-independent division. There was also a population of cells which had undergone 0-5 rounds of cell division that produced IFNγ following homologous peptide re-stimulation but did not bind the homologous tetramer. There is some evidence of an association between cell division and

the acquisition of effector functions where the cells that undergo the most proliferation are the ones that are most proficient at secreting IFNγ (Gudmundsdottir, et al., 1999). In that study, the authors examined the correlation between the degree of clonal expansion and secretion of different cytokines in OVA-specific $CD4^+$ T cells from transgenic mice and found that the division cycle not only affected the absolute numbers of cytokine producing cells but also the ability to secrete cytokines at the single cell level. Although we did not pursue the mechanisms which influence heterogeneity in the expanded populations, it is possible that $cytokine^+$ cells within the $CFSE^{mid\text{-}high}$ population are low affinity cells that have not completed their full program of cell division and hence have an incomplete activation profile.

E. Intraclonal heterogeneity and functional avidity of serotype cross-reactive T cell clones.

We simultaneously measured multiple effector functions of serotype cross-reactive T cell clones over a range of peptide doses to characterize the functional avidities of these clones for their cognate vs variant ligands. The stimulation of cross-reactive clones with variant ligands revealed an activation threshold difference in the requirements for the activation of cytolysis vs the production of various cytokines. Some variant ligands induced CTL responses even at low peptide concentrations but only induced cytokine production at high peptide concentrations or failed to induce any cytokine function. Other variants induced CTL at peptide concentrations higher than that required for cognate ligands in clones that showed very poor cytokine production. Thus there was a hierarchy in the induction of the two functions with cytolysis requiring less ligand concentration and having a lower activation threshold than cytokine production.

Our observation that all of our clones retain cytolytic ability even in the absence of other effector functions merits further discussion. For the successful elimination of dengue virus without the immunopathological after-effects, such a dichotomy between these two functions might be an advantage. Cytolysis and IFNγ production are known to have different signaling thresholds, with lower peptide concentrations being required to elicit lytic functions from a T cell clone *in vitro* (Valitutti, et al., 1996). If this is also the case *in vivo*, then dengue infections which result in low antigen load will be more easily cleared without the activation of a cytokine cascade that might result in DHF. The recovery of a large number of such clones as opposed to those with the reverse phenotype (more cytokine, less cytolysis) might either be a consequence of selecting for antigen-specific populations via CTL-based limiting dilution assays or may simply reflect the persistence of these "protective" clones within the memory PBMC population.

Other studies which examined murine flu-specific $CD8^+$ responses (La Gruta, et al., 2004), human flu-specific $CD8^+$ T cell clones (Valitutti, et al., 1996), or HIV-specific human $CD8^+$ T cell clones (Douek, et al., 2002) showed that there was a difference in the hierarchy of epitope-specific cytokine production between the primary and secondary response to the same epitope and that the concentration of peptide required for the induction of cytolysis<IFNγ<proliferation. However, the dependence of functional hierarchy on the level of T cell activation by variant ligands that we observed in our studies has only been previously reported in human $CD4^+$ T cell clones in the context of autoreactivity and activation by self-peptides and their synthetic variants (Itoh and Germain, 1997., Hemmer, et al., 1998; Itoh, et al., 1999).

A recent study which examined cytokine hierarchies in human $CD8^+$ T cell clones specific for a flu epitope and an EBV epitope showed a pattern of MIP-1β>IFNγ>TNFα (higher percentage of cells producing MIP-1β, followed by the others) response in clones cross-reactive for both epitopes but not in clones with a specificity for only one of the epitopes (Clute SC, 2005). In our studies, we observed that a similar hierarchy of cytokine response in serotype cross-reactive clones, which was both clone- and peptide-specific. However, we also found that the pattern of MIP-1β>IFNγ>TNFα seen in response to homologous peptide stimulation was altered following variant stimulation at different peptide doses. For example, the proportion of cells producing MIP-1β and IFNγ were identical. In other words, as variant ligand concentration increased, the ratios of cells producing the three cytokines changed in a clone-specific manner.

It has also been shown that the level of TCR down-modulation (an indicator of TCR engagement) has a direct correlation with the activation of T cell responses, since ligands that are more efficient at inducing functional responses induce TCR down-regulation more robustly than weaker ligands (Bachmann, et al., 1997; Hemmer, et al., 1998; Itoh, et al., 1999). We also observed this association in two dengue-specific T cell clones. The D3 variant to which both clones had the highest functional avidity induced the most profound loss of tetramer staining (Figures 24, 25). In contrast, the D1 and D2 ligands, which induced robust CTL but no cytokine response at high peptide concentrations, showed more modest decreases in tetramer binding ability. Interestingly, when we compared tetramer down-regulation between populations of cells which were $IFN\gamma^+$ versus $CD107a^+$, we found that there were more $CD107a^+$ cells which had down-regulated tetramer as compared to $IFN\gamma^+$ cells at higher peptide concentrations (Figure

27b). Since IFNγ production has been shown to be associated with significantly more ligand-induced TCR down-modulation than cytotoxicity (Valitutti, et al., 1996), we interpreted these results to mean that cells with different functional avidities could be found even within an activated clonal population of cells.

Interestingly, there was also a sizeable population of cells which had down-regulated tetramer but did not express an effector function. These cells may be temporarily anergized or may represent a distinct population of cells with effector functions other than the secretion of the three cytokines studied. We speculate that the former possibility is likely since a previous study showed that *in vitro* "resting" of CD4 T cells which had down-regulated their TCR Vβ chains but failed to produce IFNγ after sorting reversed the unresponsiveness to TCR engagement (Itoh and Germain., 1997).

F. Summary of *in vitro* results.

Overall, these *in vitro* data imply that (i) the cross-reactive T cell population that expands during secondary dengue infection will be influenced by antigen load and the functional avidities of the cross-reactive cells for the variant epitope. (ii) The altered TCR activation by variant epitopes during secondary infection can result in a polyfunctional T cell response. (iii) The relative frequencies of these T cell sub-sets will dictate the type and level of cytokines and chemokines produced and thus influence disease severity.

We were unable to compare these patterns of functional heterogeneity seen during *in vitro* analysis of secondary response in vaccinated individuals with the response seen in infected individuals due to limited numbers of patient samples available and the difficulty of maintaining the viability of cells obtained during acute illness in culture. We

therefore restricted our study to the analysis of phenotypic heterogeneity in acute disease vs convalescence in individuals with DF and DHF.

G. Alterations in the kinetics and phenotype of dengue-specific immune response during acute infection leading to severe disease.

The detection of high levels of proinflammatory cytokines and other markers of immune activation during clinical illness in DHF clearly argues for a role for dengue-specific T cells in disease mediation at the time of defervescence. Hence it is crucial to document the kinetics of T cell expansion during secondary response and understand how the onset of cross-reactive T cell function correlates with disease phenotype. We analyzed the response specific to the HLA-B*07 restricted NS3 222-231 epitope in 5 patients with DF and 5 with DHF. We found that the frequencies of tetramer$^+$ CD8$^+$ T cells were not only significantly higher in those with more severe disease, but that they were detected as early as 5 days prior to defervescence. This is in contrast to other studies which measured the frequencies of epitope-specific CD8$^+$ T cells throughout the acute phase and pegged the peak of the T cell response at 2 weeks after defervescence (Mongkolsapaya, et al., 2003; Mongkolsapaya, et al., 2005). The findings in those reports are at odds with the kinetics of disease onset and progression, since pathologic symptoms in secondary dengue-infected patients with DHF occur at the end of the viremic phase, with the severity of illness coinciding with a sudden drop in viral load (Libraty, et al., 2002). While our study as well as the previous studies included epitopes which are well conserved between serotypes, it is possible that the kinetics of response are specific both to the epitope in question as well the level of homology between its variants.

While the range of tetramer$^+$ T cell frequencies observed in our study is comparable to or higher than those seen by Mongolsapaya et al.(2003, 2005) and those reported in other studies during acute dengue illness (Loke, et al., 2001), massive and early changes in the size of the epitope-specific response seen in some of the DHF patients are novel findings. In the DHF cases where early bleeds were unavailable, the frequencies of tetramer$^+$ cells ranged up to ~1%-8% of circulating CD8$^+$ T cells even in the acute stage. While it is impossible to clearly identify the peak response in these donors, the presence of such high tetramer$^+$ populations in circulation 1-5 days before antigen clearance suggests that these cells may be responsible for initiating the immunopathology seen during secondary DHF.

H. Alterations in epitope-specific T cell phenotype after secondary infection.

Activation of memory cells during a recall response is accompanied by changes in the surface expression of various markers which track their differentiation into effector T cells. In DHF patients, these cells displayed a late activated phenotype as shown by CD38 expression on >80% of the tetramer$^+$ population while remaining high in CD69 expression. In addition, we found that DHF patients had higher frequencies of CD69$^+$tetramer$^+$ cells as compared to DF patients. This difference in the activation profile of CD8$^+$ T cells was seen in a previous study which compared total CD8$^+$ responses in DF and DHF patients (Green, et al., 1999). Our results indicate that there is earlier activation of dengue-specific T cells in those who develop severe disease.

The tetramer$^+$ populations in DF vs DHF patients also varied in the expression of homing receptors. Interestingly, in some donors, only a fraction of the tetramer$^+$ CD38$^+$ cells down-regulated CCR7. Since the CCR7$^-$ phenotype is a hallmark of effector

memory T cells as proposed by the central/effector memory hypothesis (Sallusto, et al., 1999), it is possible that there is a functional heterogeneity among the tetramer$^+$ population. Other groups have reported that only the CD38$^+$CCR7$^-$ subsets are proficient in proliferation and in the production of IFNγ and TNFα during anti-EBV and anti-parvovirus B19 responses (Hislop, et al., 2001; Isa, et al., 2005). CCR7 staining was also higher in the convalescent samples as compared to the corresponding acute samples in the DF donors. But tetramer$^+$ cells detected during convalescence in DHF patients unexpectedly showed little CCR7 expression. This suggests that there may be sustained immunologic effects in the dengue-specific memory T cell population months after recovery from DHF and the prevalence of effector memory subsets capable of cytokine production may contribute to disease exacerbation.

Earlier *in vitro* studies had showed that T cells in the acute phase of the disease were impaired in proliferation, and that this was due to functional defects in the APC (Mathew, et al., 1999). Since DCs and monocytes, which are targets of DV infection, can suppress T cell activation and proliferation via their expression of PD-L1 (programmed death receptor ligand 1), we were interested in seeing whether dengue-specific T cells would express the PD-1 receptor for this ligand. We have now shown for the first time that PD-1 is expressed on a majority of the tetramer$^+$ cells during acute illness. This is the first documentation of PD-1 expression on T cells during acute infection in humans. We also observed PD-1 expression on a large number of tetramer$^-$ cells which may be either other dengue-specific or bystander cells. Although the kinetics of PD-1 expression during acute illness are unknown, and the mechanisms and the extent to which PD-1 signaling suppresses T cell function are still unclear, studies of HIV infection showed that PD-1

expression leads to decreased IL-2 and TNFα production while not affecting the production of IFNγ (Petrovas, et al., 2006; Trautmann, et al., 2006). It is therefore conceivable that the dengue-specific T cells that have undergone massive expansion after initial antigen contact at the start of the response to secondary dengue infection are impaired in some or all of their effector functions thus producing a functionally heterogeneous T cell response which may lead to enhanced immunopathology.

I. Serotype cross-reactivity during secondary dengue infection.

As discussed previously, 'original antigenic sin' has been proposed as mechanism which contributes to DHF immunopathology due to the dominance of T cells which have low avidity for the secondary serotype and are therefore inefficient at viral clearance (Mongkolsapaya, et al., 2003). We also observed this pattern of increased avidity for the primary serotype since a higher proportion of dengue-specific T cells bound to the variant tetramer. However, we also found that the ratio of T cells with different avidities for their cognate and variant ligands was not static. In other words, the population of epitope-specific cells that was undergoing expansion during acute infection was not always dominated by low avidity cells but underwent day-to-day changes. Although we have not compared the effector functions between these two populations, we speculate that these high avidity T cells can also contribute to DHF because of their capacity to not just efficiently lyse virus-infected cells but because their expanded numbers will have resulted in a highly inflammatory environment.

J. Models of serotype cross-reactive T cell mediation of secondary immune response in heterologous dengue infection

Based on *ex vivo* analyses of phenotypic and functional cross-reactive $CD8^+$ T cell responses from dengue-infected and vaccinated individuals, this thesis strengthens the view that DHF immunopathology is a consequence of an early and increased immune response and that the T cell response to secondary dengue infection is mediated by a heterogeneous population of memory CD8 T cells that have undergone a robust program of expansion during acute infection (Figure 26A, B). The phenotypic heterogeneity, expression of PD-1, and alterations in the sensitivity to tetramer binding imply that the virus-specific population includes functionally diverse sub-sets of cells which could be activated to induce selective responses depending on the antigen load and serotype. The presence of such a functional hierarchy within a serotype cross-reactive epitope-specific population implies that a secondary infection with a serotype encoding epitope variants will change the relative ratios of different functional sub-sets within the expanded memory pool and immunopathology may result if this qualitative alteration in the secondary immune response is primarily mediated by inflammatory sub-sets (Figure 26B, C).

The ultimate goal of these studies is to establish a direct correlation between a particular profile of cross-reactive T cell response and disease state in order to design vaccines that are capable of manipulating T cell effector function to achieve immune protection rather than immunopathology. The diverse and unknown histories of viral

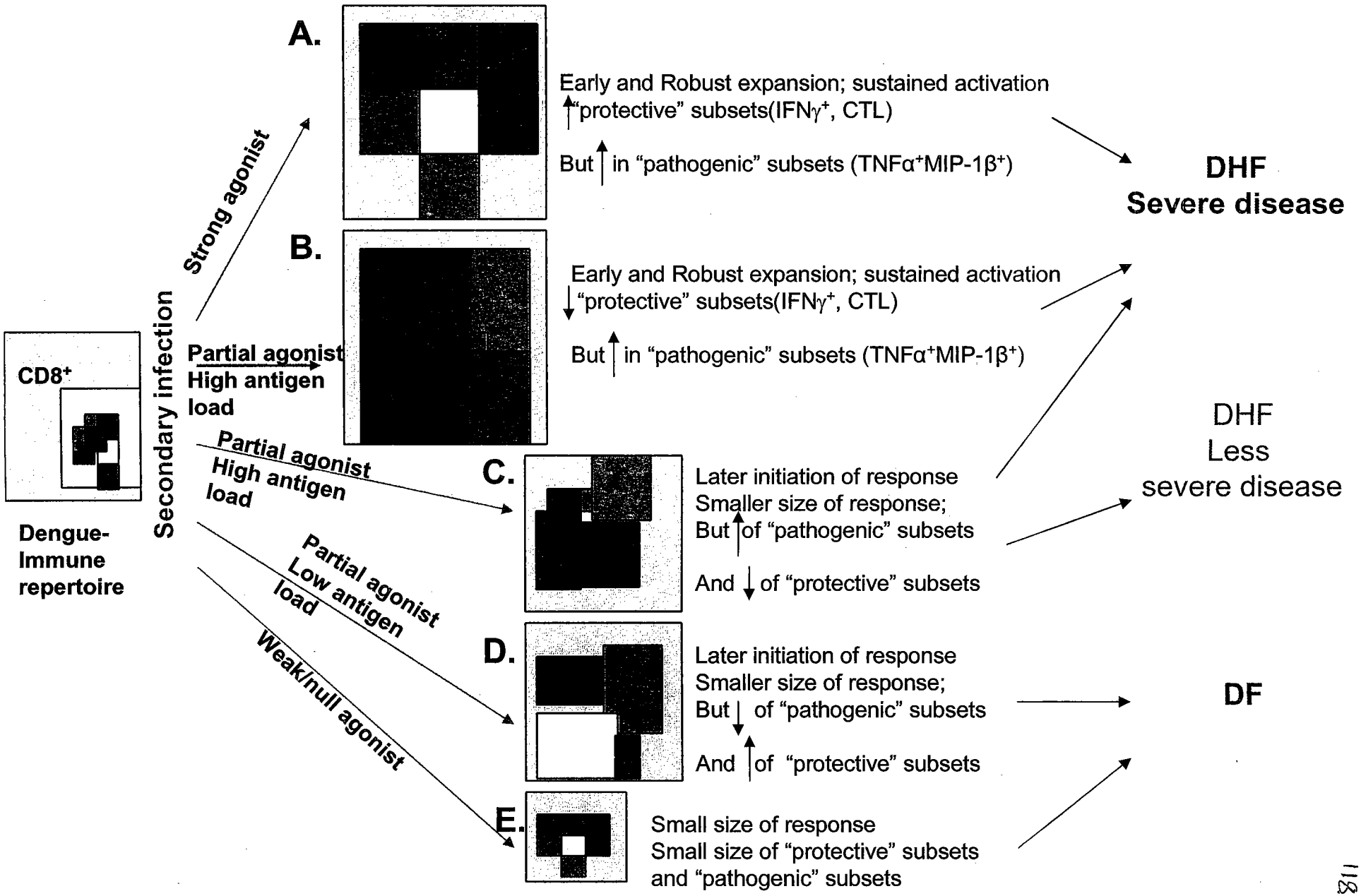


Figure 26: Models of serotype cross reactive CD8+ T cell mediated secondary response and potential contribution to disease severity

infection and the private specificities of T cell repertoires may make this a difficult task (Kim, et al., 2005). Nevertheless, examination of a broader spectrum of T cell effector functions in dengue immune subjects who have varied clinical manifestations of dengue disease should further our understanding of secondary immune responses against heterologous dengue infection. This, in turn, will hopefully facilitate the development of a vaccine strategy that would provide immunity against all 4 dengue serotypes without inducing immunopathologic cross-reactive responses.

CHAPTER VII

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