Defining the Influence of HLA Polymorphisms and T Cell Phenotype and Function in Dengue Disease Pathogenesis

Thesis

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DEFINING THE INFLUENCE OF HLA POLYMORPHISMS AND T CELL PHENOTYPE AND FUNCTION IN DENGUE DISEASE PATHOGENESIS

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Abstract

Dengue virus presents a growing threat to public health in a number of tropical and subtropical countries. Over half of the world's population lives in areas at risk of infection. In its most serious forms, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), it is also a major cause of morbidity and mortality, particularly in Southeast Asia where it is the principle reason for paediatric admission to hospital during the rainy season. There is no vaccine available and the pathogenesis and viral factors that underlie clinical disease and protective immunity remain poorly understood. Epidemiological and clinical observations have shown that both host and viral factors determine the severity of the disease.

A study of genetic susceptibility to DHF/DSS was carried out to investigate the association of HLA alleles and DHF/DSS in Vietnamese children with secondary DENV-2 infection. This study has found that HLA Class I and II polymorphism significantly influences genetic susceptibility to DSS, in particular, secondary DENV-2 infected children with HLA-B*44 were likely to have DSS, whereas secondary DENV-2 infected children with HLA-Cw*12 and HLA-DQB1*03 were at increase risk of DHF when compared with the controls. HLA-A and HLA-DRB1 polymorphisms were not associated to DSS. This implies that HLA Class I- and II-restricted immune responses may play an important role in DHF disease.

T-cell responses to dengue viruses may be important in both protective immunity and pathogenesis. The study of 51 Vietnamese adults with secondary dengue virus infections defined the breadth and magnitude of peripheral T-cell responses to 260 overlapping peptide antigens derived from a dengue virus serotype 2 (DV2) isolate. There are forty-seven different peptides evoked significant IFN-γ ELISPOT responses in 39 patients, and of these, 34 peptides contained potentially novel T cell epitopes. NS3, and particularly $NS_{200-324}$, were important T cell targets. The breadth and magnitude of ELISPOT responses to DENV-2 peptides was independent of the infecting dengue serotype. Acute ELISPOT responses were weakly correlated with the extent of haemoconcentration in individual patients, but not with the nadir of thrombocytopaenia or the overall clinical disease grade. $NS3_{556-564}$ and $Env_{414-422}$ were identified as novel HLA-A*24 and B*07-restricted $CD8^+$ T cell epitopes, respectively. Acute T cell responses to natural variants of $Env_{414-422}$ and $NS3_{556-564}$ were largely cross-reactive and peaked during disease convalescence.

By phenotyping $CD8^+$ T cells ($CD38^+/HLA\text{-}DR^+$, $CD38^+/Ki\text{-}67^+$ or $HLA\text{-}DR^+/Ki\text{-}67^+$) in serial blood samples from children with dengue, we found no evidence of increased $CD8^+$ T cell activation prior to the commencement of resolution of viraemia or haemoconcentration. Investigations with MHC class I tetramers to detect $NS3_{133\text{-}142}$- specific $CD8^+$ T cells in two independent cohorts of children suggested the commencement of haemoconcentration and thrombocytopaenia in DHF patients generally begins before the appearance of measurable frequencies of $NS3_{133\text{-}142}$- specific $CD8^+$ T cells.

The results highlight the importance of NS3 and cross-reactive T cells during acute secondary infection but suggest the overall breadth and magnitude of the T cell response is not significantly related to clinical parameters. The temporal mismatch between the appearance of surface activated and DENV-specific $CD8^+$ T cells suggests other mechanisms are responsible for triggering capillary leakage in children with DHF.

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Abbreviations

ADCC	Antibody-Dependent Cellular Cytotoxicity
Ae. Aegypti	Aedes aegypti
C	Capsid
CTLs	Cytotoxic T Lymphocytes
DCs	Dendritic cells
DC-SIGN	Dendritic cell specific ICA3M-grabbing non-integrin receptor
DENV	Dengue Virus
DF	Dengue Fever
DHF	Dengue Haemorrhagic Fever
DSS	Dengue Shock Syndrome
E	Envelope
ELISA	Enzyme-Linked Immunosorbent Sssay
ELISPOT	Enzyme-Linked Immunosorbent Spot
ER	Endoplasmic Reticulum
GWAS	Genome-Wide Association Study
HI	Hemagglutination Inhibition
HLA	Human Leukocyte Antigen
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
M	Membrane
MHC	Major Histocompatility Complex
NK	Natural Killer
NS	Nonstructural Protein
NTPase	Nucleoside Triphosphatase
OVL	Overlapping Peptide
PBMCs	Peripheral Blood Mononuclear Cells
prM	precursor Membrane
RNA	Ribonucleic Acid

RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SFU	Spot-forming units
SSP-PCR	Sequence-Specific Primer Polymerase Chain Reaction
sTNFR	soluble Tumor Recrosis Factor Receptor
TCR	T Cell Receptor
TNF	Tumor Necrosis Factor
WHO	World Health Organization

Chapter 1

Introduction

Chapter 1: Introduction – Dengue virus infection

1.1 Dengue infection-the global health problem

Dengue is currently the most important arboviral disease of human. Dengue is caused by any one of four serotypes of dengue virus (DENV). During the last few decades the global prevalence of dengue has grown dramatically. In a new estimate of burden of dengue from PDVI, the disease is now endemic in 124 tropical countries of the world, threatening more than 3.6 billion people (WHO/TDR 2009). This new estimation shows that there may be 70 million to 500 million cases of DENV infections worldwide every year, which result in 36 million cases of dengue fever and 2.1 million cases of dengue haemorrhagic fever/ dengue shock syndrome and 21,000 deaths each year. Dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), the most serious consequences of DENV infection, is also a major cause of morbidity and mortality. Particularly in Southeast Asia, dengue is a principle reason for pediatric admission to hospital during the rainy season.

Although classical dengue fever has been recorded for many centuries, DHF appears to be a more recent phenomenon. Epidemics of DHF have become more frequent since the 1950s in Southeast Asia. Before 1970, only nine countries had known epidemics of dengue haemorrhagic fever, but this number has increased up to 70 countries in 2005 (Kroeger and Nathan 2006). Dengue is now endemic in countries of Africa, the Americas, and the Eastern Mediterranean. Countries in Southeast Asia and the Western Pacific are seriously affected by the illness.

1.2 Emergence of Dengue/Dengue Haemorrhagic Fever

The earliest clinical and epidemiologic description of the potential dengue-like illness is recorded in a Chinese medical encyclopaedia in 992 (Gubler 2006). After World War II, the subsequent growth of cities in Southeast Asia and movement of people led to an increased co-circulation of multiple DENV serotypes and epidemic activity. The first major epidemics of the severe and fatal form of disease, dengue haemorrhagic fever (DHF), occurred in Manila, Philippines, from 1953 to 1954 as a direct result of this changing ecology (Mairuhu, Wagenaar et al. 2004). Within 20 years the disease in epidemic form had spread throughout Southeast Asia; by the mid-1970s, DHF had become a leading cause of hospitalization and death among children in the region (Gubler 1998). Since then, epidemics of DF/DHF have appeared in many new countries. In Asia, epidemic DHF has expanded geographically from Southeast Asian countries west to India, Sri Lanka, the Maldives, and Pakistan and east to China (Gubler 1997). Several island countries of the South and Central Pacific (Niue, Palau, Yap, Cook Islands, Tahiti, New Caledonia, and Vanuatu) have experienced major or minor DHF epidemics (Gubler 1998). In the Americas, dengue outbreaks have mainly occurred in the central and southern regions, with the first major DHF epidemic in Cuba in 1981 followed by outbreaks in Venezuela and Colombia (Kyle and Harris 2008) (Usme-Ciro, Mendez et al. 2008). Several dengue serotype 2 virus epidemics were documented, but no DHF cases were described in Africa (Diallo, Sall et al. 2005).

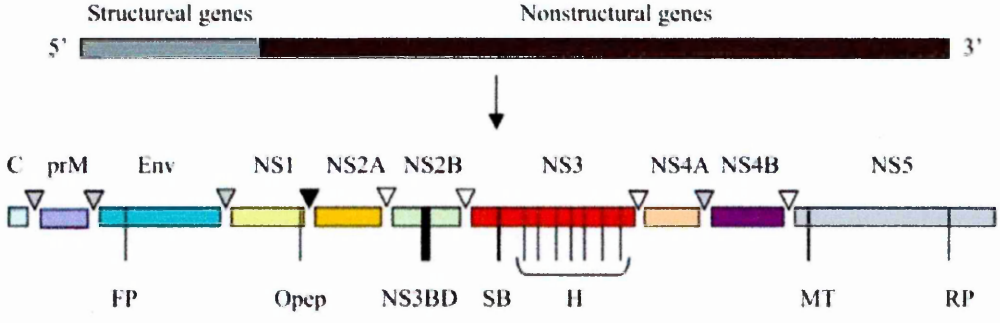
1.3 Dengue virus

1.3.1. Dengue virus structure

Dengue virus (DENV) is a positive single-sense RNA virus belonging to the Flaviviridae family, *Flavivirus* genus. This family comprises more than 70 RNA viruses, many of which are causes of important human diseases, such as yellow fever virus, Japanese encephalitis

virus, and tick-borne encephalitis virus (van Der Most, Murali-Krishna et al. 2000). There are four serotypes (DENV1–4), classified according to their antigenic features. The single-stranded RNA genome is encapsulated by an icosahedral nucleocapsid and covered by a lipid bilayer containing protein envelope and (pr) membrane. The mature virion is 40-50 nm in diameter and contains an 11-kb plus-sensed RNA genome consisting of a single open reading frame directing the synthesis of a polypeptide, which is proteolytically cleaved by host-cell and virus-specified proteases into ten viral proteins. Of these, three are structural proteins: core (C), envelope (E) and membrane (M, synthesized in precursor form prM); the remaining seven are nonstructural (NS) proteins. The order of proteins encoded is 5′-C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′ (Rothman 2004) (Rigau-Perez, Clark et al. 1998) (Kuhn, Zhang et al. 2002) (Henchal and Putnak 1990) (Figure 1.1).

Figure 1.1 Schematic diagram of DENV genome and polyprotein processing



FP: fusion peptide
Opep: octapeptide of NS1/NS2A
NS3BD: NS3 binding domain
SB: substrate-binding pocket of viral protease
H: helicase
MT: methyltransferase
RP: RNA dependent-RNA polymerase

1.3.2. Dengue virus protein function

Flavivirus RNA replication involves cyclization of the viral genome. DENV and other mosquito borne flaviviruses RNA have two pairs of complementary sequences at the 5' and 3' un-translated regions (UTRs) that play crucial roles in the initiation and regulation of translation, RNA synthesis, and viral assembly. The 5' UTR of DENV genome contains two defined elements essential for viral replication; a large stem-loop structure and 5' upstream AUG region (Lodeiro, Filomatori et al. 2009). Alvarez et al. demonstrated that deletion of individual domains of the 3' UTR of DENV affected RNA synthesis, RNA amplification and viral replication (Alvarez, De Lella Ezcurra et al. 2005).

The three structural proteins are associated with virus assembly, fusion activity, and virus-cell interactions. The envelope protein, which is exposed on the surface of the virus particle, is responsible for the main biological functions of the virion, including virus attachment and virus-specific membrane fusion in acidic pH endosomes. It binds to receptors on host cells, allowing the virus to be transported into the cell. In addition, the envelope protein is associated with haemagglutination of erythrocytes, induction of neutralizing antibodies, and protective immune responses (Guzman and Kouri 1996).

The non-structural proteins (NS1-NS5), expressed as both membrane-associated and secreted forms, are primarily involved in the replication of viral RNA as a part of the replication complex (Chambers T.J. and Rice 1990). The glycoprotein NS1 is not a part of the virion but is expressed on the surface of infected cells. Although no precise function has yet been ascribed to NS1, preliminary evidence suggests its involvement in viral RNA replication and serves an important role in the pathogenesis of severe dengue infections (Young, Hilditch et al. 2000). NS2B, NS4A, and NS4B proteins are assumed to be membrane-associated proteins as they exhibit conserved hydrophobicity profiles among flaviviruses (Chambers T.J. and

Rice 1990) (Appanna, Huat et al. 2007). NS3 protein exhibits protease, nucleoside triphosphatase (NTPase), helicase, and RNA triphosphatase activities (Falgout, Pethel et al. 1991). NS2B protein is an ER resident integral membrane protein of about 14 kDa and has been extensively studied as an essential cofactor for the activity of the NS3 serine protease (Niyomrattanakit et al., 2004). Flavivirus NS5 proteins have been shown to possess RNA-dependent RNA polymerase activity (Malet, Masse et al. 2008).

1.3.3. DENV replication in vivo

Dengue infection is initiated by the bite of a mosquito on the skin, where the virus is introduced into the host. The host cells of DENV can be dendritic cells, monocytes, macrophages or B cells. Skin dendritic cells are believed to be ten times more permissive to dengue infection than monocytes or macrophages (Wu, Grouard-Vogel et al. 2000). These host cells express dendritic cell specific ICA3M-grabbing non-integrin receptor (DC-SIGN), a mannose-specific, C-type lectin that serves as binding-receptors for all four serotypes (Navarro-Sanchez, Altmeyer et al. 2003) (Tassaneetrithep, Burgess et al. 2003). The virus binds to and enters a permissive host cell via these receptors and then endocytosed through engagement with an as yet unidentified factor (or co-receptor) (Lozach, Burleigh et al. 2005). Alternatively, Fc receptors or mannose receptors may be other kind of receptors for dengue virus-penetration. Dengue binds to antibody against a dengue serotype from a previous infection (enhancing antibody) and is then endocytosed by Fc receptor bearing cells.

During infection, DENV is internalized into the endosomal compartment where there is a conformational change in the envelope structure, which is triggered by an acidic pH, permitting fusion of the virus envelope to the endosomal membrane and release of the viral genome into the cytoplasm (Kuhn, Zhang et al. 2002) (Lozach, Burleigh et al. 2005)

(Mukhopadhyay, Kuhn et al. 2005) (van der Schaar, Rust et al. 2007). The first step of DENV assembly takes place in association with the membranes of the ER (Chambers T.J. and Rice 1990). The DENV RNA genome is translated into a polyprotein in the ER where a viral replication complex is assembled facilitating repeated replication of the genome (Lindenbach, 2001).

Immature virus particles are transported to the plasma membrane in vesicles for subsequent release by exocytosis. The surface glycoprotein prM then is proteolytically processed by a host protease, known as furin, in the exocytotic pathway of the trans-Golgi network, leaving the short polypeptide M protein on the surface of the virion and allowing the E protein to fold into its mature, homodimeric form (Yu, Zhang et al. 2008) (Zybert, van der Ende-Metselaar et al. 2008) (Duarte dos Santos, Frenkiel et al. 2000).

1.4 Vertebrate host-vector

All four serotypes of DENV have a similar natural history, including humans as the primary vertebrate host and Aedes mosquitoes of the subgenus Stegomyia (especially Ae. aegypti, Ae. albopictus and Ae. polynesiensis) as the primary mosquito vectors (Senanayake 2006). Ae. aegyti is highly anthropophilic, thriving in crowded cities and biting primarily during the day while Ae. albopictus is less anthropophilic and inhabits rural areas. In Africa, and perhaps parts of Asia, dengue viruses also exist in enzootic forest cycles with nonhuman primates as the vertebrate host (Rico-Hesse 1990) (de Silva, Dittus et al. 1999). The adult mosquito usually rest in dark indoor sites. The species is day-active, with most biting activity occurring in the early morning or late afternoon. Significant increases in mosquito larval populations are seen during the rainy season. This is an obvious reason why epidemics of dengue tend to coincide with the rainy season (Thavara, Tawatsin et al. 2001).

1.5 Transmission

For transmission to occur, the female Aedes mosquitoes must bite an infected human during the viraemic phase of the illness which can last up to 7 days (Gubler 1997). Each mosquito may be infected by several virus subtypes without affecting the virus yield. The feeding behaviour of the mosquito is characterized by easily interrupted feeding and repeated probing of one or several hosts (Stephenson 2005).

After biting an infected human, there is an obligatory extrinsic incubation period for the mosquito itself before it becomes infectious. This period is about 8 to 12 days. The virus first replicates in the midgut, reaches the haemocoel, and then infects the salivary gland. The virus is finally secreted in the saliva, causing infection during probing. Several studies suggested the genital tract is also infected and the virus may enter the fully developed egg at the time of oviposition (Joshi, Mourya et al. 2002).

1.6 Clinical manifestation

1.6.1 DF and DHF

All four serotypes of DENV are human pathogens. A person infected by one of the four DENV serotypes is believed to be immune to that serotype (homologus immunity), but only transiently (~6 months) immune to the three other serotypes (heterologus immunity). The clinical outcomes of DENV infection can vary from asymptomatic infection to dengue fever to severe and life-threatening dengue haemorrhagic fever (DHF), of which the rates of morbidity and mortality are high in areas with little clinical experience in managing the disease (Gubler 1998) (Guzman and Kouri 2003) (WHO. 1997).

After an incubation period of 3 to 7 days, people with symptomatic illness usually develop at least one of the following symptoms; high fever, headache, rash, abdominal pain, myalgia and arthralgia for 2 to 7 days. In earlier data on the epidemiology of inapparent dengue disease and dengue severity, Burke et al. found that the majority of dengue virus infection, 87 percent, were either asymptomatic or minimally symptomatic (Burke, Nisalak et al. 1988). Later, Endy et al. showed an equal incidence of occurred inapparent and symptomatic secondary dengue infection (Endy, Chunsuttiwat et al. 2002). Conversely, the disease can be complicated with symptoms such thrombocytopenia, plasma leakage, bleeding, and hypovolemic shock; this is commonly referred to as dengue hemorrhagic fever (DHF). In a prospective population-based cohort of Thai school-age children, Anderson et al. shown that sixteen percent of symptomatic confirmed dengue infections were classified as dengue haemorrhagic fever (Anderson, Chunsuttiwat et al. 2007).

1.6.2 Other severe dengue syndromes

There are some unusual manifestations of dengue. These include severe haemorrhage, hepatic damage, cardiomyopathy, and encephalopathy (Rigau-Perez, 1998 #43) (WHO. 1997) (Solomon, Dung et al. 2000) (Lawn, Tilley et al. 2003). Neurological manifestations such as altered consciousness, convulsions, and coma have been ascribed to an encephalopathy secondary to prolonged DHF/DSS (Solomon, Dung et al. 2000). Other unusual presentations of dengue infection include ocular manifestations (Haritoglou, Scholz et al. 2000) (Haritoglou, Dotse et al. 2002).

1.7 Clinical management

There are no specific therapies or licensed vaccines for dengue. However, careful case management, centered on the maintenance of circulating body fluids, saves many lives. Without treatment, the mortality rate associated with DHF can exceed 20% (Stephenson

2005). With proper care, including fluid or blood transfusion, fluid resuscitation (in DSS cases) and supportive care the mortality rate can be reduced to < 1% (Stephenson 2005).

1.8 Clinical and laboratory diagnosis

1.8.1 Clinical diagnosis

The World Health Organization (WHO) has published guidelines for the diagnosis and management of dengue infections (WHO. 1997). There are variable clinical manifestations associated with dengue infection. DF cases are characterized by an acute febrile illness with two or more manifestations (headache, retro-orbital pain, myalgia, arthalgia, rash, haemorrhagic manifestations, or leucopenia). DHF cases are characterized by three major clinical manifestations: haemorrhagic tendencies, thrombocytopenia and haemoconcentration (WHO. 1997). All three features must be present for a DHF classification. The most common haemorrhagic phenomenon is a positive tourniquet test and easy bruising and/or bleeding at venupuncture sites. The other common findings of DF/DHF are leukopenia, hypoproteinaemia, hyponatraemia, and elevated levels of serum aspartate aminotransferase.

In practice, there are difficulties in following the WHO criteria for DF/DHF classification. In many studies, application of strict WHO criteria for DHF has been problematic (Phuong, Nhan et al. 2004) (Balmaseda, Hammond et al. 2005). This has led to the suggestion that the existing WHO classification scheme needs to be refined (Balmaseda, Hammond et al. 2005) (Bandyopadhyay, Lum et al. 2006) (Deen, Harris et al. 2006). A new WHO Dengue case classification has been published to resolve this problem (WHO-TDR, 2009). This new scheme classify dengue cases into 2 main groups - severe dengue and mild dengue, which in turn is subdivided into 2 groups – dengue with and without warning signs (WHO-TDR, 2009).

1.8.2 Laboratory diagnosis

Laboratory diagnosis of DENV infection can be made by the isolation of virus, detection of viral antigen, genomic sequence, and/or virus-reactive antibodies (Guzman, Pelegrino et al. 2003) (Guzman and Kouri 1996) (Gubler 1998) (WHO. 1997). Currently, the most commonly used approaches are detection of the genomic sequence by a nucleic acid amplification technology or detection of DENV specific antibodies and DENV antigen. After the onset of illness, the virus is found in serum or plasma, circulating blood cells, and selected tissues, especially those of the immune system, for approximately 2 to 7 days, roughly corresponding to the period of fever (WHO. 1997). Recent studies suggest that assays for NS1 antigen detection could be a potential means for the early diagnosis of DENV infection (Alcon, Talarmin et al. 2002) (Koraka, Burghoorn-Maas et al. 2003) (Young, Hilditch et al. 2000) (Kumarasamy, Wahab et al. 2007) (Hang, Nguyet et al. 2009).

1.8.2.1 Antibody detection

Several methods have been described for the serological detection of DENV-specific antibodies, including the hemagglutination inhibition (HI) test (16), the neutralization test (77), the indirect immunofluorescent- antibody test (90), ELISA (6), complement fixation (30), dot blotting (10), Western blotting (51), and the rapid immunochromatography test (for which several commercial kits are available). Among these, capture IgM and/or IgG ELISAs and the HI test are the most commonly used techniques for the routine diagnosis of DENV infections. The use of IgM antibody-capture ELISA (MAC-ELISA) serves as a valuable tool for the presumptive diagnosis of acute flaviviral infections since IgM antibody titers are detectable early, peaking at about 2 weeks postinfection, and subsequently decline to lower levels over the next few months. IgM antibodies are generally less cross-reactive than IgG in

primary infections, and the serotype having the highest IgM titer is often the one responsible for current infection (WHO. 1997). Differentiation between primary and secondary infections has been suggested by determining the ratio of IgM to IgG in acute-phase sera (15, 29). In this context, the term "secondary infection" is used to describe the nature of the serological response and does not imply this was necessarily the second DENV infection experienced by the patient, since it could also be third or fourth DENV infection (Gibbons, Kalanarooj et al. 2007). Several commercial MAC-ELISA assays had been validated as both sensitive and specific methods for DENV antibodies diagnosis (Porter, Widjaja et al. 1999) (Vaughn, Nisalak et al. 1999) (Wu, Grouard-Vogel et al. 2000). The IgG ELISA is also reported as a diagnostic tool of high sensitivity and specificity (Hapugoda, Batra et al. 2007).

1.8.2.2 Antigen detection

Recent studies suggest that assays for NS1 antigen detection could be a potential means for the early diagnosis of DENV infection (Alcon, Talarmin et al. 2002) (Koraka, Burghoorn-Maas et al. 2003) (Young, Hilditch et al. 2000) (Kumarasamy, Wahab et al. 2007) (Hang, Nguyet et al. 2009) (Alcon-LePoder, Drouet et al. 2005). NS1 antigens in the form of an immune complex could be detected in the acute-phase sera of both patients with primary and secondary DENV infection up to 9 days after the onset of illness (Alcon, Talarmin et al. 2002) (Koraka, Burghoorn-Maas et al. 2003) (Young, Hilditch et al. 2000) (Kumarasamy, Wahab et al. 2007) (Alcon-LePoder, Drouet et al. 2005). Hang et al. recently reported the sensitivity and specificity of two commercial NS1 assays for diagnosing dengue, the Platelia ELISA and a lateral flow rapid test (Hang, Nguyet et al. 2009). Their results indicate these tests are most sensitive when used during the first 3 days of illness and are most likely to be positive if the patient has primary dengue. Hand et al. also showed the relationship between the sensitivity of both and the level of viraemia in patients.

1.8.2.3 Viral isolation

During the febrile phase, dengue viruses can be isolated from serum, plasma, or leucocytes. It can also be isolated from postmortem tissues such as liver, lung, spleen, lymph nodes, thymus, cerebrospinal fluid, or pleural/ascitic fluid (WHO. 1997).

Virus isolation by cell culture or in mosquitoes is a gold standard diagnostic method, although it is less sensitive and requires a longer time for a result when compared to other methods (Yamada, Takasaki et al. 2002) (Teles, Prazeres et al. 2005). The isolation of viruses from clinical samples can be carried out with cultured mosquito cells C6/36 (Yamada, Takasaki et al. 2002) or mammalian cells, such as Vero, BHK21 (Guzman and Kouri 1996).

1.8.2.4 Molecular diagnosis

DENV nucleic acid may also be detected in tissue or serum samples from human or in mosquitoes by RT-PCR techniques which were developed from the early 1990s (Lanciotti, Calisher et al. 1992) (Harris, Roberts et al. 1998) (Wang, Lee et al. 2000) (Sudiro, Zivny et al. 2001) (Raengsakulrach, Nisalak et al. 2002) (De Paula, de Melo Lima et al. 2004) (Pinheiro, Tadei et al. 2005). Among these, the two-step nested RT-PCR protocol originally reported by Lanciotti et al. is well known (Lanciotti, Calisher et al. 1992) (Harris, Roberts et al. 1998). More recently, the real-time RT-PCR assay has gradually replaced conventional PCR or RT-PCR methods for the rapid diagnosis of DENV infection as it provides quantitative measurements, a lower contamination rate, a higher sensitivity, a higher specificity, and easy standardization (Chen, Yeh et al. 2001) (Drosten, Gottig et al. 2002) (Houng, Chung-Ming Chen et al. 2001) (Shu, Chang et al. 2003) (Wang, Sung et al. 2002) (Chutinimitkul, Payungporn et al. 2005). DENV-specific primers and probes are usually designed for targeting conserved sequences among four dengue serotypes within the E, NS3,

NS5 region or recently the 3' non-coding region (Lanciotti, Calisher et al. 1992) (Houng, Chung-Ming Chen et al. 2001) (Chien, Liao et al. 2006) (Seah, Chow et al. 1995).

1.9 Immunopathogenesis to dengue infection

1.9.1 Innate immune responses against dengue virus infection

Before the proliferation of B and T cells, the initial host response to DENV infection is likely to be by the innate immune system, Mononuclear phagocyte lineages such as dendritic cells, macrophages, monocytes are considered to be the prime target cells for DENV infection (Wu, Grouard-Vogel et al. 2000) (Marovich, Grouard-Vogel et al. 2001) (Kyle, Beatty et al. 2007). Activated DCs also secrete cytokines such as TNF-α and IFN-α in response to DENV infection (Libraty, Pichyangkul et al. 2001) (Ho, Wang et al. 2001).

Cytolysis by natural killer (NK) cells may be also important in limiting DENV replication (Shresta, Kyle et al. 2004). Ex vivo phenotyping data imply a potential role for activated NK cells during early DENV infection (Green, Pichyangkul et al. 1999). A significant increase in circulating NK cell frequencies was found in patients during acute dengue disease (Azeredo, De Oliveira-Pinto et al. 2006).

In vitro infection of human cells and cell lines with DENV, or exposure to dengue antigens, results in the production of various anti-viral cytokines. For instance, neutrophils may produce TNF-α that can act directly against viral infections (Navarro-Sanchez, 2005). IFN-α might play an important role in the mechanisms of host cell defense against DENV. The release of IFN-α appeared to be critical for early immune response and resistance to DENV infection in a mouse model (Shresta, Kyle et al. 2004). A strong IFN-α inhibitory effect on

DENV replication was observed when different cell types were treated with cytokine prior to exposure to virus (Diamond, Roberts et al. 2000).

The role of complement in dengue is not well understood. Complement activation occurs during the acute and early convalescent phase of the disease, and the degree of activation appears to be correlated with disease severity. Levels of C3a, C4a, and C5a in patients with DHF were increased when compared to those with DF (Wang, Chen et al. 2006) (Nascimento, Silva et al. 2009). The complement cascade is activated by DENV antibody complex or by different cytokines to release C3a and C5a that may also have an effect on vascular permeability (Avirutnan, Punyadee et al. 2006).

1.9.2 Antibodies responses against dengue virus infection

1.9.2.1 Dengue virus-specific antibodies after primary and secondary infection

After a primary DENV infection, anti-DENV IgM becomes detectable in most patients by the time of defervescence. Levels of IgM peak at two weeks after infection, and decay over a period of 2-3 months after infection. Low titres of anti-DENV IgG are detected during the early convalescent phase (Churdboonchart, Bhamarapravati et al. 1991).

During secondary infections, IgG levels increase quickly during the acute phase, but IgM levels are low or even undetectable. IgG specific for E, NS3 and NS5 DENV proteins can usually be detected during the acute phase of infection, while IgG specific for other proteins such as C and NS1 DENV proteins can be detected during the convalescent phase (Churdboonchart, Bhamarapravati et al. 1991). These antibodies tend to be highly cross-reactive with other serotypes of the virus. Therefore, it is usually not possible to identify the infecting serotypes of DENV from the antibody response profile during acute secondary dengue.

After a primary DENV infection, individuals develop neutralizing antibody and probably, lifelong immunity to the homologous serotype. Subsequent secondary infection by one or more of the three heterologous serotypes is generally accepted as a major risk factor for DHF and/or dengue shock syndrome due to antibody dependent enhancement (ADE) (Guzman, Kouri et al. 1990) (Halstead 1988).

1.9.2.2 Role of DENV-specific antibodies in protective immunity

Antibodies to DENV have been reported to mediate three biologic functions in vitro which may contribute to prevention of or recovery from DENV infection: neutralization, complement-mediated cytolysis, and antibody-dependent cell-mediated cytotoxicity (Laoprasopwattana, Libraty et al. 2007) (Lin, Lei et al. 2001). However, antibodies might also augment DENV infection through the phenomenon called antibody-dependent enhancement. Passive protection studies in mice have shown that DENV-specific monoclonal antibodies are capable of conferring some degree of protection against further challenges (Calvert, Huang et al. 2006).

Antibody-dependent cellular cytotoxicity (ADCC) is a mechanism in which virus-specific antibodies bind to the surface of heterologous DENV-infected cells and mediate lysis by NK cells. A higher level of ADCC activity measured before secondary DENV-3 infection was associated with lower subsequent viremia levels, which suggests a protective role of antibodies mediating ADCC (Laoprasopwattana, Libraty et al. 2007).

1.9.3 Cellular immune responses in dengue infection

1.9.3.1 T-cell regcognition of DENV proteins

Most studies of DENV-specific T cell responses have occurred in the context of T-cell clones generated from live attenuated DENV vaccines, or less frequently, from dengue patients (Dharakul, 1994) (Gagnon, 1996) (Green, 1993), (Kurane, 1991) (Kurane, 1995) (Mathew,

1996). CD4+ and CD8+ T cell response to DENV is directed against multiple viral proteins, although the NS3 protein appears to be an immunodominant protein, with multiple epitopes throughout the protein. Serotype specific and cross-reactive T cells vary in their ability to recognize different DENV serotypes, depending upon the degree of conservation in the amino acid sequences of the epitopes and the TCR (Kurane, Zeng et al. 1998) (Zivny, Kurane et al. 1995).

1.9.3.2 DENV-specific T cell responses in subjects who received live attenuated DENV vaccines

PBMC from individuals who received live attenuated DENV vaccination showed proliferative responses to multiple dengue and other non-dengue flaviviruses with the highest response to homologous DENV serotype (Dharakul, 1994) (Mathew, 1996). CD8+ T cells were predominant cytotoxic cells identified in short term generated T-cell lines. Gagnon et al. isolated CD4+ cytotoxic T cells against core protein and found that these T cells could cognate target cell as well as bystander target cells (Gagnon, 1999). Most of the CD4+ and CD8+ T-cell lines generated from these donor responded to virus, viral antigen, or peptide stimulation by secreting Th1 cytokines including IFN-γ , TNF-α, TNF-β, MIP-1β, and IL-2 (Gagnon, 1999) (Kurane, 1994) (Kurane, 1989).

Serotype specificity and cross-reactivity T cells vary in their ability to recognize different DENV serotypes, depending upon the degree of conservation in the amino acid sequence of the epitopes and the TCR (Kurane, Zeng et al. 1998) (Zivny, Kurane et al. 1995). Dominance of cross-reactive T cells was observed among DENV specific memory T cells during secondary infection (Mathew, Kurane et al. 1998).

1.9.3.3 DENV-specific T cell responses in patients with natural DENV infections

There have been some studies of naturally immune people (Mathew, Kurane et al. 1998) (Gagnon, Mori et al. 2002) (Zivna, Green et al. 2002) (Mongkolsapaya, Dejnirattisai et al. 2003) (Simmons, Dong et al. 2005). Cytotoxic T-cell clones and bulk lines generated from patients following natural secondary infections were broadly serotype-cross-reactive and directed against the non-structural proteins (Mathew, 1998). Zivna et al. showed the frequency of HLA-B*07-restricted epitope specific T cells measured by ELISPOT assay was higher in patients with DHF compared with DF (Zivna, 2002). It is possible to detect DENV-specific CD4+ and CD8+ memory cytotoxic T lymphocytes (CTLs) up to a year after a natural secondary infection (Mathew, Kurane et al. 1998). A detailed analysis of a CTL response during acute dengue showed that there was an association between the magnitude of the T-cell response and disease severity (Mongkolsapaya, Dejnirattisai et al. 2003). More recently, our results showed that the T-cell response to DENV-2 peptides was independent of the infecting DENV serotype, suggesting that cross-reactive T cells dominate the acute response during secondary infection (Simmons, Dong et al. 2005).

Activated T lymphocytes and their byproducts have been detected in patients with dengue (REF). Circulating level of soluble CD4, sCD8, sIL-2R, and sTNFR are elevated in the serum or plasma of patients with servere disease (Bethel DB, 1998) (Green, 1999) (Hober, 1996) (Kurane, 1991). There were significant increases in the expression of CD69 on CD8+ T cells and NK cells in children with DHF compared with DF during the febrile period of illness (Green, 1999).

1.9.4 Cytokine responses in dengue infection

DENV infected monocytes, B-lymphocytes, and mast cells produce different cytokines. At the present there is disagreement about the predominant cytokines produced during dengue

fever and DHF. DENV-infected human peripheral blood leukocytes produce Th1 and Th2 cytokines such as TNF-α, IFN-γ , IL-2, IL-4, IL-5, IL-6, and IL-10 (Chaturvedi, Elbishbishi et al. 1999) (Chen and Wang 2002) (Mangada and Rothman 2005). Some studies have suggested that Th1 type cytokine (IL-2, IFN-γ) responses are seen in early days of illness and Th2 type cytokine (IL-5, IL-4) responses occur later. According to Chaturvedi et al., serum concentrations of TNF-α, IL-2, IL-6, and IFN-γ are highest in the first three days of illness whereas IL-10, IL-5, and IL-4 tend to appear later (Chaturvedi, Elbishbishi et al. 1999). Contrarily, some reports suggest that predominant Th2 responses occur in DHF/DSS, where Th1 responses seem to protect against severe infections. Increased levels of IL-13 and IL-18 have also been reported during severe dengue infections, with highest levels seen in patients with grade IV DHF. Serum IL-12 levels are highest in patients with dengue fever, but undetectable in patients with grade III and IV DHF. Levels of TGF-β (an inhibitor of Th1 and enhancer of Th2 type cytokines) correlate with severity of disease and show an inverse relationship with IL- 12 levels (Mustafa, Elbishbishi et al. 2001). DHF patients have higher levels of TNF-α, IL-6, IL-13, IL-18, and cytotoxic factor compared with DF patients. These cytokines have been implicated in causing increased vascular permeability and shock during dengue infections (Mustafa, Elbishbishi et al. 2001) (King, Marshall et al. 2000).

1.10 Risk factors for the development of DHF

Several risk factors have been proposed for development of DHF. These include: serotype and virulence of the infecting DENV; age, sex, immune status; and genetic background of the host.

1.10.1 Virulent factors

Viral factors are hypothesized to contribute to the progression of DHF. The risk for DHF is higher where two or more virus serotypes are circulating simultaneously (Halstead, 1997). Particular structural differences in several viral proteins and the 5' and 3' untranslated regions between DENV genotypes have been found to correlate with disease severity (Leitmeyer, Vaughn et al. 1999) (Mangada and Igarashi 1998).

All four DENV serotypes can cause DHF/DSS but DENV-2 is more frequently associated with DHF/DSS (Balmaseda, Hammond et al. 2006). DENV-4 usually caused mild clinical symptoms but sometimes it can cause severe dengue (Nisalak, Endy et al. 2003). American genotype DENV-2 viruses isolated in the Pacific islands and Venezuela are associated with DHF and DSS, and the absence of DHF and DSS in a Peruvian outbreak could be attributed to antibodies from a previous outbreak caused by DENV-1 (Stephenson 2005).

1.10.2 Host immune

Evidence shows that at the defervescent stage, viral load falls abruptly as the fever abates, associated with severe symptoms such as vascular leakage, hemorrhage, and shock (Vaughn, Green et al. 2000) (Wang, Chao et al. 2003). This observation suggested that it may be the host immune response to the virus, rather than the virus itself, that causes the pathology.

1.10.2.1 Antibody-dependent enhancement

Antibody-dependent enhancement (ADE) is hypothesized to contribute to the pathogenesis of severe dengue illness, as seroepidemiological studies have identified secondary infection as a risk factor for DHF and have shown that the presence of pre-existing anti-DV antibodies correlates with DHF (Bruke, 1988; Halstead, 1970). Halstead proposed that during secondary DENV infection, the pre-existing DENV antibodies at sub-neutralizing or non-neutralizing levels to infecting serotype may result in ADE of infection (Halstead, Lan et al. 2002).

The mechanism of action of ADE is thought to involve formation of complexes of DENV and pre-existing, non-neutralising antibody against a different DENV serotype; these complexes then bind to FcγRI and FcγRII bearing cells via the Fc portion of the antibody (Littaua, Kurane et al. 1990). This allows for the infection of greater numbers of cells which can lead to a greater viral load, particularly early in infection. High viral load during DENV infection have been shown to correlate with progression to DHF at defervescence (Vaughn, Green et al. 2000) (Libraty, Young et al. 2002).

The occurrences of DHF during primary DENV infection in the first year of life in children born to mothers with immunity to DENV, who therefore acquired antibody against DENV transplacentally, also support the idea of an in vivo role for ADE (Kliks, Nimmanitya et al. 1988) (Witayathawornwong 2001) (Pancharoen and Thisyakorn 2001) (Chau, Quyen et al. 2008). However, high viremia titers in older children and adults with primary DENV infections and in clinically mild secondary DENV infections indicate that other factors are involved (Sudiro, Zivny et al. 2001) (Vaughn, Green et al. 1997).

1.10.2.2 T-cell activation

Excessive immune activation during secondary infection has also been hypothesized to act in concert with other risk factors and promote the development of severe vascular leak. In particular, the rapid mobilisation of serotype cross-reactive memory T cells that release vasodilatory inflammatory molecules has been suggested to explain some aspects of the clinical syndrome (Kurane, Rothman et al. 1994). Accordingly, relatively higher frequencies of activated, cross-reactive CD8+ T cell (Mongkolsapaya, Dejnirattisai et al. 2003) (Zivna, Green et al. 2002), and a range of direct and indirect markers of cellular immune activation have been associated with severe disease during secondary infection (Gagnon, Mori et al.

2002) (Green, Pichyangkul et al. 1999) (Green, Vaughn et al. 1999) (Kurane, Innis et al. 1991).

There is strong evidence of T-cell activation in vivo during DENV infection, and such activation of CD4+ and CD8+ T cells is greater in patients with DHF than those with the milder dengue fever (Rothman 2003). Studies of the extent of T-cell activation in children from Thailand with DHF/DSS indicated that severe disease is associated with high levels of T-cell activation accompanied by massive apoptosis (Mongkolsapaya, Dejnirattisai et al. 2003).

1.10.3 Host genetic

Observations on dengue epidemiology in the Americas and in Africa suggest the host genetic make-up may be risk factors for DHF/DSS. In Haiti, despite hyperendemic transmission of dengue fever, DHF is not reported (Halstead, Streit et al. 2001). In Africa, where all four dengue viral serotypes circulate and epidemics of dengue fever occur, few cases of DHF are seen (Nimmannitya, 2002). Human genetic factors have been little studied in DHF. A few studies have looked at the effect of polymorphisms at the major histocompatibility complex locus on susceptibility to DHF. Loke et al. found that polymorphism at the HLA class I loci was significantly associated with DHF disease susceptibility (Loke, Bethell et al. 2001). Another study suggested HLA-A*0203 to be associated with less severe dengue, regardless of the secondary infecting virus serotype. Furthermore, HLA-A*0207 was associated with DHF in patients having secondary DENV-1 or DENV-2 infections only. This study also suggested HLA-B*51 to be associated with development of DHF in patients with secondary infections, and HLA-B*52 to be associated with dengue fever in patients with secondary DEN-1 and DEN-2 infections. Moreover, after secondary dengue infections HLA-B44, B62, B76, and B77 appeared to protect against development of disease (Stephens, Klaythong et al.

2002). Five polymorphic non-HLA host genetic (IL-4, IL-1RA, MBL, VDR, FcγRII) might influence susceptibily to DHF. The less frequent t-allele of a variant at position 352 of the vitamin D receptor (VDR) gene was associated with resistance to severe dengue. Homozygotes for the arginine variant at position 131 of the FcγRIIA gene, who have less capacity to opsonize IgG2 antibodies, may also be protected from DHF (Loke, Bethell et al. 2002).

1.11 Dengue in Vietnam

DHF first occurred in the rainy season of 1963 in South Vietnam and at the time was a leading cause of hospitalization and death in Vietnamese children (Halstead, Voulgaropoulos et al. 1965). The number of DF/DHF cases and case fatality mortality rate in South Vietnam from 1994 to 2007 is shown in Figure 1.2. In 1998, there was a widespread DHF epidemic that affected 19 provinces in southern Vietnam. In this outbreak, 123,997 cases were reported to the Dengue Control Program in South Vietnam at Pasteur Institute in Ho Chi Minh City. This epidemic comprised 119,429 cases of DHF and 342 deaths (Ha, Tien et al. 2000) (Pasteur 2007). The case fatality rate has been decreasing from 0.3% in 1998 to 0.09% in 2007 (Pasteur 2007).

Figure 1.2 The number of DF/DHF cases and dengue mortality rate in South Vietnam from 1994 to 2007 (Data was obtained from Pasteur Institute, HCMC, 2007)

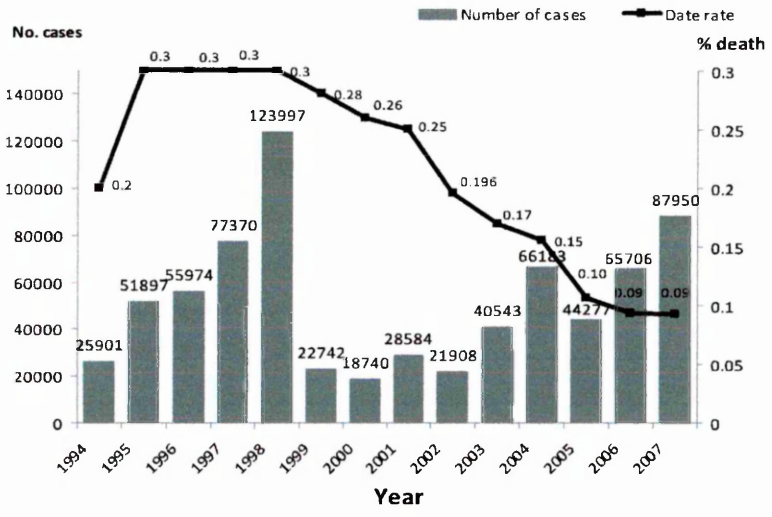
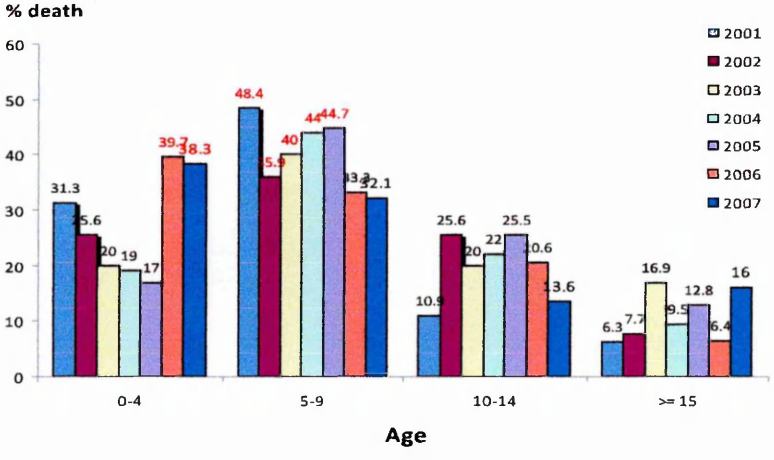
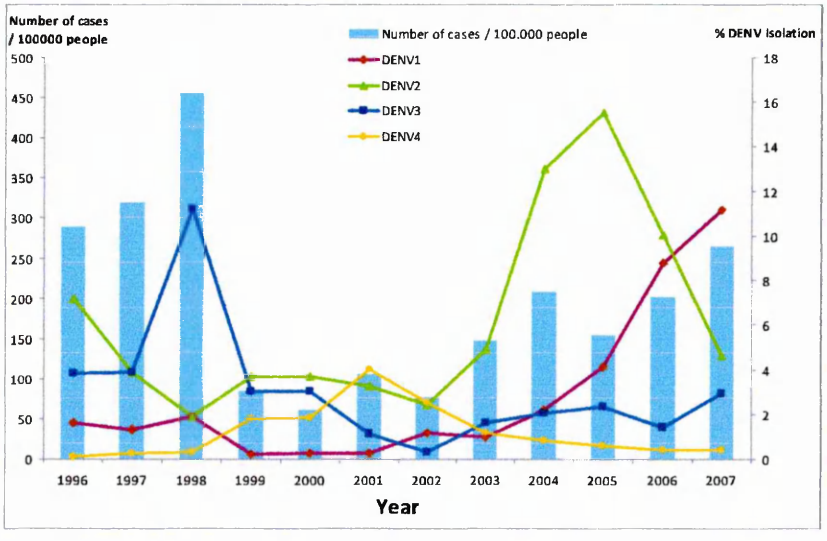


Figure 1.3 Correlation between death rate and age in DF/DHF cases in southern Vietnam (Data was obtained from Pasteur Institute, HCMC, 2007)



From 1987 to 2007, the circulation of DENV serotypes has changed depending on the period of time. DENV-2 was responsible for the 1987 epidemic. DENV-1 was predominant from 1990 to 1995 but was almost absent by 1999 (Ha, Tien et al. 2000). DENV-1 has recently re-emerged as the dominant serotype in 2006. DENV-3 was first detected in 1987 only in Ho Chi Minh City, and by 1991 it was also identified in Tien Giang Provine (Ha, Tien et al. 2000), (Pasteur 2007). In the largest DHF epidemic in Vietnam in 1998, DENV-3 became a dominant serotype in hospitalizaed dengue cases. From 2002 to 2006, DENV-2 was the major serotype isolated in hospitalized patients. DENV-4 was dominant from 2001-2002 (Figure 1.4).

Figure 1.4 Dengue cases and prevalence of DENV serotypes in patients in South of Vietnam. (Data was obtained from Pasteur Institute, HCMC, 2007)



Chapter 2

Materials and Methods

Chapter 2: Materials and Methods

2.1 Serological confirmation

2.1.1 Dengue IgM/IgG capture ELISA for the detection of secondary Dengue infection

Dengue cases were confirmed via serological testing of acute and early convalescent phase plasma with a commercial capture-immunoglobulin M IgM) and IgG enzyme-linked immunosorbent assay (ELISA) (Panbio, Brisbane, Australia). The ELISA was performed and results were interpreted according to the manufacturer's instructions. Results were determined by comparison with IgM and IgG reference sera provided (cut-off calibrators). A positive sample was defined as having a calibrator absorbance ratio greater than 1.0 and a negative sample with a ratio less than 1.0. Dengue infection was confirmed by the elevation of either IgM or IgG.

2.1.2 Dengue serological tests (MAC ELISA)

A Japanese encephalitis virus (JEV)/Dengue IgM and IgG ELISA (Venture Technologies, Sarawak, Malaysia), employing inactivated viral antigen from DENV1-4 and JEV, was used to discriminate between DENV- or JEV-specific IgM and IgG responses. The procedures were performed as described previously (Cardosa, Wang et al. 2002). Briefly, ELISA plate (Maxisorp, Nunc) was coated with 100 µl per well of anti-human IgM (A 0425, DakoCytomation) or IgG (I 2136, Sigma) with a dilution ratio of 1:2000 and incubated overnight at 4 °C. Plates were then blocked with 200 µl blocking solution (3% BSA-PBS) per well and incubated for 2 hours at room temperature (RT). After washing the plates 3 times with phosphate buffered saline with 0.05% Tween 20 (PBS-0.05%), 100 µl of diluted plasma specimens (1:100), positive control (1:100), and negative control (1:100) were added and incubated for 2 hours at RT. Specimens were added in triplicate for each sample, three sets of triplicate positive control and at least five sets of triplicate for negative control. After

washing the plates 5 times with PBS-0.05% Tween 20, 100 µl of each antigen (DENV, JEV, and control) was added to each well and incubated overnight at 4 °C. After washing, the plate was incubated with 100 µl of mouse monoclonal antibodies to DENV and JEV for 1 hour at room temperature and then detected by 100 µl of anti-mouse Ig Horseradish Peroxidase (HRP) (DakoCytomation) at a dilution ratio of 1:2000. After washing, the plate was developed by o-phenylenediamine dihydrochloride (OPD) (Sigma) and stopped by 10% hydrogen peroxide. The optical absorbance of the samples at a wavelength of 490 nm was then measured. The IgM and IgG positive control sample was a pool of acute plasma from dengue patients. The negative control sample was a mixture of plasma collected from healthy Vietnamese donors. Wells with PBS in place of DENV antigen were used to define absorbance background value. The cut-off value was defined as being 5 times higher than the mean OD of negative control samples after the subtraction of background OD.

A diagnosis of confirmed dengue was made if there was evidence of rising IgM titres to DENV antigen in paired plasma specimens and the response to DENV antigen was greater than to JEV antigen. A determination of primary dengue in patients was made if IgM levels exceeded IgG levels at the time of discharge from the hospital. Conversely, a determination of secondary dengue was made if there was detectable IgG response in patients at the time of both hospital admission and discharge.

2.2 RNA extraction and dengue viral PCR

2.2.1 RNA isolation

DENV RNA was isolated from plasma by the Boom method (Boom, Sol et al. 1990). Reagents for Boom extraction were repaired as following and stored in dark.

- L6 lysis buffer was made by adding 120 g of guanidinium isothiocyanate to 100 mL of 0.1 M Tris/HCl (pH of 6.4) and 22 mL of 0.2 M EDTA pH 8.0, and 2.6 g of Triton X100, and then stirred overnight in the dark to dissolve.
- L2 buffer was made by adding 120 g of guanidinium isothiocyanate to 100 mL of 0.1 M Tris/HCl pH 6.4 and then stirred overnight in the dark to dissolve.
- Size-fractionated silica particles were made by adding 60 g of silicon dioxide to 500 mL of distilled water in a measuring cylinder and allowed to equilibrate for 24 hours at room temperature. A 430 mL of supernatant was discarded and solids were re-suspended in 500 mL of distilled water and allowed to equilibrate again at RT for 5 hours. A 440 mL of supernatant was discarded and then added 600 ul of concentrated HCl (pH of 2.0) and aliquoted into 1.5 mL volumes.

In each sample, a standard amount of Equine Arteritis Virus (EAV) was used as an internal control on extraction and the PCR process. Briefly, for RNA extraction using the Boom method, 50 to 200 µl of plasma was added to a tube containing 1 mL of L6 lysis buffer and 20 µl of a suspension of size-fractionated silica particles; the mixture is then briefly vortexed. After incubation for 10 minutes (min) at RT, the tube was vortexed for 5 seconds and centrifuged for 1 min at 13,000 *x* G. The supernanant was then discarded and 1 mL of washing buffer L2 was added to the silica-nucleic acid complex, vortexed at 5 sec followed by two washes with buffer L2 (1 min at 13,000 *x* G), two washes with 70% (vol/vol) ethanol, and finally one wash with 1 mL of acetone (Merck). After disposal of the acetone, the tube was dried at 56 °C with open lid in a heating block for 10 min. The pellet was re-suspended in 100 µl of 1 x TE buffer, vortexed for 5 seconds, and left at 56 °C for 10 min to elute the nucleic acids. After incubation, it was vortexed again and centrifuged for 2 min at 13,000 *x* G. Only 70 µl of the supernatant containing viral RNA were gently pipetted to a new 0.5 mL

eppendorf to avoid disturbing the pellet. In most cases, RNA was immediately used for synthesis of complementary DNA (cDNA); otherwise, the RNA was stored at negative 80 °C until used.

2.2.2 cDNA synthesis

cDNA was generated from viral RNA by using Random hexamers (Roche, Lews, UK) and Superscript III reverse transcriptase (Invitrogen). A mixture of 8 µl of RNA, 1 µl of 100 ng/µl random hexamers, 0.4 mM dNTPs (Roche, Lews, UK), and sterile distilled H_2O adjusted up to 13 µl was incubated at 65 °C for 5 min and immediately chilled on ice for at least 1 min. A mixture of 4 µl of 5x First-strand buffer (Invitrogene), 2.5 mM dithiothreitol (DTT) (Invitrogene), 16 units of Rnase Inhibitor (Invitrogene) and 40 units of SuperScript III RT (Invitrogene) adjusted with sterile distilled H_2O to make up a reverse transcription reaction volume of 20 µl. The content was mixed by gentle pipetting up and down then incubated at 25°C for 10 min followed at 48 °C for 30 min, then 95°C for 5 min. The resulting cDNA was used as template in the subsequent PCR reaction.

2.2.3 SYBR Green RT-PCR dengue screening

Dengue virus genomic material was detected by RT-PCR using a range of approaches. In some experiments, a dengue virus complex-specific assay was used. Briefly, 4 ul of cDNA were used as template in a fluorogenic PCR in 20 µl of a reaction mixture containing 1.25x HotstarTaq buffer (pH 8.7), 1.2mM $MgCl_2$, 0.4mM dNTP, 0.625x SYBR Green, 0.02 µM FC, one unit HotstarTaq (Qiagen) and 0.8 µM of each forward and reverse primer. Dengue group primers were as described by Shu *et al.* with minor modification (inosines instead of degenerate positions) (Shu, Chang et al. 2003). Sequences of forward and reverse primers were:

5'-CAATATGCTGAAACGCGiGAGAAA-3': forward primer (nucleotide position 135-158)

5'-CCCCATCTiiYCAiiATYCCTGCTGT-3': reverse primer (nucleotide position 282-305)

After pre-treatment at 95 °C for 15 min, reaction mixtures were subjected to 45 cycles of denaturation at 95 °C for 20 seconds (s), annealing at 55 °C for 30 s and extension at 72 °C for 30 s, and product melt at 83 °C for 10 s. The PCR product was 176 bp in length. Following amplification, a melting curve analysis was performed to verify the correct product by the specific melting temperature (T*m* = 83°C). Melting curve analysis was comprised of a denaturation at 95 °C for 1 min, lowered to 60 °C for 10 min, and followed by 70 cycles of incubation in which the set point temperature was increased to 95 °C at a rate of 0.5 °C /10 s/cycle with continuous reading of fluorescence.

2.2.4 Dengue serotyping by TaqMan real time RT-PCR

We used a serotype-specific, fluorogenic real-time RT-PCR assay to measure dengue viraemia levels in plasma. The dengue-specific primers and probes were adapted from previously published sequences and are summarized in Table 2.1 and Table 2.2 (Laue, Emmerich et al. 1999). Hotstar Taq polymerase was used in all assays (Qiagen, USA). PCR conditions were 95 °C for 14 mins then 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s for 45 cycles. Linearised plasmids in which the target amplicon had been cloned were used to generate a standard curve against which Ct values derived from test samples were compared. Results were expressed as cDNA equivalents per milliliter of plasma. For DENV1, DENV2 and DENV3, the detection limit was 1 copy per reaction. For DENV4, the detection limit was 10 copies per reaction. All reactions were performed in duplicate and sample measurements were only valid when positive and negative control samples were valid.

Table 2.1 DENV and EAV specific primer sequence

	Forward primer sequence (5'-3')	Position	Reverse primer sequence (5'-3')	Position
D1	ATCCATGCCCACCAYCAATG	9960-9980	CAGGGATCCACACCAYTGATC	10100-10121
D2	ACAAGTCGAACAACCTGGTCC AT	9938-9941	GCCGCACCATTGGTCTTCTC	10095-10115
D3	TTTCTGCTCCCACCACTTTCAT	9719-9741	TGGCGTTGGATGCYAGTCT	9915-9934
D4	GYGTGGTGAAGCCYCTRGAT	9587-9607	AGTGARCGGCCATCCTTCAT	9744-9764
EAV	CATCTCTTGCTTTGCTCCTTAG	1847-1869	AGCCGCACCTTCACATTG	1962-1980

Table 2.2 DENV and EAV specific probe sequence

	Sequence	Position
D1	5'(Familusi, Moore et al.)- TCAGTGTGGAATAGGGTTTGGATAGAGGAA-3'(TAMRA)	10002-10032
D2	5'(Familusi, Moore et al.)- GTTTTGTCTTCCATCCA (BHQ-1)- 3'	10038-10055
D3	5'(Familusi, Moore et al.)- AAGAAAGTTGGTAGTTCCCTGCAGACCCCA-3'(TAMRA)	9761-9791
D4	5'(Familusi, Moore et al.)- ACTTCCCTCCTCTTYTTGAACGACATGGGA-3'(TAMRA)	9612-9642
EAV	5'(Cy5)- CGCGCTCGCTGTCAGAACAACATTATTGCCCACAGCGCG (BHQ3)- 3'	1888-1927

2.3 DNA extraction for human leukocyte antigen – sequence specific primers (HLA-SSP) PCR

DNA extraction from blood samples was performed using a commercial DNA extraction kit (Nucleon, Amersham Biosciences, UK) according to manufacturers' instructions. Briefly, the blood pellet was re-suspended in red cell lysis buffer, reagent A (10 mM Tris-HCl, 320 mM surose, 5 mM $MgCl_2$, 1% Triton-X100, pH of 8.0), with a ratio of 1:4. The nucleated cells were pelleted after a 10-minute centrifugation at 1300 x G. The cell pellet was re-suspended and lysed in 350 µl of reagent B (40 mM Tris-HCL, 60 mM EDTA, 150 mM NaCl, 1% SDS, pH of 8.0), and incubated for 10 min at 37°C. Then the cell lysate was mixed with 100 µl sodium perchlorate followed by 600 µl of chloroform. The tube was mixed by hand and inverted at least seven times. Without remixing the phase, 150 µl of nucleon resin was added to the tube and centrifuged at 350 *x* G for one minute. Without disturbing the nucleon resin layer, the upper phase was transferred to a new tube. Next, two volumes of cold absolute ethanol was added and mixed by inversion until the precipitate appeared. The tube was centrifuged at top speed for five minutes to pellet the DNA, and the supernatant was discarded. The pellet was washed with 1 mL of cold 70% ethanol, re-centrifuged, and the supernatant was discarded. The pellet was dried for 10 minutes before dissolving in an appropriate volume of water or TE buffer.

2.4 DNA measurement by PicoGreen assays

In this study, all genomic DNA concentrations were quantified using PicoGreen. PicoGreen is an ultra-sensitive fluorescent nucleic acid stain for quantifying double-stranded DNA (dsDNA). DNA concentration determinations were performed according the manufacturer's instructions in MicroAmp Optical 96-well plates (Costar, Corning Incoporated, USA) and using a fluorescent microplate reader at 485/535 nm (GENios or SPECTRAFluor Plus,

TECAN, United States). Each plate contained a duplicate set of lambda DNA standards ranging from 0 to 800 ng/ml. Each sample was assayed in duplicate. The level of fluorescence in each well was measured and the concentration of each sample was then calculated based on the standard curve on a Microsoft Excel spread sheet or was automatically calculated by the reader software. If the standard curve was not linear, the process was repeated (Figure 2.1).

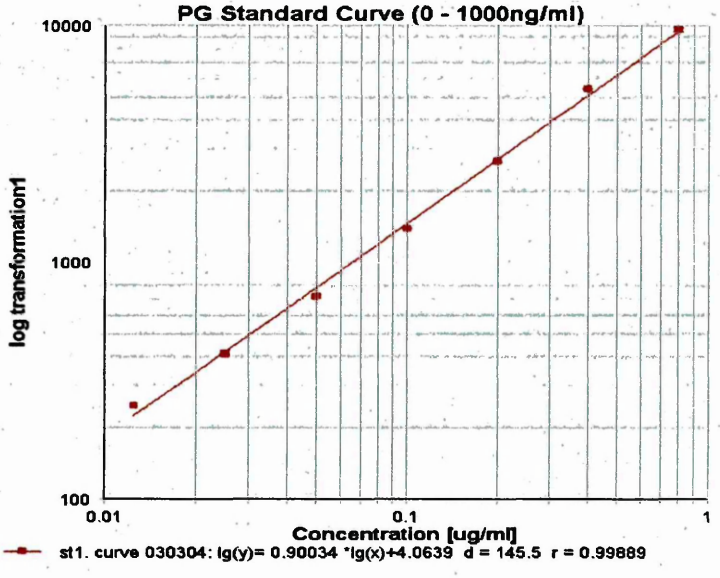


Figure 2.1 The standard curve for estimation of DNA concentration. Red line indicates the DNA standard curve. Y-axis corresponds to logarithm of fluorescent intensity. X-axis corresponds to DNA concentration.

2.5 PCR using Sequence-Specific Primers (PCR-SSP) for HLA typing

Typing of HLA class I and class II alleles was performed using the amplification refractory mutation system PCR (ARMS-PCR) with sequence specific primers as described previously (Bunce, O'Neill et al. 1995). One hundred and ninety two PCR reactions were performed to genotype HLA-A, B, Cw, DRB1 and DQB1 alleles was performed using 96-well polycarbonate plates (Thermo-Fast 96, Low profile, ABgene, UK) and a Peltier thermal cycler machine (DNA Engine Tetrad 2, BIO-RAD). Each well of a 96-well PCR plate contained a unique set of allele-specific PCR-primers and a set of internal control primers (amplifying a conserved region from the third intron of the HLA-DRB1 locus). PCR cycling conditions are listed in Table 2.3.

Table 2.3 PCR cycling conditions for PCR-SSP

Stage	Cycling condition	No. of cycles
1	96 °C - 1 minute	1
2	96 °C - 20 s, 70 °C - 45 s, 72 °C - 25 s	5
3	96 °C - 25 s, 65 °C - 50 s, 72 °C - 30 s	21
4	96 °C - 30 s, 55 °C - 60 s, 72 °C - 90 s	4

Each PCR reaction was carried out in 13 µl volume containing primers (1-4 µM for allele-specific primers and 0.1 µM for DRB1 control primers), PCR buffer (6 mM $MgCl_2$, 1.63x buffer, 0.3 mM dNTP, 0.45M Betaine). BIOTAQ™ polymerase (Bioline) was used for these PCR reactions.

PCR products were visualized using 2% Ethidium Bromide (EtBr) stained agarose/TBE gel (Tris/borate/EDTA buffer: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA). The presence of an allele is defined as when the allele-specific PCR product is present and negative when only the internal control product is present. 192 PCR reaction was performed to genotype HLA-A, B, Cw, DRB1 and DQB1 alleles. Primer pairs and the HLA class I and class II alleles they detected are listed in **Appendix 1**.

The frequency (*f*) of HLA class I and II antigens was determined by the formula $f = n/N$, where n is the number of individuals that are positive for a particular HLA (either heterozygotes or homozygotes) and N the number of individuals tested.

2.6 Separation of peripheral blood mononuclear cells from peripheral blood

2.6.1 The Ficoll method

Peripheral blood mononuclear cells (PBMCs) were separated using Lymphoprep (Axis-Shield Poc AS, Oslo, Norway). Initially, Lymphoprep™ was placed in a 50 mL Falcon tube. Then whole blood was added to the Lymphoprep™ at a 1:1 ratio and centrifuged at 3000 revolutions per minutes (rpm) for 20 to 25 minutes at room temperature with no brake. After centrifugation, the translucent interface layer (the PBMCs layer) was transferred into other another 50 mL tube. PBMCs were washed twice with RPMI or PBS+3% heat-inactivated FCS. PBMCs were re-suspended in cRPMI (RPMI 1640 medium containing 10% HI-FCS, 2 mM L-glutamine, 100 µg/ml Streptomycin, 100 U Penicillin) for direct use in enzyme-linked

immunospot (ELISPOT) assay. PBMCs were also cryopreserved in HI-FCS containing 10% dimethyl sulfoxide (DMSO) and placed in liquid nitrogen container for future use.

2.6.2 CPT Vacutainer method

Peripheral blood was collected into CPT Vacutainer tubes (Becton Dickinson) containing sodium heparin. The tubes were centrifuged in a swing-out centrifuge at 3000 rpm for 20 minutes at room temperature with no brake. PBMCs and plasma remained above the gel. The PBMCs were transferred into a Falcon tube then washed once with RPMI containing 1% FCS and used directly or cryopreserved as described above.

2.7 Counting PBMCs for Elispot Assay and Cryopreservation

PBMCs were separated from peripheral blood and counted before cryopreservation or use in Elispot assays. Trypan blue (final concentration 0.36%) was used to discriminate dead from viable cells. An aliquot of diluted PBMC in Trypan blue were enumerated on KOVA® glastic slides with standard grids (Hycor Biomedical, UK).

2.8 Cryopreservation of PBMCs

PBMC were cryopreserved at a cell concentration of 5×10^6 to 10×10^6 viable cells per milliliter in freezing medium (HI-FCS + 10% DMSO) and aliquoted to cryovials (Corning Incorporated, CORNING, Mexico). The cryovials were immediately placed in a slow-freeze container (NALGENE™ Cryo 1°C Freezing Container, USA) which was then placed in a -70 °C freezer for 4 to 24 hours. After 4 to 24 hours, the cryovials were transferred into a liquid nitrogen-rated box and placed into the vapor phase of liquid nitrogen (-135°C) containers for long-term storage.

2.9 Thawing of cryopreserved PBMCs

Cryovials containing frozen PBMCs were removed from liquid nitrogen storage and placed in a 37 °C water bath until the majority of the suspension had thawed. The suspension was then transferred to a 15 mL centrifuge tube containing 5 mL of chilled cRPMI medium. The thawed cells then were washed and counted as described in section **2.7**.

2.10 Peptides for IFNγ ELISPOT assay

We designed 260 peptides spanning the structural antigens (capsid, preM/M, and Env) and nonstructural viral antigens (NS3 and NS4a) from a dengue virus serotype 2 sequence (strain 16681). Peptides were synthesized by standard, solid-phase 9-fluorenylmethoxy carbonyl chemistry. Purity ranged from 30 to 90% as determined by high-performance liquid chromatography. Peptides consisted of 220 15mers and 40 20mers peptide. All peptides overlapped by 10 amino acids. The sequences of peptides are shown in **Appendix 3.**

2.11 IFNγ ELISPOT assay

The IFNγ ELISPOT assay was performed according to the manufacturer's instructions (Mabtech AB, Stockholm, Sweden). The 96-well polyvinylidene difluoride (PVDF)-backed plates (MAIP, Millipore, USA) were coated with coating antibody 1-D1K (50 ul at 15 ul/ml), incubated at room temperature for 2 hours or overnight at 4 °C. The wells were then washed twice with 200 ul of sterile PBS followed by blocking with 100 µl of cRPMI for at least one hour at room temperature. PBMC samples (1×10^5 to 3×10^5 cells/well) were stimulated with individual peptides or peptide pools. Individual peptides were used at a final concentration of 2 µg/mL. Peptide pools were used at a final concentration of 5 µg/mL for each peptide. Two hundred and sixty peptides were arranged into a matrix of 32 peptide pools containing 16

individual peptides. Each peptide was present in two different pools. A peptide was identified as evoking a response in the IFNγ ELISPOT assay as it drove a response in two different pools. In every positive case, peptides identified as being antigenic in this manner were retested as individual peptides. Thus, each positive response was confirmed twice: first in the matrix pools then in confirmatory individual assays. PBMCs were incubated overnight with peptides at 37 °C in a humidified incubator containing 5% CO_2 in air.

After overnight incubation, wells were washed twice with PBS containing 0.05% Tween 20 (Sigma) to remove cells and media. Next, detection monoclonal antibody, 7-B6-1-biotin (50 ul at 1 μg/ml Mabtech, Sweden) was added to each well for 2 hours at 37 °C. The wells were washed again twice with PBS containing 0.05% Tween 20 before incubating with 50 μl of streptavidin-alkaline phosphate (1 μg/ml Mabtech, Sweden) for one hour at 37 °C. After washing again twice with PBS-0.05%, streptavidine-alkaline phosphate conjugate (Mabtech) was added at a dilution of 1:1000 in PBS to visualize the spot-forming cells. The number of spot-forming units (SFU) in each well was counted with the aid of a dissecting microscope and the background (no antigen stimulation) was subtracted. For each assay, the phytohemagglutinin (Sigma, Poole, UK) was used as the positive control.

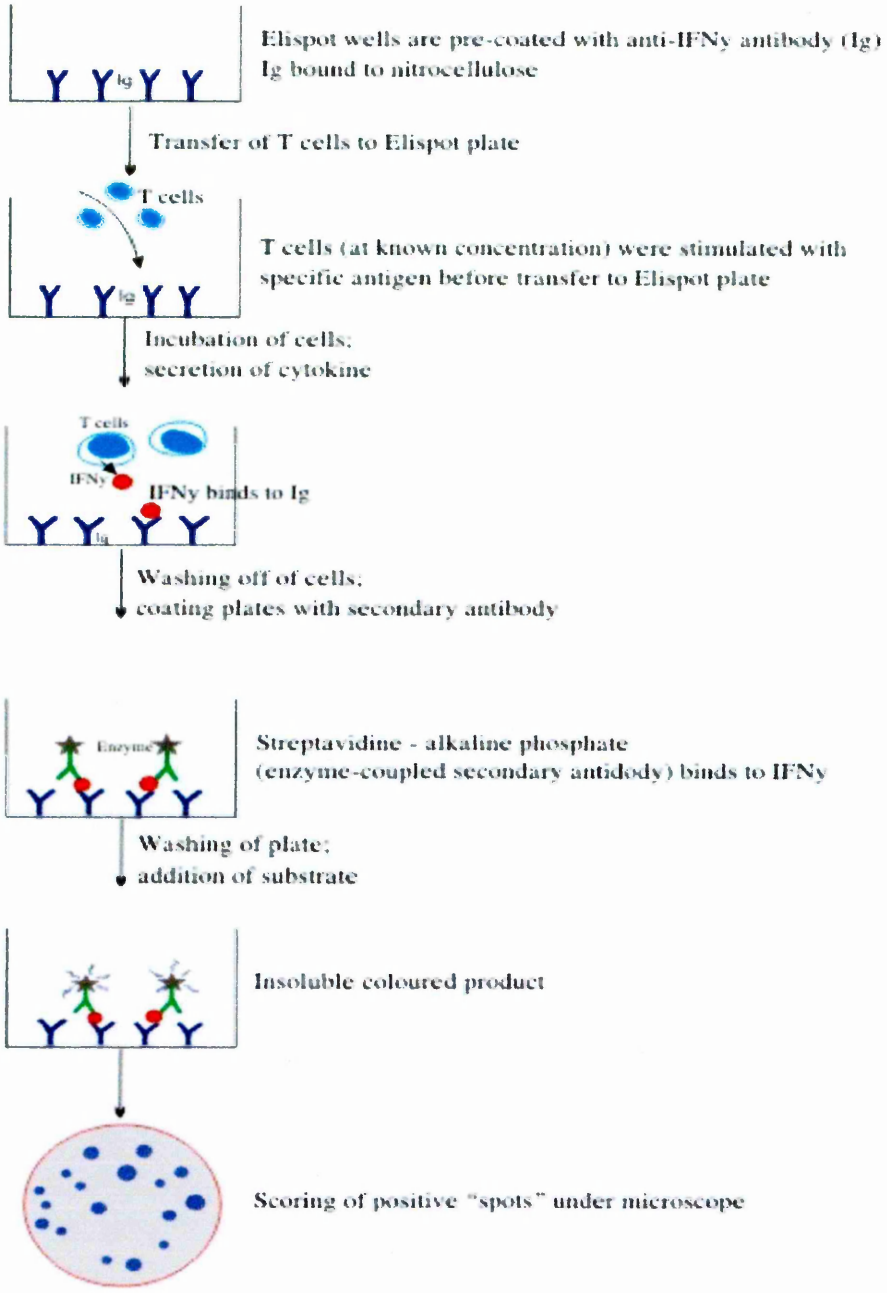


Figure 2.2 Elispot assay to quantify secretion of cytokines by T lymphocytes

2.12 $CD4^+$ and $CD8^+$ T cell depletions

$CD4^+$ and $CD8^+$ T cells were depleted from PBMCs using anti-CD4 or anti-CD8 antibody-coated immunomagnetic beads (Dynal, Oslo, Norway). The PBMCs were incubated with the appropriate immunomagnetic beads in 0.5 mL of cRPMI, at a ratio of five beads per cell, at 4 °C for 30 minutes with gentle mixing. Cells attached to immunomagnetic beads were removed using magnetic particle concentrator (Dynal), and the remaining cells were collected for further analysis. Flow cytometry analysis indicated this approach routinely achieved over 95% depletion of the target cells.

2.13 Cytotoxicity assay

T-cell lines were generated by pulsing 2×10^6 PBMCs with 100 μM of $Env_{414-422}$ peptide for one hour. Cells were cultured in cRPMI supplemented with 25 ng of IL-7/mL for 3 days. Thereafter, 100 U of IL-2/mL was added every 3 to 4 days. After 14 days, cells were harvested and used as effectors in a 51-chromium (Cr)-labeled release assay. Target cells in the chromium release assay consisted of a 51-Cr-labeled B-cell line (BCL) that was HLA matched with effectors only at the B*07 locus. The BCL was pulsed for 1 hour with the $Env_{414-422}$ peptide, washed, and aliquoted in microtiter plates (5,000cells/well). Then effector cells were added in a range of effector-to-target ratios.

Un-pulsed cells were used as negative controls. The 51-Cr release was calculated from the following equation:

$$C = \frac{Cr_{exp} \cdot Cr_{spon}}{Cr_{max} \cdot Cr_{spon}} \cdot 100\%$$

([experimental release spontaneous release]/[maximum release spontaneous release]) x 100%.

Nonspecific killing of un-pulsed target cells was subtracted from that of pulsed target cells.

2.14 Reagents and monoclonal antibodies for $CD8^+$ T cell phenotype

Cell phenotype for T cell activation was determined by cell surface and intracellular staining. Cell surface staining was routinely performed on 100 µL of fresh whole blood. The mouse anti-human surface monoclonal antibodies (mAbs) used in this study included: APC-conjugated anti-CD3, PE-conjugated anti-CD8, PE-Cy7-conjugated anti-CD38, and PerCP-conjugated anti-HLA-DR. Intracellular staining marker used FITC-conjugate anti-Ki-67. All antibodies were purchased from Becton Dickinson, CA, USA. Data acquisition was performed on a BD FACS Canto II flow cytometer (Becton Dickinson, San Diego, CA, USA). The data analysis was using FlowJo software (Tree Star, Inc., OR, USA).

2.15 MHC tetramers and FACS analysis

Tetramer were prepare as described previously (Altman, Moss et al. 1996). Briefly, the recombinant HLA-A*1101 heavy chain contains the BirA-recognition site, which has been incorporated into the carboxyl terminus of the MHC molecule. The monomer adopts an appropriate conformation following the addition of β_2 microglobulin (β_2m) and synthetic peptide (NS3 133-142 peptide). Then the enzyme BirA is used to attach a biotin molecule to the specific BirA-recognition sequence. Four MHC-biotin complexes are link to a single streptavidin molecule, using the specific biotin-avidin interaction, to form a tetramer. The streptavidin molecule here is tagged with phycoerythrin fluorochrome.

HLA*A-1101 MHC class I tetramers containing the $NS3_{133-142}$ epitope from all four DENV serotypes were synthesized as previously described (Mongkolsapaya, Dejnirattisai et al. 2003). The four tetramers (DV1$NS3_{133-142}$ GTSGSPIVNR, DV2.1$NS3_{133-142}$ GTSGSPIIDK, DV2.2$NS3_{133-142}$ GTSGSPIVDK, DV3-4$NS3_{133-142}$ GTSGSPIINR) were pooled at equal concentrations and used to stain fresh whole blood. Briefly, whole blood was stained with

pooled PE-labeled $NS3_{133-142}$ tetramers at 37°C for 30 min in the dark and then for surface makers at 37 °C for 15 min by addition of APC-conjugated anti-CD8 Ab. Stained blood were then lysed, washed, fixed, and analyzed by flow cytometry.

Chapter 3

Genetic susceptibility to DHF/DSS – HLA class I and II alleles

Chapter 3: Genetic susceptibility to DHF/DSS – HLA class I and II alleles

3.1 Genetic susceptibility to infectious disease

The interaction between the microbe, the host and the environment is a major selective force in evolution. Interest in the role that host genetic variation plays in susceptibility to infection has occurred from both the individual severity of disease and its progression, as well as infection rate at a population level. Genetic associations might therefore provide answers to fundamental questions about the pathogenesis of an organism and the host response. The best known example of such genetically determined factors is found in malaria and haemoglobinopathy (Hill, Bennett et al. 1992). For example, heterozygosity for sickle cell haemoglobin is strongly associated with protection against death and severe disease.

The outcome of infection with several diseases such as tuberculosis and human immunodeficiency virus (HIV) was subsequently correlated with polymorphisms in some host genes (Hill 1996). Additional evidence of the selective genetic pressure of infectious agents can be demonstrated by the higher level of polymorphisms seen in the human leukocyte antigen (HLA) region when compared with other regions in the human genome. Positive and negative genetic associations between particular HLA variants and a variety of major infectious diseases, such as HIV/AIDS, hepatitis, leprosy, tuberculosis, malaria, leishmaniasis, and schistosomiasis have been reported to include (reviewed in Blackwell, 2009).

3.2 Case and control studies for studying genetic susceptibility to infectious disease

There are two main approaches to determine the contribution of genes to disease susceptibility, involving either linkage or association studies, or both. While linkage studies can only use familial data, association studies can be family or case-control based. The collection of case-control series is often easier than multi-case families. Case-control studies need to be well-matched in terms of ethnicity (genetic background) and environment. Matching for age and sex is less important as allele frequencies seldom differ between sexes nor change with age. A further advantage of the case-control study is that the completion time for the study is relatively short, and multi-risk factor can be compared in the same study population. However, large sample sizes are usually required for these studies so that sufficient power is obtained to detect effects in the order of two-fold change in risk. Moreover, the results do not provide direct information about the mechanism of disease risk; it only provides an estimate of relative risk.

To determine if a gene is a contributing risk factor to the disease phenotype, its polymorphisms are examined for association in case-control studies. Significant differences between genotype frequencies in the cases and controls might suggest a role for genetic factors in disease outcome. In the simplest form, the association between a particular risk factor and the disease outcome can be assessed using a 2x2 contingency table (χ^2 test of association). The Odds Ratio (OR) is the ratio of odds of disease among people with the risk factor to the odds of disease among people without the risk factor.

3.3 Studies of Genetic influences on Dengue virus infections

Central in the pathogenesis of DHF and DSS is the loss of endothelial integrity that is believed to be the result of an "abnormal" immune response and a disturbance in immune regulation. There are several possible reasons why some infected individuals might produce a greater inflammatory response than others. Retrospective analysis of the Cuban epidemic of dengue hemorrhagic fever in 1981 suggested that those of African descent were hospitalized less frequently with DHF and DSS than those of European descent (Kouri, Guzman et al. 1989). In addition, despite the hyperendemic transmission of multiple DENV serotypes in the Haitian population, neither DHF/DSS outbreaks nor sporadic cases has been reported (Halstead, Streit et al. 2001). The results of these studies suggest that human genetic factors may influence susceptibility to DHF. Several studies have confirmed that some genetic polymorphisms may protect or predispose an individual to DHF or DSS.

3.4 HLA and dengue

One of the functions of the human leukocyte antigens (HLAs), encoded by the major histocompatibility complex (MHC) and whose genes are on chromosome 6, is to display antigenic peptides to receptors on host T-lymphocytes in order to activate cellular host immune responses. HLA genes show great variability and specific polymorphisms seen in human HLA gene regions influence peptide epitope binding (Cooke and Hill 2001). Several studies have looked at the variation in the HLA genes and found some of them to be associated with the severity of dengue virus infection (Table 3.1).

Table 3.1 Summary of associations between human leukocyte antigen class I and II alleles and DF/DHF

Class	Allele	Population	Type of association[a]	Reference
Class I	A1	Cubans	−	(Paradoa Perez, Trujillo et al. 1987)
	A2	Thai	−	(Stephens, Klaythong et al. 2002)
	A*0203	Thai	+	(Stephens, Klaythong et al. 2002)
	A*0207	Thai	−	(Stephens, Klaythong et al. 2002)
	A24	Vietnamese	−	(Loke, Bethell et al. 2001)
		Vietnamese	−	(Nguyen, Kikuchi et al. 2008)
	A29	Cubans	+	(Paradoa Perez, Trujillo et al. 1987)
	A33	Vietnamese	+	(Loke, Bethell et al. 2001)
	B blank	Thai	−	(Chiewsilp, Scott et al. 1981)
		Cubans	−	(Paradoa Perez, Trujillo et al. 1987)
	B13	Thai	+	(Chiewsilp, Scott et al. 1981)
	B14	Cubans	+	(Paradoa Perez, Trujillo et al. 1987)
	B15	Cubans	−	(Sierra, Alegre et al. 2007)
	B31	Cubans	−	(Sierra, Alegre et al. 2007)
	B44	Thai	+	(Stephens, Klaythong et al. 2002)
	B46	Thai	−	(Stephens, Klaythong et al. 2002)
	B51	Thai	−	(Stephens, Klaythong et al. 2002)
	B52	Thai	+	(Stephens, Klaythong et al. 2002)
	B62	Thai	+	(Stephens, Klaythong et al. 2002)
	B76	Thai	+	(Stephens, Klaythong et al. 2002)
	B77	Thai	+	(Stephens, Klaythong et al. 2002)
Class II	DRB1*04	Cubans	+	(Sierra, Alegre et al. 2007)
		Mexicans	+	(LaFleur, Granados et al. 2002)
	DRB1*07	Cubans	+	(Sierra, Alegre et al. 2007)
	DRB1*0901	Vietnamese	+	(Nguyen, Kikuchi et al. 2008)
	DQ1	Brazil	−	(Polizel, Bueno et al. 2004)
	DR1?	Brazil	−	(Polizel, Bueno et al. 2004)

[a] +, protection against DF/DHF; −, susceptibility to DF/DHF

3.4.1 HLA class I and dengue

The HLA class I region can be divided into eight exons encoding the different domains, and three individual loci, HLA-A, -B, -C, -E, -F, -G (Figure 3.1). HLA-B is the most polymorphic, followed by HLA-A, and HLA-C (Steven G.E. Marsh 2000). Each individual expresses up to two alleles for each locus. Alleles at each of these loci are related phylogenetically and can be separated into families. HLA class I products have wide distribution and are present on the surface of all nucleated cells and on platelets. Antigens associated with HLA class I products will interact with $CD8^+$ T cells during an immune response.

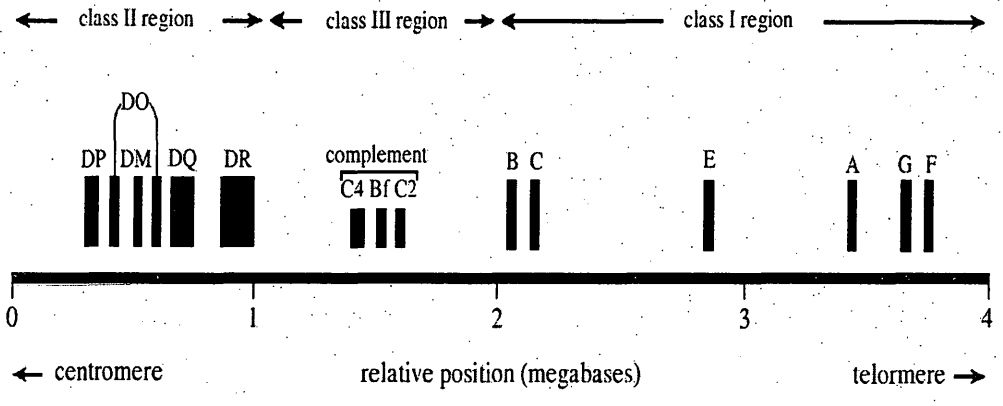


Figure 4.1 Simplified map of the HLA complex (Steven G.E. Marsh 2000)

Chiewlip *et al.* was the first to report an association between HLA class I and the severity of dengue virus infection (Chiewsilp, Scott et al. 1981). The frequency of the HLA types in unrelated Thai children who had clinical DHF/DSS deviated from those of controls, with $p < 0.05$, for six antigens: HLA-A1, HLA-A2, HLA-A9, HLA-B13, HLA-B17, and HLA-B. Later, a study in Cuban patients who had been hospitalized with DHF/DSS also showed a significant difference in the frequency of HLA antigens when compared with a control group (Paradoa Perez, Trujillo et al. 1987). This Cuban study reported that HLA-A29 was associated with protection against DHF, whereas HLA-A1 and HLA-Cw1 were associated with susceptibility to DHF.

Subsequently, a larger case control study in Vietnamese demonstrated that polymorphisms in the HLA class I region, particularly of the HLA-A gene, were significantly associated with susceptibility to DHF (Loke, Bethell et al. 2001). Of the 26 alleles of HLA class I and II studied, two particular alleles were relevant: patients with HLA-A33 were less likely to develop DHF (P = 0.01; odds ratio, 0.56; 95% confidence interval, 0.34-0.93), whereas those with HLA-A24 allele were at an increased risk to develop DHF ($p = 0.02$; odds ratio, 1.54; 95% confidence interval, 1.05-2.25). HLA-B alleles were not associated with DHF disease susceptibility.

Another case-control study, in Thai children, suggested that the HLA-A2 locus serotype was associated with disease susceptibility (Stephens, Klaythong et al. 2002). HLA-A*0203 was increased in all DF patients with secondary infections when compared with either the control or DHF with secondary infections. In this study, HLA-A*0207 was associated with susceptibility to the more severe DHF in patients with secondary DENV1 and DENV2 infections only. In the same study, they also observed dengue virus-specific associations within the HLA-B5 group of related alleles, whereby molecularly determined HLA-B*51

alleles were associated with DHF after secondary dengue virus infections. By contrast, HLA-B*52 showed a strong association with less severe DF secondary DENV-2 infections. Moreover, the reduced frequency of the HLA-B15-related alleles (B62, B76, and B77) was observed in the patients with secondary dengue virus infections, suggesting that they may be protected against developing clinical disease in immunologically primed individuals. By contrast, HLA-B46 was increased in the DHF patients with seconday infections. Since HLA-B46 is in strong linkage disequilibrium HLA-A*0207, it is believed that the effect of B46 was likely to be an adjunct to that of A*0207. Finally, HLA-B44 appeared to be protective against the development of severe disease in patients with secondary dengue virus infections. Recently, a hospital-based case-control study in two provinces in Southern Vietnam showed an increase of frequency of HLA-A*24 in both DHF and DSS patients (Nguyen, Kikuchi et al. 2008). The frequency of HLA-A*24 with histidine at codon 70, based on main anchor binding site specificity analysis in DSS and DHF patients, was significantly higher than that in the population background groups.

3.4.2 HLA class II and dengue

The organization of class II genes is similar to class I genes, where each of the exons encode a different domain. HLA class II products consist of HLA-DO, -DR, -DP, and –DQ (Figure 3.1). The HLA class II products have more limited distribution on B-cell, macrophages, dendritic cells, Langerhans cells and activated T cells. HLA class II molecules are involved in the presentation of exogenous antigens to $CD4^+$ T helper cells.

A study of HLA-DR antigen frequencies in Mexican patients with dengue found that the frequency of HLA-DRB1*04 was lower in DHF patients than in DF patients and that this allele was negatively associated with DHF risk (LaFleur, Granados et al. 2002). These findings are in contrast to the finding of Loke *et al.*, who studied polymorphisms in the HLA-

DRB1 gene in Vietnamese DHF patients but did not find an association (Loke, Bethell et al. 2001).

Recently, a significantly increased frequency of HLA-A*31 and HLA-B*15 has been demonstrated in Cuban individuals with a history of symptomatic dengue virus infection compared with controls (Sierra, Alegre et al. 2007). HLA-DBR1*07 and HLA-DRB1*04, on the other hand, showed an evaluated frequency in control subject compared with dengue case patients suggesting these alleles are associated with protection (Sierra, Alegre et al. 2007). In a study of HLA alleles and severe dengue in Southern Vietnam, the frequency of HLA-DRB1*0901 in particular was significantly decreased in DSS when compared with DHF in DENV-2 infection (Nguyen, Kikuchi et al. 2008).

Notwithstanding the previous studies of HLA and dengue, the interpretation of previous case-control studies is potentially confounded by a number of factors including heterogeneity in the case classification, serological and virological features of the patients being investigated leading to small subgroup analysis and limited power.

3.5 Study design

Human leukocyte antigens expressed on the cell surface function as antigen presenting molecules and that polymorphism can shape an individuals' adaptive immune response.
In this study, I first aimed to investigate the HLA class I and II polymorphisms present in a population of Vietnamese children with DSS resulting from secondary infections with DENV-2, compared to a group of population controls (cord bloods). Then we aimed to confirm the correlation appearing in the second independent case-control study in wider range of clinical presentation.

3.6 Patient recruitment

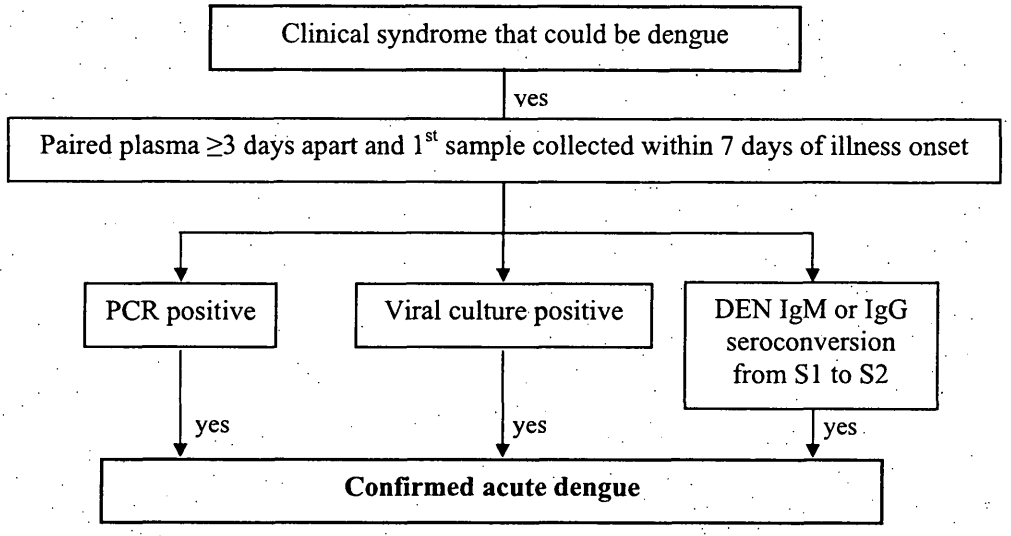
There are two prospective studies described. In the first study, pediatric patients with dengue shock syndrome (≤15 yrs of age) were recruited into a prospective study at the Pediatric Intensive Care Unit of the Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam, between 1999-2002. All patients were of Vietnamese Kinh ethnicity and had a clinical diagnosis of DHF/DSS Grade III or IV. Blood samples were collected at study entry (day 1) and then on day 2 and day 4 for research and hematological investigations.

The second study was a prospective study of children with mild dengue at Ward Pediatric A of the Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam, between 2002 and 2006. Any Vietnamese Kinh child between 2-15 years of age hospitalized with clinically suspected dengue and fever for < 72 hours was eligible for study entry. Any child who developed Shock (DSS) was transferred to the Pediatric Intensive Care Unit. All patients had daily haematocrit and platelet count. Blood samples were collected at study entry and then on the 6^{th} day of illness for research purposes.

For both studies, DNA was extracted from the blood pellets using a commercial DNA extraction kit (Nucleon, Amersham Biosciences, UK) according to the manufacturer's instructions. Clinical symptoms were recorded in standardized case record forms and laboratory investigations (see Figure 3.2) were used to confirm the diagnosis.

Written informed consent was obtained from a parent or guardian of each study participant. The study protocol was approved by the Scientific and Ethical committees at the Hospital for Tropical Diseases and the Oxford University Tropical Research Ethical committee.

Figure 3.2 Dengue diagnostic algorithm



3.7 Control samples

Umbilical cord blood samples were used as the control samples for this study. Cord blood samples were collected from the umbilical cord of healthy neonates at the time of delivery to mothers of Vietnamese Kinh ethnicity at Hung Vuong Obstetric Hospital, HCMC. After centrifugation of cord blood, the cells and the plasma were separated and stored at –20°C. Written informed consents were obtained from mothers. The study protocol was approved by the Scientific and Ethical committees at Hung Vuong Hospital and the Hospital for Tropical Diseases and the Oxford University Tropical Research Ethical committee.

3.8 Statistical analysis

HLA class I and II typing was performed to establish the frequencies of the major HLA alleles. The frequency of HLA class I and II antigens was determined by the formula *n/N*,

where n equals the number of individuals that are positive for a particular HLA (either heterozygotes or homozygotes) and N equals the number of individuals tested. 2x2 $\chi 2$ test and P values derived from StatCalc (EpiInfo) software were used for comparing allele frequencies. P values of less than 0.05 were considered significant. The odds ratio (OR) and 95% confidence interval were used to assess the risk of disease associated with a specific class I and II phenotype.

3.9 Results

3.9.1 Characteristic of patient population

As the first purpose of this study was to investigate the association of HLA alleles and dengue shock syndrome in Vietnamese children with secondary DENV-2 infections, we selected DNA samples from a subset of patients enrolled in a prospective study of DSS at the Pediatric Intensive Care Unit of the Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam. Between 1999 and 2002, there were 120 patients who were experiencing a secondary DENV2- infection. After applying the former WHO case classification criteria (WHO. 1997) to each dengue case, there were 113 DHF grade III patients and 7 DHF grade IV patients. The characteristics of the patient population are described in Table 3.2.

Table 3.2 Characteristics of the patient population selected for HLA typing

Variable	DHF grade III N=113	DHF grade IV N= 7
Male sex, no. (%)	62 (54%)	3 (42%)
Age (years)	10 (3-14)	11 (5-13)
Day of illness	5 (3-10)	4 (3-5)
Petechiae	95/95(100%)	5/7(71%)
Skin bleeding	59/112 (52%)	4/7 (57%)
Other bleeding*	25/109 (22%)	1/7 (14%)
Effusion	40/107 (37%)	1/7 (14%)
Ascites	32/107 (29%)	1/7 (14%)
Hepatomegaly (mean)	1.8 ± 0.1	2.1 ± 0.3
Platelet nadir, cells/μL	22,000 (8,310-90,000)	20,000 (12,000-50,000)
Maximum hemoconcentration, %	38.9 (21.5-66.7)	50 (32.4-53.4)

* Other bleeding including Gum bleeding, Epistaxis, Purpura, GI bleeding, PV bleeding

3.9.2 HLA class I and II genotyping by PCR sequence-specific primers

All DSS patients (n=120) and cord blood control samples (n=120) were typed for HLA class I and II using amplification refractory mutation system PCR (ARMS-PCR) with sequence specific primers (SSP) (Brunce *et al.*, 1995). One hundred and ninety two PCR reactions were performed to simultaneously detect HLA-A, B, Cw, DRB1 and DQB1 alleles. At the time this study was conducted, this approach was recognized as a high resolution technique and better at distinguishing HLA alleles subtypes compared to serological methods. Indeed HLA typing by PCR-SSP has proven to confer significantly greater sensitivity, accurate, and resolving power than serology typing methods (Petersdrorf EW, 1999).

The results from this integrated class I and II method can be visualized on a single photographic or electronic image and hence is described a "Phototyping". A phototype is determined successful when the control amplifications are positive and at least one allele or group of alleles is present in each locus. Amplification of control primers gives a product of 796 base pairs fragment from the third intron of HLA-DRB1 locus. An example of HLA phototype of dengue patient DF398 is shown in Figure 3.3.

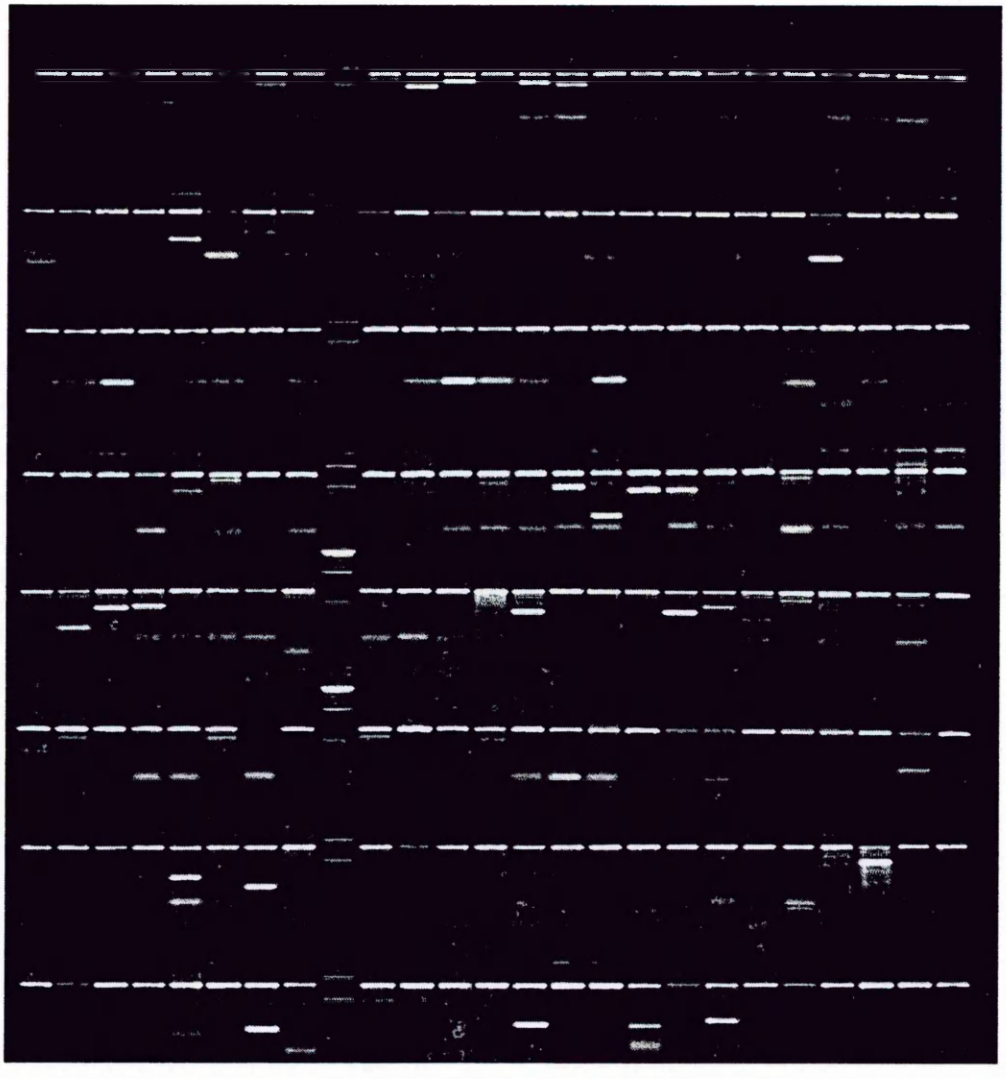


Figure 3.3 HLA PCR SSP phototyping of patient DF389. The sample type was A*24, A*33, B*15, B*35, Bw6, Cw*04, DRB1*04, DRB3, DRB5, DQB1*04, DQB1*06

3.9.3 Phenotype frequencies of HLA class I and II alleles

We identified 14 HLA-A alleles, 21 HLA-B alleles, 10 HLA-Cw alleles, 13 HLA-DRB1 alleles and 5 HLA-DQB1 alleles in the study subjects. Major alleles (phenotype frequencies having more than 5% in either the patient or control groups) are shown in Table 3.3-3.7, accounted for about 80-90% of the total phenotypes.

The most common HLA-A, -B, -Cw, -DRB1 and DQB1 alleles were A*02, A*11, A*24 and A*33; B*15 and B*46; Cw*01 and Cw*07; DRB1*12 and DRB1*15; DQB1*03 and DQB1*05, respectively. Alleles of HLA- A*74 (2.5%), -B*48 (0.8%), -B*52 (1.7%), -B*54 (0.8%, -Cw*16 (0.8%), -DRB1*01(2.5%) were observed in DSS patients but not in control subjects.

Table 3.3 Frequency of HLA-A alleles in DSS patients with secondary DENV-2 infection and control subjects

HLA-A antigen	Case patient (*N*=120)[a]	Control subject (*N*=120)[a]	Odds ratio (95% CI)	χ^2	*P*
*01	8 (6.7)	6 (5)	1.36(0.41-4.58)	0.30	0.581
*02	57 (47.5)	62 (51.7)	0.85(0.49-1.45)	0.42	0.518
*11	55 (45.8)	60 (50)	0.85(0.49-1.45)	0.42	0.518
*24	40 (33.3)	38 (31.7)	1.08(0.61-1.92)	0.08	0.783
*33	27 (22.5)	34 (28.3)	0.73(0.39-1.37)	1.08	0.299

χ2 test and *P* values derived from Statcalc (EpiInfo) sofware.

[a] Data are no. positive for antigen (frequency of antigen).

Table 3.4 Frequency of HLA-B alleles in DSS patients with secondary DENV-2 infection and control subjects

HLA-B antigen	Case patient (N=120)[a]	Control subject (N=120)[a]	Odds ratio (95% CI)	χ^2	P
*07	4 (3.3)	9 (7.5)	0.43(0.11-1.57)	2.03	0.153
*13	13 (10.8)	8 (6.7)	1.7(0.63-4.7)	1.3	0.253
*15	60 (50)	65 (54.2)	0.85(0.49-1.45)	0.42	0.518
*27	11 (9.2)	5 (4.2)	2.32(0.71-7.95)	2.41	0.120
*35	8 (6.7)	7 (5.8)	1.15(0.36-3.68)	0.07	0.789
*38	11 (9.2)	9 (7.5)	1.24(0.46-3.42)	0.22	0.640
*40	11 (9.2)	11 (9.2)	1(0.38-2.6)	0.00	1.000
*44	4 (3.3)	14 (11.7)	0.26(0.07-0.89)	6.01	**0.014**[b]
*46	21 (17.5)	27 (22.5)	0.73(0.37-1.45)	0.94	0.333
*51	5 (4.2)	7 (5.8)	0.7(0.19-2.56)	0.35	0.553
*56	11 (9.2)	13 (10.8)	0.83(0.33-2.08)	0.19	0.666
*57	7 (5.8)	6 (5)	1.18(0.34-4.1)	0.08	0.775
*58	12 (10)	15 (12.5)	0.78(0.32-1.86)	0.38	0.539

$\chi 2$ test and P values derived from Statcalc (EpiInfo) sofware.

[a] Data are no. positive for antigen (frequency of antigen).

[b] Statistically significant.

Table 3.5 Frequency of HLA-Cw alleles in DSS patients with secondary DENV-2 infection and control subjects

HLA-Cw antigen	Case patient (*N*=120)[a]	Control subject (*N*=120)[a]	Odds ratio (95% CI)	χ^2	*P*
*01	34 (28.3)	43 (35.8)	0.71(0.4-1.27)	1.55	0.21
*03	27 (22.5)	17 (14.2)	1.76(0.86-3.62)	2.78	0.095
*04	21 (17.5)	26 (21.7)	0.77(0.38-1.52)	0.66	0.416
*06	9 (7.5)	6 (5)	1.54(0.48-5.06)	0.64	0.423
*07	46 (38.3)	49 (40.8)	0.9(0.52-1.56)	0.16	0.692
*08	23 (19.2)	14 (11.7)	1.8(0.83-3.92)	2.59	0.107
*12	12 (10)	4 (3.3)	3.22(0.93-12.25)	4.29	**0.038**[b]
*15	22 (18.3)	17 (14.2)	1.36(0.65-2.87)	0.77	0.381

$\chi 2$ test and *P* values derived from Statcalc (EpiInfo) sofware.

[a] Data are no. positive for antigen (frequency of antigen).

[b] Statistically significant.

Table 3.6 Frequency of HLA-DRB1 alleles in DSS patients with secondary DENV-2 infection and control subjects

HLA-DRB1 antigen	Case patient (*N*=120)[a]	Control subject (*N*=120)[a]	Odds ratio (95% CI)	χ^2	*P*
*03	8 (6.7)	6 (5)	1.36(0.41-4.58)	0.30	0.581
*04	23 (19.2)	29 (24.2)	0.74(0.38-1.44)	0.88	0.347
*07	15 (12.5)	25 (20.8)	0.54(0.25-1.15)	3.00	0.083
*08	16 (13.3)	14 (11.7)	1.16(0.51-2.68)	0.15	0.696
*09	25 (20.8)	35 (29.2)	0.64(0.34-1.2)	2.22	0.136
*10	18 (15)	12 (10)	1.59(0.69-3.71)	1.37	0.241
*12	59 (49.2)	57 (47.5)	1.07(0.62-1.83)	0.07	0.796
*13	4 (3.3)	8 (6.7)	0.48(0.12-1.83)	1.40	0.236
*14	14 (11.7)	9 (7.5)	1.63(0.63-4.28)	1.20	0.272
*15	29 (24.2)	23 (19.2)	1.34(0.69-2.61)	0.88	0.347

$\chi 2$ test and *P* values derived from Statcalc (EpiInfo) sofware.

[a] Data are no. positive for antigen (frequency of antigen).

Table 3.7 Frequency of HLA-DQB1 alleles in DSS patients with secondary DENV-2 infection and control subjects

HLA-DQB1 antigen	Case patient (N=120)[a]	Control subject (N=120)[a]	Odds ratio (95% CI)	χ^2	P
*02	19 (15.8)	27 (22.5)	0.65(0.32-1.3)	1.72	0.189
*03	79 (65.8)	52 (43.3)	2.43(1.4-4.23)	11.35	**0.0007**[c]
*04	15 (12.5)	9 (7.5)	1.76(0.69-4.58)	1.67	0.196
*05	52 (43.3)	52 (43.3)	1(0.58-1.72)	0.00	1.000
*06	40 (33.3)	48 (40)	0.75(0.43-1.31)	1.15	0.283

$\chi 2$ test and P values derived from Statcalc (EpiInfo) sofware.

[a] Data are no. positive for antigen (frequency of antigen).

[b] Statistically significant.

3.9.4 Association of HLA-B, HLA-Cw, and HLA-DQB1 alleles and DSS

We analyzed only these major alleles for the evaluation of the risk of disease severity because rare alleles might have little impact on population risk. Studying fewer alleles, would also mitigate the problem of multiple comparisons.

A summary of the statistical comparisons is shown in Table 3.3-3.7. There was an increase in the frequency of the antigen HLA-DQB1*03 in the DSS patient group (65.8% vs 43.3%, P = 0.0007), with an OR of 2.43 (95%CI = 1.4-4.23), indicating that individuals carrying HLA-DQB1*03 have a higher chance of developing DSS than individuals not expressing this antigen. The antigen frequency of HLA-Cw*12 was also moderately increased in DSS patients when compared with the control (10% vs 3.3%, P = 0.038, OR = 3.22, 95%CI = 0.93-12.25). Conversely, HLA-B*44 decreased in frequency in the DSS patient group when compared with the control (3.3% vs 11.7%, P = 0.014, OR = 0.26, 95%CI = 0.07-0.89).

There were no other statistically significant associations between the typed HLA alleles and DSS.

3.9.5 Association of HLA-DQB1*03 alleles and dengue

To explore more fully the correlationship between the HLA-DQB1*03 and DSS in Vietnamese children with secondary DENV-2 infections, we selected DNA samples from a independent subset of patients and cord blood controls. Patients who were experiencing secondary DENV-2 infections enrolled in two prospective studies on either the Pediatric Ward A (uncomplicated patients) and the Pediatric intensive Care Unit (DSS patients) of the Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam represented the case population. The controls were cord blood samples collected at the same time as the previously described cord blood samples but from different newborns. The characteristics of the dengue patient populations are described in Table 3.8.

Table 3.8 Characteristics of the DENV-2 infected patient population selected for HLA-DQB1*03 typing

Variable	DSS (n=98)	uncomplicated dengue (n=147)
Male sex, no. (%)	48	90
Age (years)	10 (3-14)	12 (5-16)
Day of illness	4.5 (3-7)	3 (2-5)
DF		37
DHF		
Grade I		39
Grade II		71
Grade III	85	
Grade IV	13	
Platelet nadir, cells/μL	24,400 (6,000-80,000)	49,900 (10,800-290,000)
Maximum hemoconcentration, %	37.8 (20.0-71.5)	19 (0-55.7)

A summary of the statistical comparisons between the two patient groups and cord blood controls, which are independent of the above control group, are shown in Table 3.9 – 3.11. The HLA-DQB1*03 allele was significantly increased in frequency in DSS patients (P = 0.0001, OR = 10.54, 95%CI = 2.25-6.82) and DF/DHF patients (P = 0.0007, OR = 4.39, 95%CI = 1.64-12.15) when compared to the controls. There was no different in frequency of HLA-DQB1*03 alleles between two patient groups (DSS patients versus DF/DHF patients).

Table 3.9 Frequency of HLA-DQB1 alleles in DSS patients with secondary DENV-2 infection and control subjects

HLA-DQB1 antigen	DSS patients *(N=98)*[a]	Control subject *(N=100)*[a]	Odds ratio (95% CI)	χ^2	*P*
*02	19 (19.4%)	17 (17)	1.17(0.54-2.57)	0.19	0.663
*03	96 (98%)	82 (82)	10.54(2.25-67.82)	13.88	**0.0001**[b]
*04	8 (8.2)	6 (6)	1.39(0.42-4.74)	0.35	0.552
*05	44 (44.9)	43 (43)	1.08(0.59-1.97)	0.07	0.787
*06	14 (14.3)	25 (25)	0.50(0.23-1.09)	3.59	0.058

$\chi 2$ test and P values derived from Statcalc (EpiInfo) sofware.

[a] Data are no. positive for antigen (frequency of antigen).

[b] Statistically significant.

Table 3.10 Frequency of HLA-DQB1 alleles in uncomplicated dengue patients with secondary DENV-2 infection and control subjects

HLA-DQB1 antigen	uncomplicated dengue patients *(N=147)*[a]	Controls (*N*=100)[a]	Odds ratio (95% CI)	χ^2	*P*
*02	22 (15)	17 (17)	0.86 (0.41-1.81)	0.19	0.666
*03	140 (95.2)	82 (82)	4.39 (1.64-12.15)	11.46	**0.0007**[b]
*04	4 (2.7)	6 (6)	0.44 (0.10-1.81)	1.65	0.199
*05	63 (42.9)	43 (43)	0.99 (0.58-1.72)	0.00	0.982
*06	30 (20.4)	25 (25)	0.77 (0.40-1.47)	0.72	0.394

χ2 test and *P* values derived from Statcalc (EpiInfo) sofware.

[a] Data are no. positive for antigen (frequency of antigen).

[b] Statistically significant.

Table 3.11 Frequency of HLA-DQB1 alleles in DSS and uncomplicated dengue patients with secondary DENV-2 infection

HLA-DQB1 antigen	DSS patients *(N=98)*[a]	uncomplicated dengue patients *(N=147)*	Odds ratio (95% CI)	χ^2	*P*
*02	19 (19.4)	22 (15)	1.37 (0.66-2.83)	0.83	0.363
*03	96 (98)	140 (95.2)	2.4 (0.44-17.11)	1.23	0.267
*04	8 (8.2)	4 (2.7)	3.18 (0.84-12.97)	3.74	0.053
*05	44 (44.9)	63 (42.9)	1.09 (0.63-1.88)	0.10	0.752
*06	14 (14.3)	30 (20.4)	0.65 (0.31-1.37)	1.50	0.221

χ2 test and *P* values derived from Statcalc (EpiInfo) sofware.

[a] Data are no. positive for antigen (frequency of antigen).

Collectively, this study has found that HLA Class I and II polymorphism significantly influences genetic susceptibility to DSS, in particular, secondary DENV-2 infected children with HLA-B*44 were likely to have DSS, whereas secondary DENV-2 infected children with HLA-Cw*12 and HLA-DQB1*03 were at increase risk of DHF when compared with the controls. HLA-A and HLA-DRB1 polymorphisms were not associated to DSS.

3.10 Discussion

There is still much to be learnt about the possible influence of host genotype in resistance or susceptibility to DSS. For most HLA/DENV studies performed to date, the HLA allele frequencies in DF, DHF and sometimes DSS patients were compared to frequencies observed in control age-matched healthy individuals of the same ethnicity. Allelic frequencies that significantly deviate from frequencies observed in the control population suggest a negative or positive association between the HLA genotype and DENV disease status. Previous studies have focused on polymorphisms of HLA class I and II (Table 3.1). Nonetheless, none of the reported associations has been replicated in different populations. The inconsistency of these HLA associations may be the result of differences in ethnicity, differences in the predominant virus serotype during the study periods and HLA typing technique (Chaturvedi, Nagar et al. 2006). In our study, we tested the hypothesis that some HLA class I and II alleles may be associated with the occurrence of a particular clinical disease form resulting from DENV-2 infection. Our data demonstrated a higher frequency of the antigen HLA-B*44 in controls compared with DSS patients. Conversely, HLA-Cw*12 was associated with the development of DSS in patients with secondary DENV-2 infection. Our finding was similar to the findings of Stephens *et al.* (Stephens, Klaythong et al. 2002) who showed the antigen frequency of HLA-B44 were reduced in all DHF patients with secondary infections compared with the controls, although the frequency of HLA-B*44 and HLA-Cw*12 in our results are smaller than 5% in case patients and controls, respectively. The association here is more difficult to characterize in HLA and disease association studies due to the small frequencies of the HLA antigens in study population. A second independent study with a larger patient sample may resolve this issue.

These Vietnamese data on HLA class I association with DHF support the relevance of MHC class I variation suggested by some previous studies (Table 3.1), even though the allelic associations in each study differ, perhaps as a result of viral, clinical outcome and other non-MHC host genetic variation polymorphism. A recent case-control study of HLA associations in Vietnamese DHF patients revealed evidence for HLA-A*24 and HLA-A*33 in susceptibility and resistance respectively DHF (Loke, Bethell et al. 2001). We did not confirm these associations in our current study. A possible reason why we did not confirm an association between HLA-A*24 or HLA-A*33 and DSS is that in the study by Loke et al, DENV infections were confirmed serologically in only a small proportion of DHF patients (Loke, Bethell et al. 2001), and there was no information on the infecting serotype. In contrast, our DSS patient set comprised only laboratory confirmed secondary DENV-2 infection. In addition, DENV-2 was the prevalent serotypes in South of Vietnam from 1999 to 2006 while DENV-1 was predominant from 1990 to 1995 when Loke et al. collected their samples (Ha, Tien et al. 2000) (Pasteur 2007). The study performed by Nguyen et al is also found association between DHF/DSS patients and HLA-A*24 but not HLA-A*33 (Nguyen, Kikuchi et al. 2008). Infact, there were inconsistent association results between their three patient groups as they devided patient into primary/secondary. There was also no information on the infecting DENV serotype in Nguyen et al study.

HLA-DQB1*03, which was the most frequent class II allele in our study population and appeared to be associated with increased susceptibility to DSS, with OR 2.43 and *P* value of 0.0007 (Table 3.7). This association was confirmed in another independent population of cases with either DSS or DF/DHF grade I and II. We hypothesize that the HLA-DQB1*03 association observed might be explained by its higher efficacy in presenting DENV-2 epitopes to $CD4^+$ T cells. In this model, HLA-DQB1*03 might facilitate a greater level and

breadth of the immune response to virus peptides and this may contribute to the pathogenesis of dengue (King, 2003). Although HLA class I restricted cytolytic CD8 T lymphocytes are also likely to be most important for clearing virus infected cells, dengue specific HLA class II restricted $CD4^+$ T cell clones with cytolytic activity have been raised from individuals infected or vaccinated with dengue virus (Kurane, Okamoto et al. 1995) (Livingston, Kurane et al. 1994) (Green, Kurane et al. 1993) (Luhn, Simmons et al. 2007). Such DENV-specific $CD4^+$ T lymphocytes may contribute to immunopathology by lysing virus-infected MHC class II expressing cells, e.g. macrophages, and secreting pro-inflammatory cytokines that might lead to capillary leakage (Kurane, Innis et al. 1990). For example, $CD4^+$ T cell clones are capable of secreting a range of cytokines including IFNγ, TNFα, and IL-2 upon re-stimulation and it is conceivable that these molecules have an impact on normal vascular endothelium function.

HLA-DQB1*03 could also be just a marker for variants of other genes linked to the HLA or other HLA linked gene that has the potential to influence the diseases outcome. The DRB1*0401-DQA1*03-DQB1*0302 haplotype is reportedly more common in type I diabetes patients (Volpini, Testa et al. 2001) and DRB1*0402-DQA1*03-DQB1*0302 in Insulin-dependent diabetes mellitus patients (Saruhan, 2000). Thus to determine whether the association identified above are because of the marker being part of one specific halotype has to be address in further studies.

Due to the complexity of the immune system, genetic susceptibility to infectious diseases is most likely to be the combined effects of many genes. With the candidate gene approach, we are looking for an effect at one of the many genes. There are some strength of the study design. (i) Our DSS patient set comprised only laboratory confirmed scondary DENV-2 infection. At the time samples were collected, DENV-2 was the most prevalent serotypes in

South of Vietnam (1999 to 2006). (ii) All our patients and control were intensively typed for HLA class I and II alleles.

There are obvious weaknesses with this study design. If the effect of the individual genes is small or the alleles of a polymorphism are rare, extremely large studies would be needed to see an effect. It is only possible to study genes and polymorphisms that have been mapped and sequenced and this will become easier with the Human Genome Project. Improvements in technology that permit high-throughput genotyping of genetic polymorphisms have allowed a genome-wide approach to investigating host genetic susceptibility. Essential to this approach are large sample sizes in order to make statistical correction of the number of comparisons being made. Replication of the genetic association in a second population is now considered an essential validation step.

Another methological issue is that of selection of study population and sampling strategy. Most candidate gene studies use a case-control approach, and unrecognized ethnic differences between the groups are likely to be reflected in fake genetic associations that are unrelated to the disease of interest. This issue of "population stratification" can be minimized if cases and controls are carefully matched and its presence can also be quantified from the genetic data themselves. The majority of Vietnamese people (85%) belong to the Kinh ethnic, whereas the remaining (15%) represent 60 minorities (Vu-Trieu, Djoulah et al. 1997). In fact, there was a first GWAS to look at host susceptibility to dengue shock syndrome (Long *et al*, unpublished data) demonstrated for the first time that Vietnamese Kinh is distinct from other Asian populations such as the Chinese and Japanese. This genotyping data of SNPs in all the Vietnamese Kinh cases and control were compared with those of SNPs in Caucasian (CEU), African (YRI) and Asian populations (HCB and JPT) indicated that the case and the control

samples are homogeneous. To these evidences, we are confident that our Vietnamese Kinh cases and controls are ideal population for case-control genetic association studies.

Collectively, these genetic data underscore the potential importance of HLA class II-alleles in DHF/DSS, however, it is not possible to conclude from this evidence the actual role of HLA class II-restricted responses in pathogenesis. Association between HLA, DENV serotype, and DENV infection outcome are complex and require further explanation and research. Association-based observations require confirmation with functional studies that validate interaction between HLA-associated genotypes and dengue. The only way to understand the role of HLA restricted responses in dengue infections and DHF pathogenesis is to initiate studies in naturally immune individuals.

Chapter 4

Spectrum and kinetic of T cell responses to epitopes in dengue viruses

Chapter 4: Spectrum and kinetic of T cell responses to epitopes in dengue viruses

4.1 Background

Epidemiological studies suggest that individuals who experience a secondary infection with a heterologous dengue serotype are at significantly greater risk of developing DHF (Burke, 1988) (Graham, Juffrie et al. 1999) (Thein, Aung et al. 1997). In endemic countries, children carry most of the symptomatic disease burden but young adults are also significantly affected. Several hypotheses have been proposed for the risk factors of severe dengue. Viral, host genetic, age and nutritional factors have been associated with susceptibility to DHF (Gamble, Bethell et al. 2000) (Leitmeyer, 1999) (Loke, 2001) (Loke, 2002) (Stephens, 2002) (Thisyakorn) (Stephenson, 2005). Viral risk factors include infection with a more virulent dengue virus and the size of the viral load. Antibody-dependent enhancement (ADE) is also hypothesized to contribute to the pathogenesis of severe dengue illness. Halstead proposed that during secondary DENV infection, the preexisting DENV antibodies at sub-neutralizing or non-neutralizing levels to infecting serotype may result in ADE of infection (Halstead, 2002).

Profound immune activation during secondary infection contributes to other risk factors and promotes the development of severe plasma leakage. There are evidences of $CD4^+$ and $CD8^+$ T cell activation *in vivo* during dengue infection. Accordingly, frequencies of $CD8^+$ T cells are higher in patients with DHF than in those with mild dengue fever (Rothman, 2003) (Green, 1999) (Mongkolsapaya, 2003) (Zivna, 2002). Following the viral replication and presenting antigen to T cells, activation of $CD4^+$ and $CD8^+$ memory T cells in secondary infection leads to proliferation and release of proinflammatory cytokines (Mangada, Endy et al. 2002). Several plasma cytokines of cellular immune activation, including tumor necrosis

factor-alpha, gamma interferon, interleukin-2 receptors, interleukin-6, interleukin-13 and interleukin-18, are elevated in DHF compared to DF (Rothman, 2003) (Kurane, 1991) (Juffrie, 2001) (Mustafa, 2001). These cytokine can act directly upon vascular endothelial cells to elicit increased vascular permeability. Together, these studies suggest a vital role of immune response in immunopathogenesis of viral infection. However, the mechanism of how T cells contribute to DHF pathogenesis is less clear since only a few studies have directly examined the responses of T cell during acute disease (Green, 1999) (Mongkolsapaya, 2003). Thus there exists a need to study the cellular immune responses of T cells and serotype cross-reactivity in a patient population with a mixed HLA background to determine the relationship between T cell response and disease severity. Therefore, the aim of the current work is to identify novel T cell epitopes in dengue viruses that are restricted through HLA alleles common in Vietnamese population. Furthermore, we seek to determine the kinetics and cross-reactivity of the responses to novel epitopes.

4.2 Study design

During primary infection, inflammatory cytokines are released from infected cells, including dendritic cells, B cells, monocytes, and hepatocytes that can attract adaptive immune cells including T cells, NK cells, and B cells. Naïve T cells specific for the infecting serotype are preferentially expanded. In the secondary infection, the level of T lymphocyte activation is markedly increased, reflecting the increased antigen presentation, the increased frequency of DENV-specific and cross-reactive T lymphocytes in secondary, and the more rapid activation and proliferation of memory T lymphocytes. Infected cells present 8-11mers or 15-20mer peptides, dependent on the class of HLA molecules bound to the peptides, to specific T lymphocytes. Hence, identification of antigenic peptides recognized by dengue virus-specific T lymphocytes may suggest new way to suppress viral replication and prevent prolonged

infection. Recently, studies of dengue patients have successfully identified several peptides that are specific for $CD8^+$ and $CD4^+$ T lymphocytes (Table 4.1). In this study, we sought to identify the novel epitopes and measured *ex vivo* responses to a spectrum of dengue virus antigens by studying T cell responses to peptide antigens spanning the DENV-2 genome.

Due to several limitations, we were unable to collect enough blood from patients to study the T cell responses to peptide antigens spanning the entire genome. Therefore, we focused our investigations on structural antigens (capsid, preM/M, and Env) and nonstructural viral antigens previously nominated as being T cell imunogens (NS3 and NS4a). A total of 260 peptides (including two hundred and twenty 15mers and forty 20mers) span the capsid, preM, M, Env, NS3, and NS4a sequences from a DENV serotype 2 isolate (strain 16681) (Appendix 3). PBMC were tested in IFN-γ ELISPOT assay against the total of 260 peptides arranged into a matrix of 32 peptide pools, with 16 peptides in each pool and each peptide present in two different pools. The exceptions were pool 16 with 20 peptides and pools 29, 30, 31, and 32 with 18 peptides (Appendix 4). A peptide is identified as being antigenic if it drives a response in two different pools in IFN-γ ELISPOT assay. In every case, identified peptides were retested as individual peptides.

Table 4.1 Epitopes on dengue viral proteins recognized by virus-specific human T cells

Protein	Amino acids	Sequence	Subset	HLA	Reference
C	47-55	VLAFITFLR	CD4	DPw4	(Gagnon, Zeng et al. 1996)
	83-92	GFRKEIGRML	CD4	DR1, DPw4	(Gagnon, Zeng et al. 1996)
NS3	71-79	SVKKDLISY	CD8	B62	(Zivny, DeFronzo et al. 1999)
	130-144	GTSGSPIIDKK	CD8	A1101	(Mongkolsapaya, 2003)
	146-154	VIGLYGNGV	CD4	DR15	(Kurane, 1995)
	202-211	RKYLPAIVRE	CD4	DR15	(Kurane, 1995)
	222-230	APTRVVAAE	CD8	B7	(Mathew, 1998)
	224-234	TRVVAAEMEEA	CD4	DR15	(Kurane, 1998)
	235-243	AMKGLPIRY	CD8	B62	(Zivny, 1995)
	241-249	IRYQTTATK	CD4	DR15	(Kurane, 1995)
	255-264	EIVDLMCHAT	CD4	DPW2	(Okamoto, Kurane et al. 1998)
	352-362	WITDFVGKTVW	CD4	DR15	(Zeng, 1996)
	500-508	TPEGIIPTL	CD8	B35	(Livingston, 1995)

4.3 Patient recruitment

Adult patients with a clinical suspicion of dengue virus infection were recruited from the intensive care unit of the Hospital for Tropical Disease, Ho Chi Minh City, Vietnam. Venous blood samples were collected from patients on the first day after admission (Study Day 1), on Study Day 3 and on Study Day 5 unless the patient was discharged earlier. Convalescent samples were obtained at 2 weeks and 1 month post-admission. WHO classification criteria (World Health Organization, 1997) were applied to each case after review of the study notes.

Platelet counts and haematocrit values were recorded regularly during hospitalization. The extent of hemoconcentation during symptomatic illness was determined by comparing the maximum haematocrit recorded during hospitalization with the value recorded at convalescence.

Written informed consent was obtained from the patients. The study protocol was approved by the Scientific and Ethical Committee at The Hospital for Tropical Diseases and by the Oxford Tropical Research Ethical Committee.

4.4 Results

4.4.1 Characteristics of patient population

This study was carried out at The Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam. Between June of 2002 and April of 2003, we recruited 51 patients suspected of having a dengue virus infection from the adult intensive care unit into a prospective study of cellular immune responses. After applying WHO criteria (World Health Organization, 1997) to each case, all patients were classified as DHF cases, including DHF Grade I (n=13), DHF Grade II (n=17), and DHF Grade III (n=20); only 1 patient was diagnosed with DHF Grade IV. Two subjects had clinical manifestation of plasma leakage (e.g., pleural effusion) and mucosal bleeding, but platelet counts were between 100,000 and 120,000. We classified these cases as DHF grade II. The patients' mean age was 19 years (SD, 3 years; range, 15 to 27 years). The mean duration of illness was 5 days (SD, 1 day; range, 1 to 10 days). Serology of paired plasma samples indicated that all patients were experiencing a secondary dengue virus infection. For the period of hospital admission, the average maximum haematocrit recorded was 49% (SD, 4.7%, range, 41 to 60%) and the mean nadir of the platelet count was 47,700 $\times$ 10^3/ml (SD, 33,900$\times$ 10^3/ml, range, 13,900 $\times$ 10^3/ml to 115,000 $\times$ 10^3/ml). Amongst the 51 investigated patients, 39 patients (76%) had a response to at least one peptide in the IFN-γ ELISPOT assay. The characteristics of the patients with positive SFU in response to at least one peptide are shown in Table 4.2.

Table 4.2 The characteristic of the patients respond to DENV-2 peptides

Patient	Age (years)	Sex[a]	Day of illness	Serology	Diagnosis[b]	Dengue serotype[c]	HLA class I type		
							A	B	Cw
BC309	24	F	5	Secondary	II	1	24/34	15/46	03/03
BC310	17	F	3	Secondary	III	1	11/24	35/51	04/14
BC311	19	M	4	Secondary	III	4	24/26	38/15	07/08
BC313	19	M	5	Secondary	III	0	24/26	38/15	07/03
BC314	18	M	4	Secondary	III	2	02/11	46/15	01/08
BC315	19	F	10	Secondary	II	2	29/33	58/81	03/15
BC316	22	M	5	Secondary	III	0	29/30	07/13	06/07
BC317	22	M	6	Secondary	III	1	11/26	38/15	07/08
BC318	15	F	6	Secondary	II	2	24/33	44/15	04/07
BC320	18	F	5	Secondary	III	0	02/33	46/58	01/03
BC322	26	M	3	Secondary	III	2	11/30	07/54	01/15
BC323	31	M	4	Secondary	III	0	11/24	46/51	01/14
BC324	20	F	5	Secondary	IV	2	29/68	07/51	15/-
BC325	17	M		Secondary	II	0	02/11	56/15	01/-
BC327	20	M	4	Secondary	I	1,4	02/31	51/39	07/-
BC331	17	M	5	Secondary	II	1	11/24	40/35	03/03
BC332	16	M	5	Secondary	III	0	02/11	38/37	07/06
BC333	22	M	5	Secondary	II	0	11/26	38/15	07/08
BC334	31	M	5	Secondary	II	0	02/11	07/57	06/07
BC336	21	F	4	Secondary	II	2	02/31	46/51	01/14
BC337	16	M	4	Secondary	III	1	11/24	15/46	01/08
BC338	22	F	3	Secondary	II	2	11/33	13/46	01/03
BC343	22	M	5	Secondary	II	1	24/29	07/15	
BC347	20	M	5	Secondary	III	2	02/11	15/46	01/03
BC348	16	M	3	Secondary	III	0	24/33	15/58	03/08
BC349	17	F	4	Secondary	III	2			
BC350	17	F	5	Secondary	II	2	24/34	15/46	01/03
BC352	15	F	5	Secondary	II	2	24/29	07/48	08/15
BC353	15	F	6	Secondary	III	2	11/-	39/56	01/07
BC354	17	F	4	Secondary	II	1	11/-	15/-	08/-
BC500	26	M	2	Secondary	I	4	01/11	15/57	06/08
BC501	20	M	5	Secondary	I	4	02/11	51/54	01/-
BC502	16	M	5	Secondary	I	0	02/29	07/13	03/15
BC503	23	M	4	Secondary	I	1	11/-	07/46	01/15
BC504	27	M	4	Secondary	I	4	11/29	07/46	01/15
BC507	26	M	5	Secondary	I	4	02/24	15/15	04/07
BC508	15	M	5	Secondary	II	4	02/03	46/15	01/08
BC509	20	M	5	Secondary	I	0	02/03	38/35	07/12
BC510	15	M	5	Secondary	I	1	33/-	18/44	07/07

[a] F, female; M, male.
[b] WHO classification: I-DHF grade I; II-DHF grade II; III-DHF grade III; IV-DHF grade IV.
[c] DENV serotype: 1-DENV1; 2-DENV2; 3-DENV3; DENV4

4.4.2 IFN-γ ELISPOT responses against a panel of peptides spanning the capsid, preM, M, Env, NS3 and NS4a viral antigens

To measure the breadth and magnitude of T cell responses to multiple dengue virus antigens, PBMC samples from dengue virus-immune Vietnamese adults were tested in IFN-γ ELISPOT assays against overlapping peptides spanning the capsid, preM, M, Env, NS3, and NS4a viral antigens from a DENV-2 isolate. Amongst the 51 DHF patients, we have defined the IFN-γ ELISPOT response to dengue peptides in PBMC samples from 39 patients. In these 39 patients, one or more individual peptides that reproducibly evoked an IFN-γ response in the ELISPOT assay exceeded 25 spot forming units (SFU) per million PBMC. In total, T cells from the 39 patients recognized 47 different peptides. In terms of the percentage of peptides recognized of the total number tested for each viral antigen, the peptides from the capsid and M protein were most frequently recognized in ELISPOT assays (Figure 4.1). However, in categorical terms, there were more peptides (n=30, 64%) from the NS3 protein that evoked T cell responses in IFN-γ ELISPOT assay compared to 11% for capsid, 6% for prM/M, 17% for Env and 2% for NS4a.

The magnitude of responses to individual peptides ranged from 27 SFU to 500 SFU per million of PBMC. The breadth of the response in individual patients ranged from 1 to 10 peptides. The breadth and magnitude of responses from all patients are shown in Appendix 5. A summary of the individual peptides that reproducibly evoked responses in IFN-γ ELISPOT assays is provided in Table 4.3. In several cases, two adjacent and overlapping peptides evoked a response in the same patient, suggesting that they contain a shared epitope, or alternatively, the presence of overlapping epitopes. Thirteen of the 47 peptides (28%) recognized by PBMC contain sequences that have previously been characterized as dengue- or flavivirus-specific $CD4^+$ or $CD8^+$ T cell epitopes (Table 4.3).

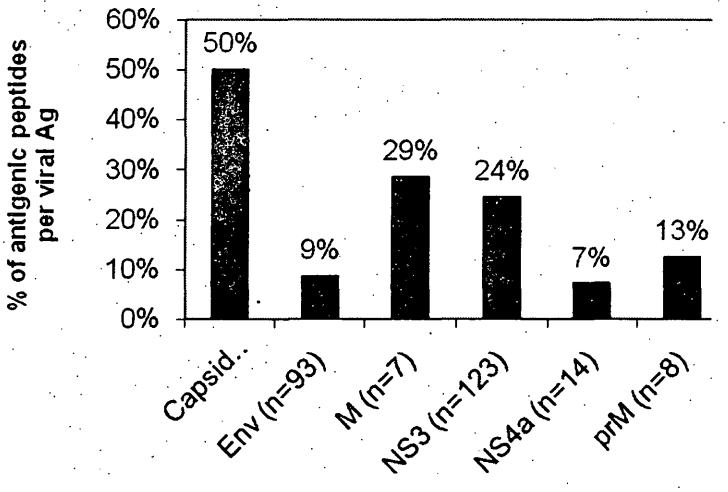


Figure 4.1 T cell responses by viral antigen. *Shown are the numbers of peptide antigens from different viral antigens that evoked T cell responses in convalescent dengue patients (Study days 5-14).*

Table 4.3 Summary of antigenic peptides recognized by T cells from secondary dengue patients

Sequence[a]	Location	N[b]	ELISPOT frequency[c]	Sub-set[d]	*Minimum epitope, HLA restriction*[e]
PFNMLKRERNRVSTVQQLTK	$Capsid_{12-31}$	1	100		
RVSTVQQLTKRFSLGMLQGR	$Capsid_{22-41}$	1	70		
TAGILKRWGTIKKSKAINVL	$Capsid_{62-81}$	3	97(60-130)	CD4	
IKKSKAINVLRGFRKEIGRM	$Capsid_{72-91}$	5	58(30-120)	CD4	$Capsid_{83-92}$ (GFRKEIGRML)/DPw4
RGFRKEIGRMLNILNRRRRS	$Capsid_{82-101}$	5	58(40-80)	CD4	$Capsid_{83-92}$ (GFRKEIGRML)/DPw4
LGELCEDTITYKCPLLRQNE	$preM_{41-60}$	3	203(28-500)	CD4	
MSSEGAWKHVQRIETWILRH	M_{20-39}	2	95(60-130)		
QRIETWILRHPGFTMMAAI	M_{30-49}	1	120		
FVEGVSGGSWVDIVL	Env_{11-25}	1	500		
SGGSWVDIVLEHGSC	Env_{16-30}	2	230(60-400)		
LRKYCIEAKLTNTTT	Env_{56-70}	2	115(80-150)		
VTMECSPRTGLDFNE	$Env_{181-195}$	1	120		
MENKAWLVHRQWFLD	$Env_{201-215}$	1	80		
TLVTFKNPHAKKQDV	$Env_{236-250}$	1	66		
KKQDVVVLGSQEGAM	$Env_{246-260}$	2	96(75-117)		
RMAILGDTAWDFGSL[f]	$Env_{411-425}$	8	142(60-300)	CD8	
TFHTMWHVTRGAVLM	$NS3_{45-59}$	5	151(55-390)	CD4	
IEPSWADVKKDLISY	$NS3_{65-79}$	3	151(100-194)	CD8	$NS3_{71-79}$ (SVKKDLISY)/B*62
ADVKKDLISYGGGWK	$NS3_{70-84}$	1	97		$NS3_{71-79}$ (SVKKDLISY)/B*62
AVSLDFSPGTSGSPI	$NS3_{125-139}$	1	140		
FSPGTSGSPIIDKKG	$NS3_{130-144}$	5	167(53-240)	CD8	$NS3_{133-142}$ (GTSGSPIIK)/A*11
KVVGLYGNGVVTRSG	$NS3_{145-159}$	3	140(140-140)	CD4	$NS3_{146-154}$ (VIGLYGNGV)/HLA-DR15
TKRYLPAIVREAIKR	$NS3_{200-214}$	3	63(40-85)	CD4	$NS3_{202-211}$ (RKYLPAIVRE)/ HLA-DR15
GLRTLIAPTRVVAA	$NS3_{215-229}$	3	92(75-100)		
ILAPTRVVAAEMEEA	$NS3_{220-234}$	7	150(40-350)	CD8	$NS3_{221-232}$ (LAPTRVVAAEME)/B*07
EMEEALRGLPIRYQT	$NS3_{230-244}$	3	213(120-260)	CD8	$NS3_{235-243}$ (AMKGLPIRY)/B*62
LRGLPIRYQTPAIRA	$NS3_{235-249}$	2	47(45-48)		
IRYQTPAIRAEHTGR	$NS3_{240-254}$	4	109(27-210)		
EHTGREIVDLMCHAT	$NS3_{250-264}$	1	200	CD4	$NS3_{255-264}$(EIVDLMCHAT)/HLA-DPw2
EIVDLMCHATFTMRL	$NS3_{255-269}$	2	150(100-200)	CD4	$NS3_{255-264}$(EIVDLMCHAT)/HLA-DPw2
LSPVRVPNYNLIIMD	$NS3_{270-284}$	3	173(90-230)		
VPNYNLIIMDEAHFT	$NS3_{275-289}$	3	213(190-230)		
LIIMDEAHFTDPASI	$NS3_{280-294}$	3	172(85-310)		
EAHFTDPASIAARGY	$NS3_{285-299}$	4	160(80-200)	CD8	
EMGEAAGIFMTATPP	$NS3_{305-319}$	1	70		
AGIFMTATPPGSRDP	$NS3_{310-324}$	3	105(80-153)		
KKVIQLSRKTFDSEY	$NS3_{380-394}$	1	118		
NDWDFVVTTDISEMG	$NS3_{400-414}$	1	100		
LDNINTPEGIIPSMF	$NS3_{495-509}$	2	155(80-230)	CD8	$NS3_{500-508}$ (TPEGIIPTL)/B*35
TPEGIIPSMFEPERE	$NS3_{500-514}$	3	167(70-240)	CD8	$NS3_{500-508}$ (TPEGIIPTL)/B*35
GDLPVWLAYRVAAEG	$NS3_{540-554}$	1	60		
VAAEGINYADRRWCF[f]	$NS3_{550-564}$	4	222(100-310)	CD8	
INYADRRWCFDGVKN	$NS3_{555-569}$	1	310		
EGERKKLKPRWLDAIY	$NS3_{585-599}$	2	68(38-97)		
KLKPRWLDARIYSDP	$NS3_{590-604}$	1	85		
WLDARIYSDPLALKE	$NS3_{595-609}$	1	250		
LATVTGGIFLFLMSGRGIGK	$NS4a_{61-80}$	1	58		

[a] sequence is derived from DENV2 isolate 16681

[b] 'N' indicates the number of patients who responded to each peptide

[c] shown are the mean (range) number of SFU/million PBMC evoked by each peptide

[d] the T cell subset responding to peptide stimulation was determined by bead depletion of $CD4^+$ and $CD8^+$ T cells

[e] the peptide sequence and HLA restriction of published epitopes that are present in the larger, overlapping 15 or 20mer peptide is shown. References link to table 3.1

[f] Peptides shown in the boldface were studied further

4.4.3 Antigen specificity of IFN-γ ELISPOT responses and their relationship to recognized structural motifs

All identified peptides recognized by T cells from dengue patients were evaluated for their relationship to recognized conserved structural motif. The spectrum of identified antigenic peptides was examined in the context of known biological features in each of the respective viral antigens. Several antigenic peptides were identified in the capsid protein. primarily located near to the conserved cleavage point of the C-terminal hydrophobic signal sequence (Table 4.3 and Fig. 4.2.A). Antigenic peptides were also detected in preM/M (Table 4.3 and Fig. 4.2.B), Env (Table 4.3 and Fig. 4.2.C), and NS4a (Table 4.3 and Fig. 4.2.D) proteins. These antigenic peptides were not associated with any recognized conserved structural motifs. NS3, a 618 amino acid long serine protease and helicase, contained the highest number (n=30) of antigenic peptides (Table 4.3 and Fig. 4.2.E). Fourteen from 30 (47%) of these antigenic peptides were clustered within a 124 amino acid long stretch of NS3 ($NS3_{200-324}$) (Fig. 4.2.E). Alignment of consensus amino acid sequences from all four dengue serotypes indicated that this region of NS3 is more conserved (78%) than NS3 as a whole (68%). NS3 was the most frequently recognized viral antigen, with 56% of all tested patients responding to at least one antigenic peptide compared to 27% for Env, 23% for the capsid, 10% for prem/M and 2% for NS4a.

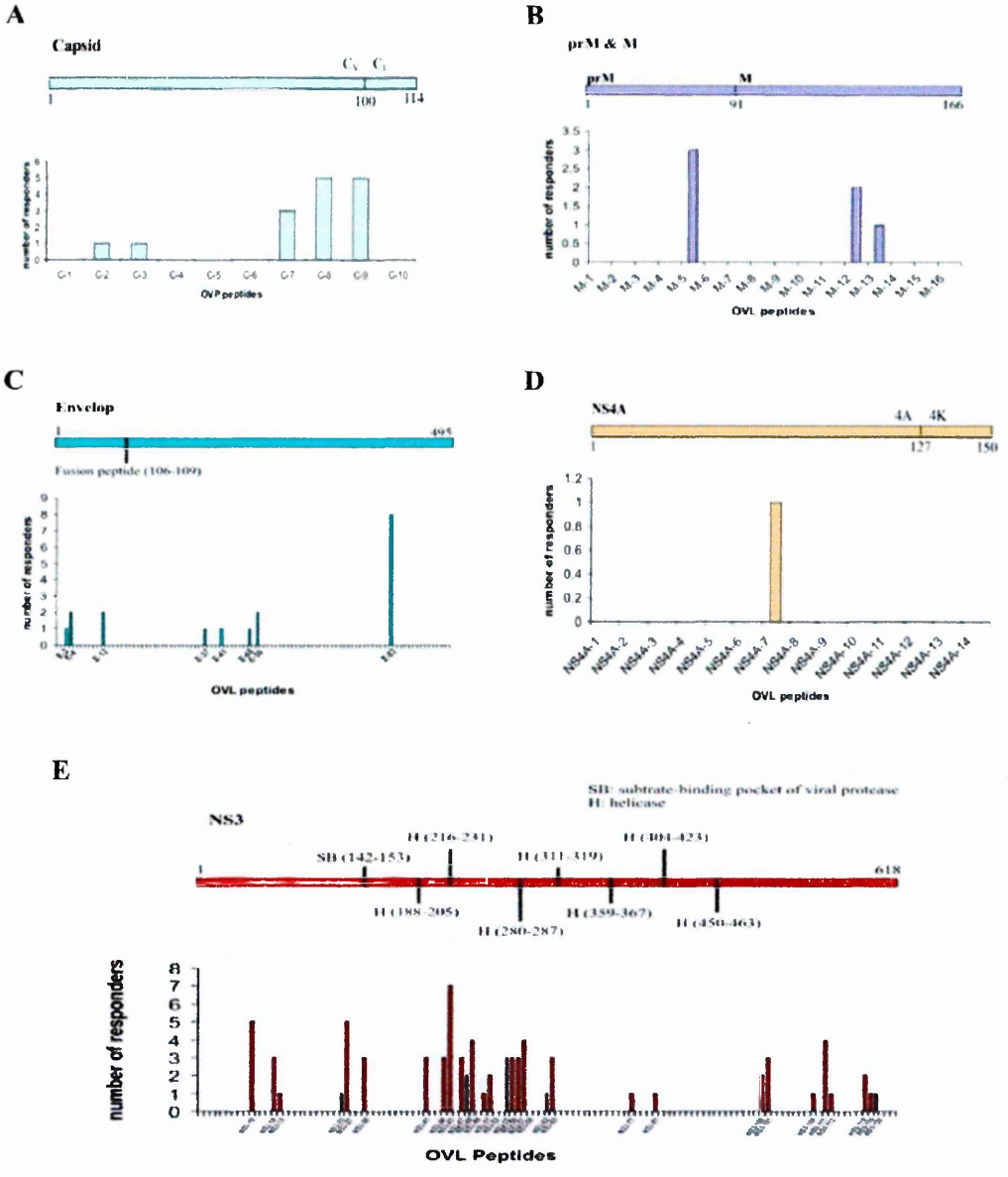


Figure 4.2 T cell responses by viral antigen. *Shown is the frequency with which individual antigenic peptides in the (A) capsid, (B) preM/M, (C) Env, (D) NS4A and (E) NS3 viral antigens were recognized by PBMC collected on Study Days 5-14 from 51 patients with secondary dengue. Shown within each viral protein are recognized structural features and in parentheses their amino acid location with respect to the start of the protein.*

4.4.4 Relationship between the serotype of the infecting virus and the breadth and magnitude of responses to overlapping DENV2 peptides in IFN-γ ELISPOT assays

A dengue serotype-specific PCR performed on acute plasma samples collected from all 51 patients identified the serotype of the infecting virus in 35 individuals (68%). Dengue viral RNA was detected in 28 (72%) of the 39 patients who made responses to one or more individual peptides. Amongst these 28 patients dengue serotype 1 (DV1) was detected in 9 (32%), DV2 in 12 (43%) and DV4 in 6 (21%). One patient had a mixed infection comprising DV1 and DV4 (4%). Dengue viral RNA was detected in 7 of the 12 patients (58%) who failed to respond to any of the peptide antigens. Interestingly, the spectrum of viruses detected in these 7 PCR-positive individuals was narrow, with DV2 detected in 6 (86%), and DV3 detected in 1 (14%).

We sought to determine whether a relationship existed between the breadth and magnitude of the T cell response to individual peptides and the serotype of the infecting virus. We did not include the patient with a mixed DENV1/DENV4 infection in this analysis. In cases when patients responded to adjacent peptides, we included responses to both in the analysis. Amongst the 47 patients analysed, the breadth of the responses was not significantly associated with the serotype of the infecting virus. Thus, patients infected with DENV2 (n=18) did not respond to significantly more peptides (median=2, range 0-10) than patients infected with either DENV1 (n=9, median; 2, range 1-7), DENV3 (n=1, median; 0), DENV4 (n=6, median; 2, range 1-4) or patients in whom a dengue virus could not be detected (n=16, median; 1, range 0-5) (Figure 4.3.A). Like the breadth of the response, the mean sum of ELISPOT frequencies to individual DENV2 peptides in patients infected with DENV2 did not significantly exceed (mean±standard deviation; 62±57 SFU/million PBMC) those in patients infected with either DENV1 (189±127 SFU/million PBMC), DENV4 (112±61

SFU/million PBMC) or patients in whom a dengue virus could not be detected (90±86 SFU/million PBMC) (Figure 4.3.B).

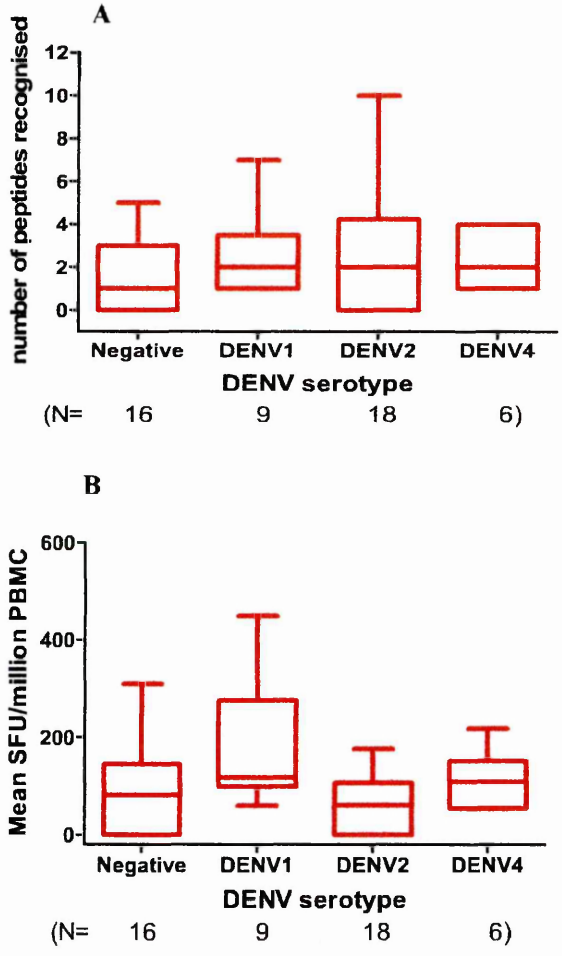


Figure 4.3 Relationship between the serotype of the infecting virus and the breadth and magnitude of T cell responses to DENV2 peptides. *Shown are the breadth of IFN-γ ELISPOT responses (A) and the mean of the sum of IFN-γ ELISPOT responses recorded (B) to peptide antigens in dengue patient by infecting DENV serotype.*

One possible explanation for the absence of significantly stronger responses to DENV2 peptides in patients infected with a serotype 2 virus is that the DENV2-specific response had not sufficiently matured at the time of analysis (Study Day 5). To test this hypothesis, ELISPOT responses to pools of overlapping DENV2 peptides were measured in serial PBMC samples collected from 5 prospectively recruited patients infected with DENV2. Responses detected against pooled peptides were verified using individual peptides. In the 4 patients who made responses to one or more peptide antigens, the breadth of the response actually contracted rather than expanded with time since infection elapsed (Figure 4.4). The narrowing of the breadth of responses coincided with a reduction in the magnitude of responses to each antigenic peptide. This suggests a significant DENV2-specific response does not evolve with increased time since secondary infection. The DENV2-specific response had sufficiently matured at convalescence.

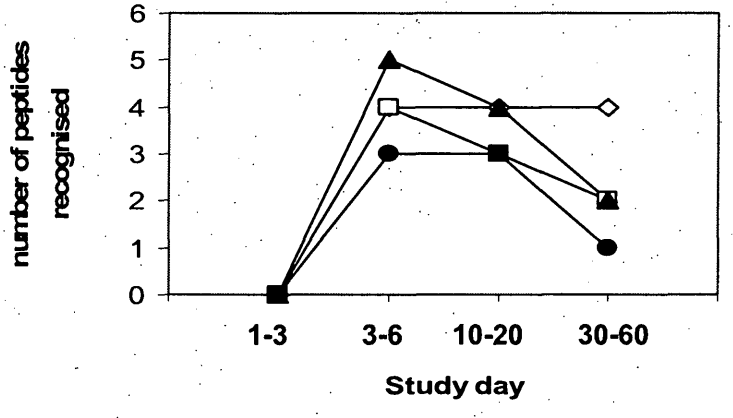


Figure 4.4 Breadth of the ELISPOT response to DENV2 peptides in patients infected with a dengue serotype 2 virus at different times post-presentation. *The number of individual peptide antigens that evoked significant responses (>50 SFU/million PBMC) in PBMC samples collected from four patients infected with DENV2 was determined at different times post-infection. Shown are the number of peptides recognized in each patient at different times post-diagnosis.*

4.4.5 Relationship between clinical parameters and the breadth and magnitude of responses to overlapping DENV2 peptides in IFN-γ ELISPOT assays

Clinical disease grade had no apparent relationship with the breadth or magnitude of the T cell response. With respect to the breadth of responses, patients with DHF grade I (n=13) recognized on average 1.9 peptides (range 0-8) in ELISPOT assays, which was virtually identical to patients in grade II (n=17, 2.4 peptides (range 0-7)) or III (n=20, 2.1 peptides, (range 0-6)). Only one patient with DHF grade IV was recruited to the study, although interestingly, PBMC from this individual recognized 10 different peptides. Like the breadth of the response, there were no significant differences between the mean sum of ELISPOT responses recorded for patients in each of the disease grades (Grade I: mean±standard deviation: 103±127, Grade II: 117±87, Grade III: 85±76, Grade IV: 110; see Figure 4.5).

The magnitude of the responses to individual peptides in each patient was also compared to the minimum platelet count and maximum relative increase in haemoconcentration recorded during hospitalization. The extent of acute haemoconcentration was determined by comparing the maximum haematocrit recorded during hospitalization to the haematocrit recorded at follow-up in each patient. This data was available for 24 patients, all of whom had detectable acute ELISPOT responses. Correlation analysis revealed a significant relationship ($r=0.41$, $P=0.04$) between the magnitude of IFN-γ ELISPOT responses measured on Study Day 5 and the extent of haemoconcentration in individual patients (Figure 4.6). Conversely, the nadir of thrombocytopaemia did not significantly correlate with the sum of responses to peptides in individual patients ($r=0.13$, $P=0.56$).

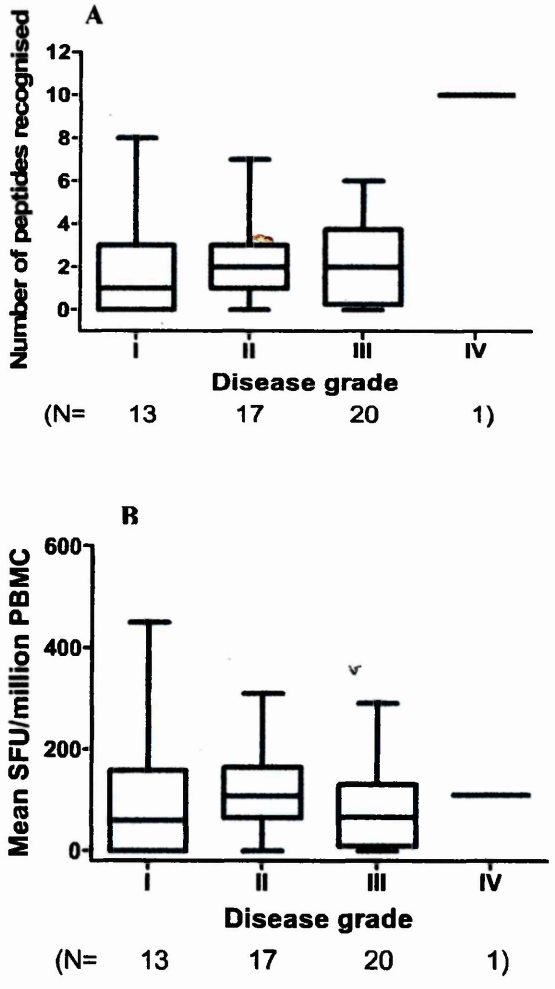


Figure 4.5 Relationship between clinical disease grade and the breadth and magnitude of T cell responses to DENV2 peptides. *Shown are the breadth of IFN-γ ELISPOT responses (A) and the mean of the sum of IFN-γ ELISPOT responses recorded (B) to peptide antigens in dengue patient by disease grade.*

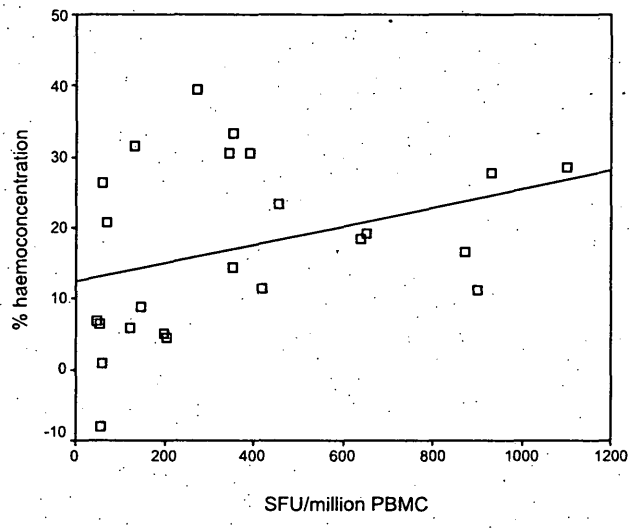


Figure 4.6 T cell responses and haemoconcentration. *Shown is the correlation between the sum of individual peptide-specific T cell responses detected in IFN-γ ELISPOT assays and the maximum percentage haemoconcentration recorded in each patient (P=0.04, Pearson correlation). The maximum percentage haemoconcentration occurring in each patient was deduced by comparison of haematocrit values obtained during hospitalization to those obtained from paired convalescent blood samples collected from 14 to 31 days after hospital admission.*

4.4.6 The minimum determinant and HLA restriction of T cells responding to $Env_{411-425}$

For several of the peptide antigens from 47 different peptides that was found to evoke T cell responses, we employed IFN-γ ELISPOT assays with PBMC depleted of either $CD4^+$ or $CD8^+$ T cells to identify the responding T cell subset. These experiments identified several peptides ($capsid_{62-81}$, $preM_{41-60}$, $Env_{411-425}$, $NS3_{45-59}$, $NS3_{285-299}$, $NS3_{550-564}$) that contain novel $CD4^+$ or $CD8^+$ T cell determinants (Table 4.3). Where possible, we attempted to predict the

HLA restriction of $CD8^+$ T cell responses by comparing the HLA types of all individuals who responded to the peptide in question. In cases with only one HLA antigen in common between all the responders, we nominated this allele as the restricting determinant.

The peptide RMAILGDTAWDFGSL ($Env_{411-425}$) was amongst the most frequently recognized by patient T cells in ELISPOT assays. Cells from 8 of 39 patients recognized $Env_{411-425}$ with a mean response of 153±76 SFU per million PBMC (Fig. 4.7A). In five patients, depletion of $CD4^+$ or $CD8^+$ T cells from PBMC prior to ELISPOT assay revealed that both $CD4^+$ and $CD8^+$ T cell enriched fractions responded to $Env_{414-422}$ peptide stimulation (Fig. 4.7B). In two patients, truncated synthetic peptides and ELISPOT assays were used to determine the minimum epitope within $Env_{411-425}$ that was recognized by $CD8^+$ T cells. Peptides with the sequences MILGDTAWDF, ILGDTAWDF, ILGDTAWDFG and RMAILGDTAWDF were recognized equally well by PBMC enriched for $CD8^+$ T cells, suggesting the sequence ILGDTAWDF ($Env_{414-422}$) may represent the minimum determinant for $CD8^+$ T cell responses (Fig. 4.8). HLA typing of patients possessing $CD8^+$ T cells that recognized peptide $Env_{414-422}$ revealed that HLA-B*07 was the only class I HLA antigen shared by all responders. We confirmed the HLA restriction of $Env_{414-422}$ via a CTL killing assay. A short term T cell line generated from $Env_{414-422}$ peptide-stimulated PBMC from BC503 was lytic to a peptide-pulsed BCL that shared only B*07 in common with the effector cells (25% specific lysis at an effector to target ratio of 10:1 for $Env_{414-422}$ peptide pulsed targets versus -2.5% for targets alone) (Fig. 4.9).

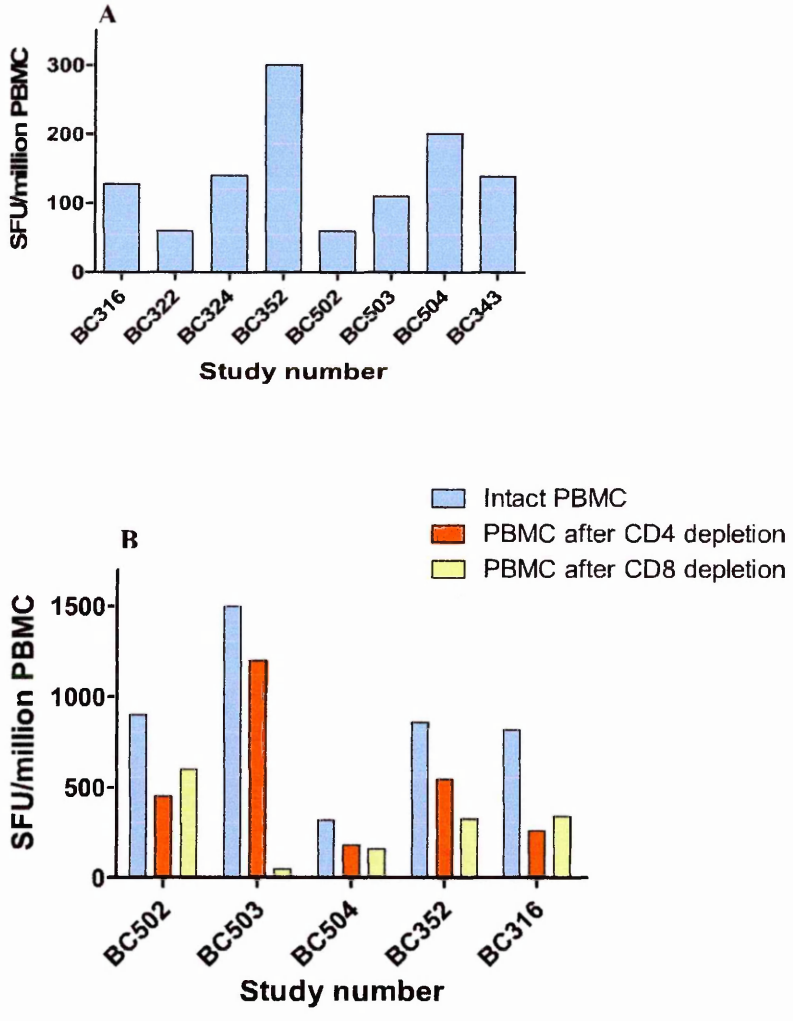


Figure 4.7 T cell responses evoked by RMAILGDTAWDFGSL ($Env_{411-425}$) stimulation. *(A) T cell responses evoked by RMAILGDTAWDFGSL ($Env_{411-425}$) stimulation from seven adult dengue patients. (B) PBMC from five adult dengue patients who recognized $Env_{411-425}$ were depleted of CD4 or CD8 T cells via magnetic beads. In each case, depletion removed >95% of targets cells. Untouched PBMC and cells remaining after CD4 or CD8 T cell depletion were co-cultured with $Env_{411-425}$ in IFN-γ ELISPOT assays.*

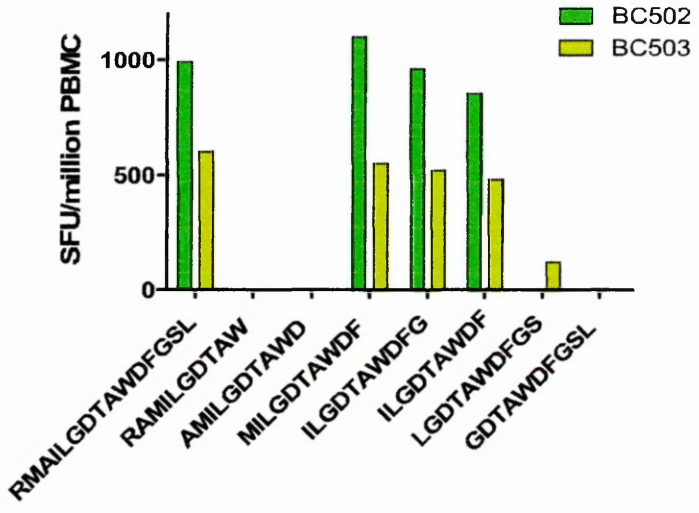


Figure 4.8 Recognition by CD8+ T cells of truncated peptide variants of RMAILGDTAWDFGSL ($Env_{411-425}$). *Shown are the frequencies of IFN-γ SFU elicited by truncated $Env_{411-425}$ peptides in IFN-γ ELISPOT assays against PBMC from BC502 and BC503 after enrichment for $CD8^+$ T cells.*

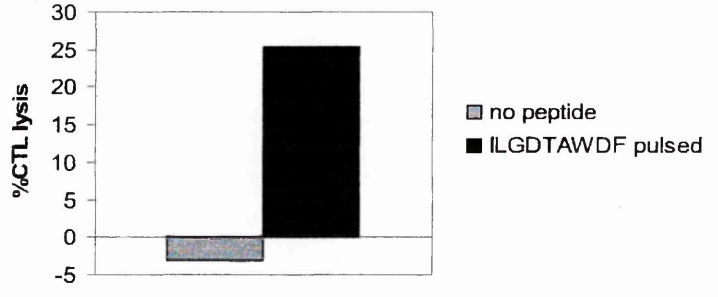


Figure 4.9 HLA-B*07 restricted recognition of the $Env_{414-422}$ peptide. *A CTL line from BC503 was co-cultured in a 4hr ^{51}Cr release assay with an $Env_{414-422}$-pulsed B lymphoblastoid cell line that matched the effector cells only at the B7 locus. Shown is the specific killing attained at a 10:1 ratio of effectors to targets.*

4.4.7 Kinetic and cross-reactivity of responses to $Env_{414-422}$

To measure the dynamic and serotype specificity of T cells specific for $Env_{414-422}$, we prospectively identified six HLA-B*07-positive patients with secondary dengue infections. The dengue serotype responsible for the current infection was determined in 4 patients: BC385, BC389 and BC395 were infected with DV2 and BC504 was infected with DV4. Virus was not detected in BC400 or BC414. Patients BC385, BC389, BC400, BC395 and BC504 were classified as DHF grade II whilst BC414 was DHF grade III. Initially, the extent of sequence variation present at $Env_{414-422}$, across all four dengue serotypes was examined. The sequence of $Env_{414-422}$ in DENV1-3 was invariant (ILGDTAWDF) whilst DENV4$Env_{414-422}$ differed by only one amino acid (ILGETAWDF). The dynamic of T cell responses to these variant peptides were then measured in 6 prospectively identified HLA-B*07-positive patients. All 6 patients responded to the HLA-B*07-restricted epitopes $Env_{414-422}$ (Fig. 4.10.A and 4.10.B) in ELISPOT assays, with the peak response measured at Study Day 3 or 14. ELISPOT responses to each of the epitope variants were generally similar. In samples from patient BC414, however, we observed much stronger responses (6-fold) to the DENV1 to DENV3 Env414–422 (ILGDTAWDF) epitope variant than to DENV4 Env414–422 (ILGETAWDF). Stronger responses to DENV1 to DENV3 Env414–422 were also observed in patient BC504 (3.5-fold at days 3 and 14) and patient BC395 (2-fold at day 14) but were less pronounced.

The generally concordant responses to the $Env_{414-422}$ peptide variants were confirmed in peptide titration assays using PBMC from BC400. These experiments indicated that the frequency of T cells responding to each of the B*07-restricted peptide variants was virtually identical across all peptide concentrations, including low peptide concentrations that are more likely to reflect *in vivo* conditions (Fig. 4.10.C).

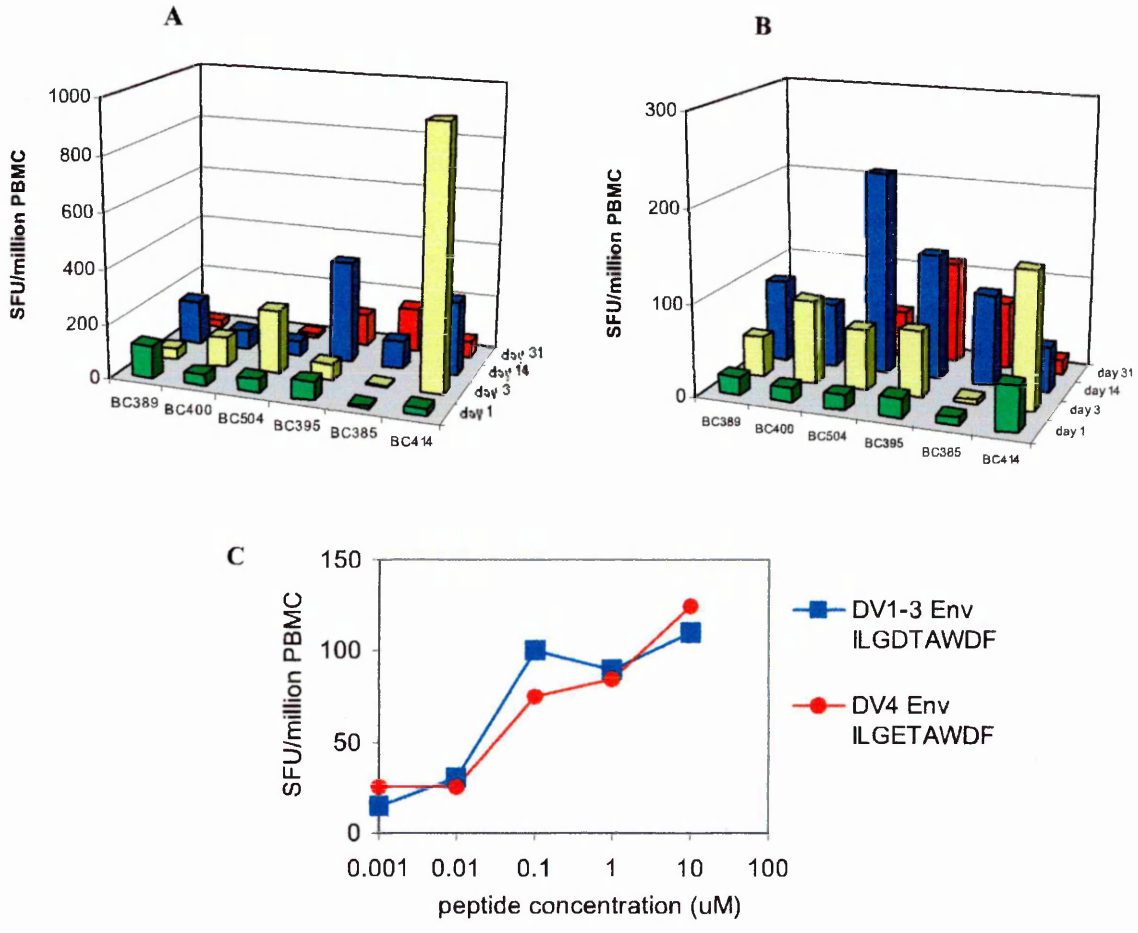


Figure 4.10 Kinetic and cross-reactivity of responses to Env$_{414-422}$. *The data depict the number of IFN-γ SFU detected in PBMC enriched for CD8$^+$ T cells from 6 HLA-B*07-positive patients with secondary dengue infections. PBMC were incubated with peptides corresponding to (A) DENV1-3Env$_{414-422}$ and (B) DENV4Env$_{414-422}$. Patients BC385, BC389, BC400, BC395 and BC504 were DHF grade II. BC414 was DHF grade III. Patients BC389, BC395 and BC385 were infected with DENV2. BC504 was infected with DENV4. Virus was not detected in BC400 or BC414. (C) A peptide titration experiment suggested T cells from BC400 were cross-reactive for the variant peptides DENV1-3Env$_{414-422}$ and DENV4Env$_{414-422}$ even at low peptide concentrations.*

4.4.8 The minimum determinant and HLA restriction of T cells responding to $NS3_{550-564}$

In IFN-γ ELISPOT assays, patient BC313 (HLA-A*24, A*26, B38, B15, Cw7, Cw3) responded to three non-overlapping NS3 peptides: VAAEGINYADRRWCF ($NS3_{550-564}$), EAHFTDPASIAARGY ($NS3_{285-299}$) and IEPSWADVKKDLISY ($NS3_{65-79}$). Depletion of $CD4^+$ or $CD8^+$ T cells from PBMC prior to ELISPOT assay indicated the responding cells were $CD8^+$ T cells (Fig. 4.11.A). The peptide spanning $NS3_{65-79}$ contained a previously defined HLA-B*15-restricted epitope, $NS3_{71-79}$ (Zivny, DeFronzo et al. 1999). Since patient BC313 was also HLA-B*15-positive, it was not further investigated. Two lines of evidence suggested the $NS3_{550-564}$ peptide contained an HLA-A*24 epitope. Firstly, a peptide containing this sequence had previously been shown to evoke T cell responses in HLA-A*24-positive Vietnamese dengue patients (Loke, Bethell et al. 2001). Secondly, the sequence NYADRRWCF ($NS3_{556-564}$) conformed to the consensus A*24 sequence, which predicts peptides with a tyrosine or phenylalanine at the 2nd position and an uncharged amino acid at the C-terminal anchor position. The minimum determinant in $NS3_{550-564}$ that elicited a response from patient BC313, who was HLA-A*24-positive, was identified by using truncated peptides spanning the predicted epitope in ELISPOT assays. INYADRRWCF ($NS3_{555-564}$) was the minimum peptide that evoked the strongest response at the lowest peptide concentration from BC313 (Fig. 4.11.B).

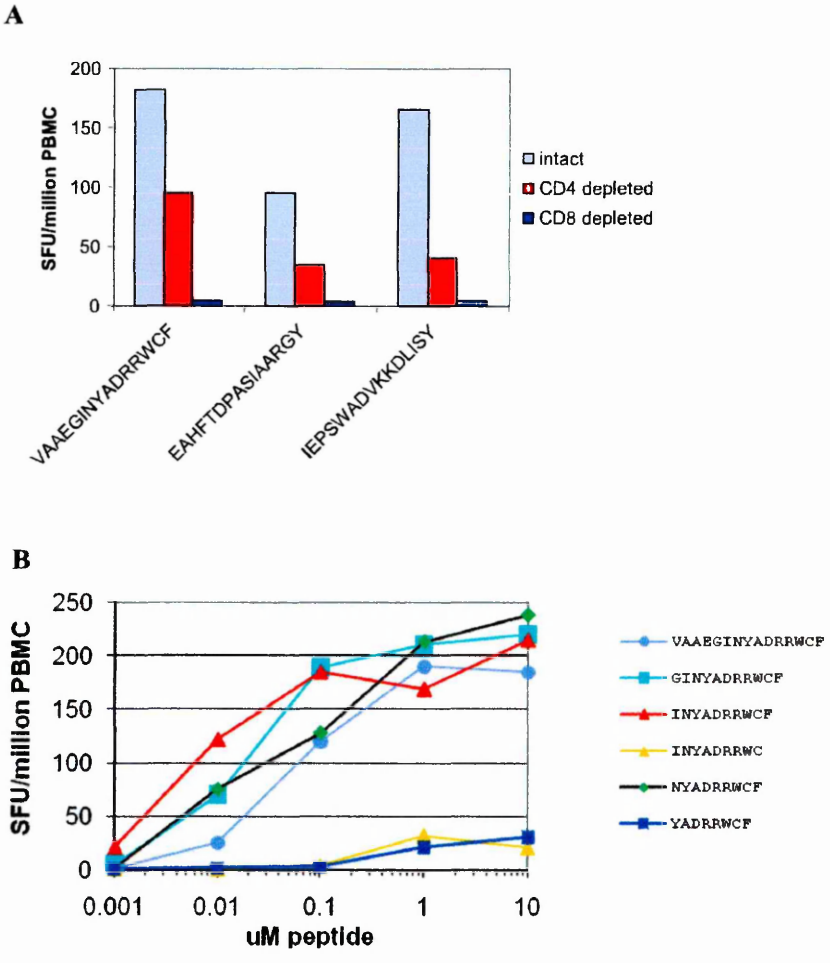


Figure 4.11 $NS3_{555-564}$ is a CD8+ T cell epitope. *In IFN-γ ELISPOT assays, PBMC (study day 3) from subject BC313 responded to the DENV2 NS3 peptide VAAEGINYADRRWCF ($NS3_{550-564}$) in a CD8 dependent fashion (A). The truncated peptide INYADRRWCF ($NS3_{550-564}$) was the minimum peptide that evoked the strongest ELISPOT response at the lowest peptide concentration (B).*

4.4.9 Kinetic and cross-reactivity of responses to $NS3_{556-564}$

Responses of T cells to peptides, corresponding to all known natural variants of the A*24-restricted $NS3_{556-564}$ epitope, were measured in 16 prospectively recruited, HLA-A*24-positive dengue patients during early convalescence (hospital discharge). The sequence of $NS3_{556-564}$ was variant from four serotypes. T cell responses to any one of the $NS3_{556-564}$ variants were detected in 4 of 16 (25%) subjects, suggesting this is not a dominant epitope. The dengue serotype responsible for the current infection was determined in 2 patients: BC311 (infected with DENV4) and BC398 (infected with DENV2). In all patients, the peak response to each of the epitope variants was recorded on Study Day 14 (Fig. 4.12). The spectrum of responses detected in each of the four patients varied according to the peptide antigen (Fig. 4.12). Thus, some patients made monotypic responses (BC313) whilst others had responses against 2 peptide variants (BC311, BC398, BC404). In both patients where information on the infecting viral serotype was available (BC311-DENV4 and BC398-DENV2), there was an ELISPOT response to the $NS3_{556-564}$ epitope matching the serotype of virus mediating the current infection, but also a response to a second natural $NS3_{556-564}$ epitope sequence (Fig. 4.13). Patients BC311 and BC398 also responded to the second $NS3_{556-564}$ epitope sequence at low peptide concentrations that is reflective of *in vivo* conditions (Fig. 4.13).

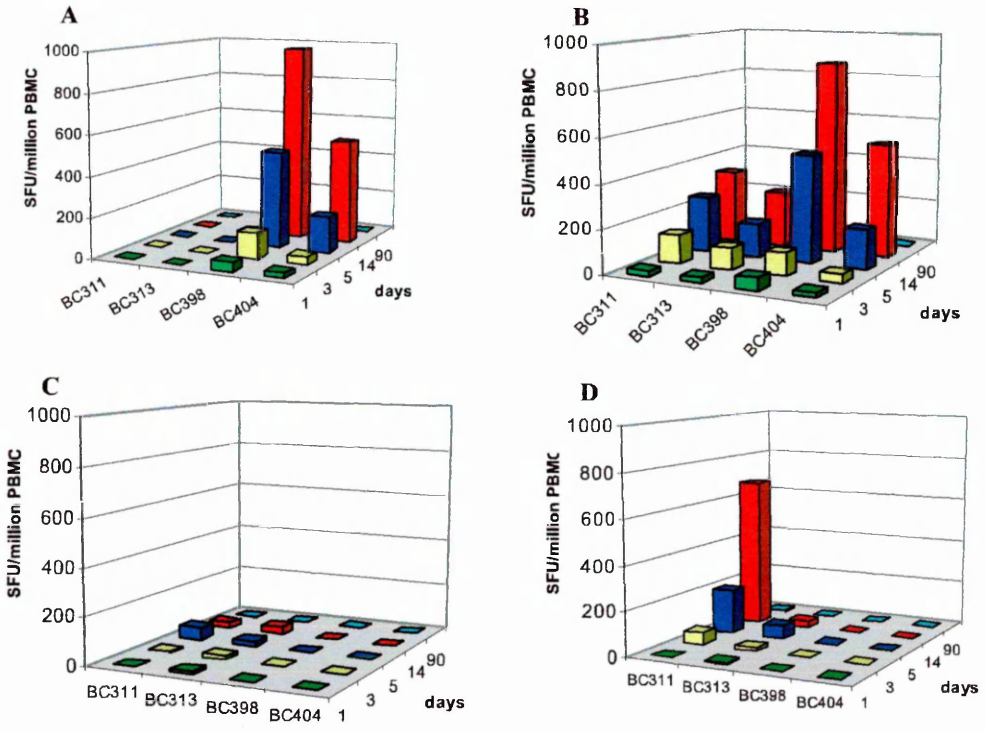


Figure 4.12 Kinetic of T cell responses to $NS3_{555-564}$. *Shown are the kinetic and magnitude of IFN-γ ELISPOT responses in 4 patients with secondary dengue infection to each of the recognized peptide variants of $NS3_{555-564}$. PBMC from each patient was stimulated with (A) $DENV1NS3_{555-564}$ (FQYSDRRWCF), (B) $DENV2NS3_{555-564}$ (INYADRRWCF) (C) $DENV3NS3_{555-564}$ (IKYTDRKWCF) and (D) $DENV4NS3_{555-564}$ (ISYKDREWCF). Subject BC311 was infected with DENV4 and subject BC398 with DENV2.*

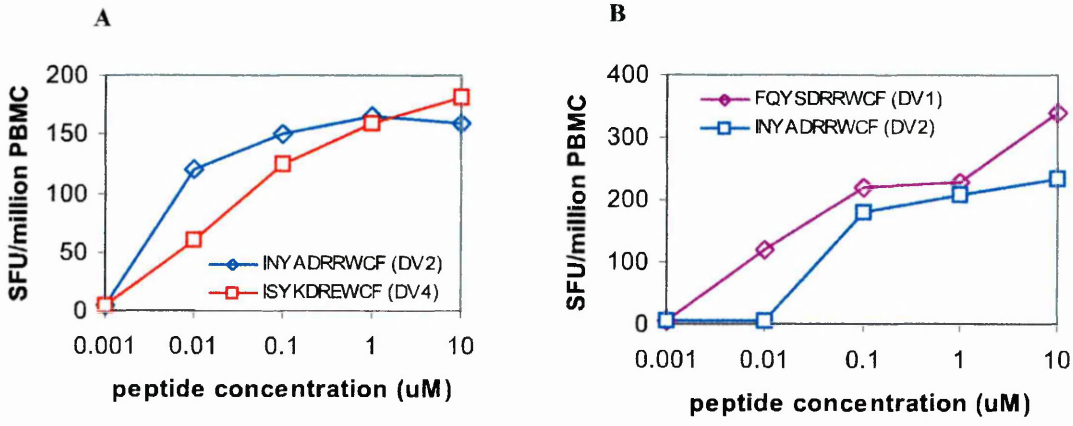


Figure 4.13 T cell responses to $NS3_{555-564}$ variants during acute secondary dengue infection. *Shown are (A) peptide titration curves in IFN-γ ELISPOT assays for PBMC collected acutely (study day 5) from BC311 (infected with DENV4) and stimulated with DENV4$NS3_{555-564}$ (ISYKDREWCF) or DENV2$NS3_{555-564}$ (INYADRRWCF). Show in (B) are peptide titration curves in IFN-γ ELISPOT assays for PBMC collected acutely (study day 5) from BC398 (infected with DENV2) and stimulated with DENV2$NS3_{555-564}$ (INYADRRWCF) and DENV1$NS3_{555-564}$ (FQYSDRRWCF).*

4.5 Discussion

T lymphocytes (CTL) play an important role in the elimination of dengue virus-infected cells (Kurane, 1994). Identification of antigenic peptides recognized by dengue virus-specific T lymphocytes may suggest novel ways to suppress viral replication and prevent persistent infection. Multiple peptides from the conserved regions of the dengue virus may be essential in the development of a universally immunogenic vaccine (Pervikov 2000). Over the last few years, studies of dengue patients have successfully identified several peptides that are specific for $CD8^+$ and $CD4^+$ T lymphocytes. The breadth and specificity of the responses of T cell to dengue viral antigens in populations of areas where dengue is endemic remain relatively poorly characterized. This serological and virological study of Vietnamese adults with secondary dengue virus infection measured the breadth and frequency of the responses of T cells to 260 overlapping peptides from a DENV2 virus. A strength of the study is the large numbers of subjects and peptides included. One of the key findings is that neither the breadth nor magnitude of the recorded peptide-specific T cell responses was significantly associated with clinical disease grade or the serotype of dengue virus mediating the current infection. Another key finding was the identification of 34 different peptide sequences that potentially contained many novel T cell epitopes. These assays facilitated the identification of a novel HLA-B* 07-restricted epitope in Env and an HLA-A*24-restricted epitope in NS3.

Most studies of dengue virus-specific T cells was conducted using T cell clones generated from live attenuated dengue virus vaccines, or less frequently, from dengue patients(Dharakul, Kurane et al. 1994) (Gagnon, 2002) (Kurane, 1991) (Kurane, 1995) (Mathew, Kurane et al. 1996) (Mathew, 1998). This study is the first attempt to define the relative antigenicity of peptides from multiple dengue viral antigens in a large number of patients from a hyperendemic country. Peptides from the NS3 region were recognized by

cells from more than half of all Vietnamese adult patients. In addition, they contained the largest number of antigenic peptides to which T cell preferentially appear to respond. These results are consistent with previous studies that have emphasized the importance of NS3 as the target of T cells (Mathew, Kurane et al. 1996). Fourteen of the 30 NS3 antigenic peptides (47%) were clustered within a 124 amino acid long stretch of $NS3_{200\text{-}324}$ that represents 20% of the whole protein. $NS3_{200\text{-}324}$ is highly conserved (78%) across all four serotypes of dengue virus, containing motifs and charged residues that are essential for helicase activity and viral replication (Matusan, 2001) (Matusan, 2001). Given the conserved nature of NS3 (68%), and particularly $NS3_{200\text{-}324}$, it seems likely that many of the NS3-derived antigenic peptides identified in this study were targets of reactivated, dengue virus cross-reactive T cells that were primed by a previous dengue virus infection. In addition, the breadth and magnitude of the responses to DENV2 peptides in patients infected with either DENV1 or DENV4 were equivalent to patients infected with DENV2. This observation supports the contention that serotype cross-reactive, rather than serotype-specific, T cells dominate the acute response during secondary infection. In patients infected with DENV2, there was no evidence that the T cell response to DENV2 peptides increased in breadth with time since secondary infection. All patients in this study experienced a secondary infection, suggesting that a previously mounted immune response against the virus is being boosted, a phenomenon referred to as "original antigenic sin" in the antibody response. This suggests the early, cross-reactive T cells' response remains dominant in late convalescence when the memory T cell pool has been established. This is supported by a previous study done with Thai schoolchildren in which dominance of cross-reactive T cells was observed among DENV-specific memory T cell 12 months after secondary DENV infection (Mathew, 1998). It is thought that peptides of non-structural proteins recognized by both serotype-specific and serotype cross-reactive

$CD8^+$ CTL will have important implications for the design of effective subunit vaccines (Livingston, 1995).

The purpose of peptide titration (figure 4.13) is to show whether serotype cross-reactive exists in parallel with serotype-specific T cells at the acute response during secondary infection. The response of DENV infected patients to the second NS3 epitope sequence was virtually identified at all peptide concentrations, including low-peptide concentrations that is likely reflective of in vivo conditions. Although the figure shows lesser response to infecting strains at lower concentration, the difference is not significant. The ELISPOT responses to infecting and second epitope peptides are equivalent in breadth and strength.

In this study, several patients also responded to the proteins capsid, preM/M, Env, and NS4a. The capsid protein contains a C-terminal hydrophobic domain and is present in infected cells. This hydrophobic domain may serve to localize an assembly of nucleocapsid at the membrane site and function as a signal sequence for prM (Bulich and Aaskov 1992). The E protein is thought to contain peptides that predominantly induce virus-neutralizing antibody responses. This protein is responsible for virus attachment to susceptible cells and consequently virus growth. This antigen also mediates virus-specific membrane fusion, which presumably allows the newly infecting virus to escape the endocytic vesicle and initiate its intracellular replication cycle (Gubler, 1999). NS4A and NS4B may be involved in membrane localization of NS3 and NS5 replication complexes via protein-protein interaction, since the NS3-NS5 complex is weakly associated with the membrane in spite of its hydrophilic characteristics (Chambers T.J., 1990). Unexpectedly, identified antigenic peptides were not associated with any recognized conserved structural motif (protein C, E, prM/M).

The mechanisms underlying the increased vascular permeability that occurs in some individuals following secondary infection with a dengue virus remains poorly understood. In this study, no significant differences were observed in either the breadth or magnitude of the total T cell response between patients with different clinical disease grades. We did, however, observe that the sum of peptide-specific responses measured in ELISPOT assays correlated, albeit weakly, with the extent of hemoconcentration recorded in each patient. These data fit with the general observation of relatively greater immune activation in patients with severe dengue disease. For example, relatively higher frequencies of activated, crossreactive $CD8^+$ T cells (Mongkolsapaya, 2003) (Zivna, 2002) and a range of direct and indirect markers of $CD4^+$ and $CD8^+$ T cell activation have been associated with increasing disease severity during secondary infection (Gagnon, 2002) (Green, 1999) (Green, 1999) (Green, 1999) (Juffrie, 2001) (Kurane, 1991). An important distinction of this study is that we performed a comprehensive analysis of the functional T cell response in adult patients to 260 peptides spanning five viral antigens as opposed to an analysis of responses in children to a single $CD8^+$ T cell epitope in other studies (Mongkolsapaya, 2003) (Zivna, 2002). Other confounding influences, such as ethnicity, HLA background, and viral factors, may also explain differences between this study and those that have been reported previously.

Two novel $CD8^+$ T cell epitopes were identified in this study. The peptide $Env_{411-425}$ was among peptides evoked the highest responses of T cells in ELISPOT assays from dengue patients. Responses to the HLA-B*07-restricted epitopes $Env_{411-425}$ were detected in all prospectively identified HLA-B*07-positive patients, suggesting this is a dominant epitope. The rationale for studying the $NS3_{550-564}$ peptide was threefold. First, previous studies had suggested this peptide might contain an HLA-A*24 epitope (Loke, 2001). Second, A*24 is one of the more common alleles present in the Vietnamese population (33%) and hence an

A*24 epitope might be widely recognized. Third, A*24 has been associated with susceptibility to DHF in Vietnamese children (Loke, 2001), suggesting immune responses restricted through this allele might be poorly protective and/or pathogenic. Responses to the A*24-restricted $NS3_{556-564}$ epitope were detected in a minority of HLA-A*24- positive patients, suggesting that it is not a dominant epitope. Consistent with other studies on unrelated epitopes (Mongkolsapaya, 2003) (Zivna, 2002), responses to the $CD8^+$ T cell epitopes defined in this study were generally strongest after the patient had been discharged from hospital (Study day 14), suggesting that the antigen may continue to persist, possibly in the form of immune complexes, well after the resolution of viremia. Other explanation for this that there might be higher levels of apoptosis in PBMC of patients with DHF, indicating that T cell expansion during acute DV infection may be balanced by apoptosis so the peak response to the $CD8^+$ T cell epitopes was not recorded at discharged day (study day 3). The establishment of a library of defined T cell epitopes from dengue viruses will help to define the role of T cells in immunity and disease pathogenesis. They should also prove useful in measuring the immunogenicity of live dengue virus vaccine candidates (Edelman, 2003) (Gwinn, Sun et al. 2003) (Sabchareon, 2004).

Chapter 5

Timing of CD8+ T cell responses in acute dengue infection

Chapter 5: Timing of CD8+ T cell responses in acute dengue infection

5.1 Rationale

Results from the genetic susceptibility study (see chapter 3) suggest that HLA class I and II restricted cellular immune responses influence susceptibility to DHF. However, this genetic study does not provide any functional evidence for the mechanism by which HLA-restricted responses might take effect. In order to understand the role of T cells in natural dengue infection, be it recovery or pathogenesis, we would need to study cellular immune responses resulting from natural dengue infection.

Data on DENV-epitope specific responses during the febrile phase is sparse. We previously showed that DENV-epitope specific responses are difficult to detect in the acute febrile phase by ELISPOT assay and that the magnitude or breadth of early convalescent responses was not associated with the clinical phenotype (chapter 4). Similarly, Mongkolsapaya *et al* showed CD8$^+$ T cells specific for the dominant HLA-A*11-restricted epitope $NS3_{133-142}$ were present only at very low frequency during the febrile phase of dengue in Thai children, but were readily detected in early convalescence (Mongkolsapaya, Dejnirattisai et al. 2003). Other studies to characterise DENV-epitope specific T cell responses have used PBMC collected in late convalescence and so it is difficult to know whether these findings can be extrapolated to the febrile phase (Zivna, Green et al. 2002; Bashyam, Green et al. 2006). A thorough characterization of DENV-epitope specific immune response in people experiencing natural dengue infections, especially those who live in dengue-endemic regions, may contribute towards the understanding of DHF pathogenesis and the role of cellular immune responses in dengue infections.

A systematic description of the kinetics of $CD8^+$ T cell activation, and particularly DENV-specific $CD8^+$ T cells, in the context of dynamic changes in viraemia and haemoconcentration would provide further insights into the role of these cells in immunity and pathogenesis. To this end, the aim of the current study was to measure the timing of specific and non-specific $CD8^+$ T cell responses and virological and clinically important haematological events during the febrile phase of dengue.

5.2 Patient recruitment

Two prospective studies are described. In the first study, consecutive children (<15 yrs) with suspected dengue and less than 72hrs of illness were enrolled at a private clinic in HCMC, Viet Nam between October 2007 and March 2008. All patients were examined by the same physician (TVN). Blood samples were collected daily during the febrile period for measurement of viraemia and routine haematological investigations. Convalescent blood samples were obtained 2-3 weeks after first presentation.

The 2nd study was a prospective study of children with dengue at Paediatric Hospital #1 and #2 between August 2006 and March 2007. Inpatients above 6 months of age with suspected dengue and fever for less than 7 days were enrolled in the study. All patients were assessed daily by a study physician and had daily serial haematological measurements and an ultrasound within 24hrs of defervescence.

For both studies, if patients received laboratory confirmation of dengue then World Health Organization clinical classification criteria (Organisation 1997) were applied to each case. Written informed consent was obtained from a parent or guardian of each study participant. The study protocols were approved by the Scientific and Ethical committees at each participating hospital.

5.3 Summary of study method

In study #1, T cell phenotyping staining was routinely performed on fresh whole blood. Staining for intracellular markers of cellular proliferation (nuclear antigen Ki-67) and surface markers of activation ($CD38^+$ and $HLA\text{-}DR^+$), were performed at enrolment and every 2^{nd} day on fresh whole blood samples. To examine DENV-specific responses, whole blood samples from each study participant was stained at enrolment and then every second day with a pool of HLA-class I tetramers carrying the HLA-A*1101-restricted $NS3_{133\text{-}142}$ peptide from DENV-1, DENV-2 and DENV-3/4. The limit of detection was set at 3 times the standard deviation of the mean percentage of tetramer positive events in 23 patients with no virological or serological evidence of acute dengue.

In study #2, T cell tetramer staining was performed to paired (study enrolment and early convalescent) peripheral blood samples by using same pooled tetramers as study #1. Patients with blood samples were ever positive with the pooled NS3133-142- specific tetramers were confirmed as being HLA-A*11-positive by SSP-PCR.

5.4 Definition

In study #1, the extent of haemoconcentration in dengue cases was determined by comparing the maximum haematocrit recorded during the acute phase with the baseline haematocrit. Baseline haematocrit was defined as the lowest of either the value recorded before day 2 of illness if platelet count was >= 200,000 (available for 6% of patients), or the value recorded at follow-up 2-3 weeks after illness onset (available for 82% of cases), or a mean of age- and sex-matched healthy population value (13% of cases).

In study #2, the extent of haemoconcentration was determined by comparing the maximum haematocrit recorded during hospitalisation with a mean age- and sex-matched healthy population baseline haematocrit. These population values were determined from Hct values measured in healthy children attending the Hospital for Tropical Diseases for follow-up study visits. The reference Hct values for males were; age 1-4 yrs, 34.4 (n=5); age 5-9 yrs, 36.0 (n=116); age 10-13 yrs, 37.7 (n=190); age 14-15 yrs, 40.2 (n=54). The reference Hct values for females were; age 1-4 yrs, 33.0 (n=4); age 5-9 yrs, 36.2 (n=111); age 10-13 yrs, 37.8 (n=188); age 14-15 yrs, 37.0 (n=34).

The platelet nadir in study #1 and #2 studies was defined as the lowest platelet count recorded in patients with a minimum of 4 platelet counts. The day of defervescence was defined as the day the fever dropped below <37.5°C (axillary) and remained so for 48hrs. The day of illness was as self-reported by the patient's parent or guardian.

5.5 Statistical analysis

The nonparametric Mann-Whitney test was used to evaluate differences in frequencies of HLA-A*1101-restricted $NS3_{133-142}$-specific $CD8^+$T cells during the course of disease. *P*-values lower than 0.05 were considered statistically significant. The program Prism (version 5.0) was employed for analyses (GraphPad Software, San Diego, CA, USA).

5.6 Results

5.6.1 Characteristics of study population

Children with dengue are not usually hospitallised until the 3-4th day of illness so all clinical finding from dengue infected children have not been recorded in the very early phase of diseases. To focus on the early events of dengue patients, we performed a community based study. This allows us to follow a cohort of patients from first presentation through all the various stages of disease. This study was carried out at private clinic, district 8, Ho Chi Minh City, Vietnam.

The characteristics of the 126 patients in whom $CD8^+$ T cells were phenotyped are described in Table 5.1. These patients were a subset of 138 consecutively enrolled patients at an outpatient primary care clinic of whom there were 12 patients who were not investigated due to inadequate samples (clotted or insufficient sample). Amongst the 126 enrolled and investigated patients there were 103 patients with laboratory confirmed DENV infection and 23 patients with other febrile illnesses (OFI) that were not dengue (Table 5.1). There were 92 patients who returned for follow-up 15-30 days after enrolment.

After applying WHO criteria (Organisation 1997) to each dengue case, there were 17 DHF patients and 86 DF patients. During the period time of study, the mean nadir of the platelet count of the DHF patients was considerable lower than of the DF patients, 47,100 (range, 11,600 to 88,300) vs. 116,000 (range, 35,200 to 270,000) respectively. And the average maximum percentage change in hematocrit attained of the DHF patients was significant higher than of the DF patients, 27% (range, 22 to 53%) vs. 8% (range, -13 to 54%) respectively.

Table 5.1 Characteristics of the patient population in whom serial blood samples were investigated for CD8+ T cell phenotype and frequency of $NS3_{133-142}$- specific T cells.

Variable	DF (n = 86) Median (range) or No. (%)	DHF (n = 17) Median (range) or No. (%)	Other febrile illness (n=23) Median (range) or No. (%)	*P* value [a]
Male sex, no. (%)	40 (46.5)	12 (70.6)	14 (60.9)	
Age (years)	11 (4-15)	13 (8-15)	11 (5-14)	
Day of illness[b]	2 (1-4)	3 (1-4)		
Fever day[b,c]	-3 (-5 to -1)	-3 (-6 to -1)		
Infecting serotype, no. (%)				
DENV-1	39 (51.3)	10 (58.8)		
DENV-2	10 (13.2)	3 (17.6)		
DENV-3	26 (34.2)	4 (5.3)		
DENV-4	1 (1.3)			
Secondary infection, no. (%)	60 (69)	14 (76)		
Log10 of peak viremia, mean(range), cDNA copies/mL	7.37 (4.45-9.90)	7.81(5.88-9.63)		
Platelet nadir, cells/μL	116,000 (35,200-270,000)	47,100 (11,600-88,300)		< 0.001
Maximum hemoconcentration, %	8(-13 to 54)	27 (22 to 53)		< 0.001

NOTE. Data are median (range) values, unless otherwise indicated.

[a] Mann-Whitney *U*-test.

[b] at time of study enrolment

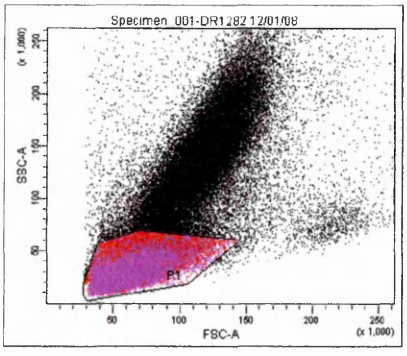
[c] the day of defervescence (axillary temperature) was regarded as fever day 0.

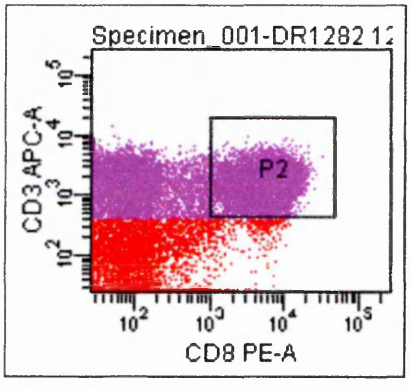
5.6.2 The timing of overall activated CD8+ T cells during dengue infection

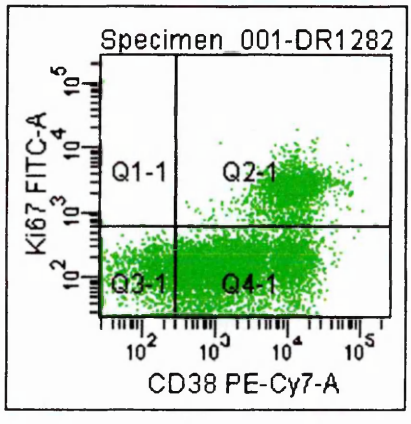
Cellular immune responses in children with dengue in the early events of dengue diseases have not been described elsewhere. To study the timing of overall CD8+ T cell activation in the relation to the course of disease and correlate response to viraemia and clinical outcomes. We therefore studied the phenotype of activated, proliferated CD8+ T cells in both acute and

early convalescence dengue infection of paediatric patients with intracellular and surface marker such as Ki-67, CD38, HLA-DR. The study was primarily carried out directly on fresh whole blood, which provides a more sensitive staining than the use of purified peripheral blood mononuclear cells.

Our studies show that the frequency of double marker activated CD8+ T cells was higher in dengue infected patients as compared to control during the course of acute dengue infection. The population of activated CD8+ T cells appeared low level at febrile phase but increased in afebrile phase. As we expect, the frequencies of activation markers were reduced in the follow-up samples. We observed a significant increase in the activated CD8+ T cells in dengue patients after defervescent day, peak at fever day 0-3 (Figure 5.1A-C).







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Specimen Name:	Specimen_001
Tube Name:	DR1282 12/01/08
Record Date:	Jan 13, 2008 7:25:25 PM
GUID:	39e587e3-a363-47c8-8f...
$OP:	Administrator

Population	#Events	%Parent
All Events	105,350	####
P1	52,419	49.8
P2	11,660	22.2
Q1	1,273	10.9
Q2	1,998	17.1
Q3	7,200	61.7
Q4	1,189	10.2
Q1-1	3	0.0
Q2-1	3,236	27.8
Q3-1	1,587	13.6
Q4-1	6,834	58.6
Q1-2	12	0.1
Q2-2	3,257	27.9
Q3-2	1,683	14.4
Q4-2	6,708	57.5

Figure 5.0 *Flow-cytometry plot showing an example of double markers staining for CD8+ T cell phenotype (CD38/Ki67).*

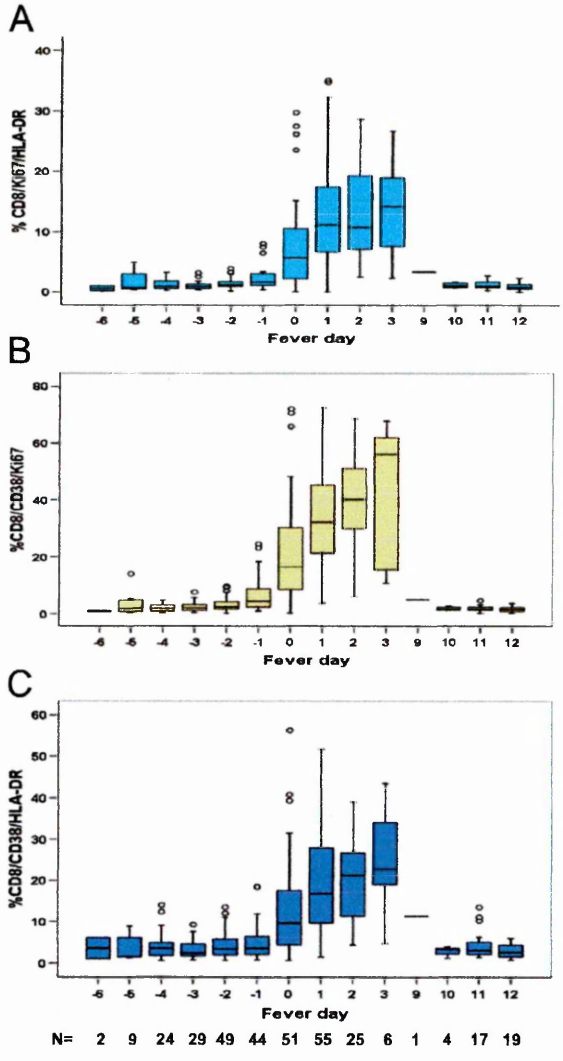

Figure 5.1 Frequencies of activated CD8+ T cells during the course of acute dengue infection and follow-up. *Shown in each box and whisker plot is the percentage of CD8$^+$ T cells that were double positive for Ki-67 /HLA-DR (A), CD38/Ki-67 (B) or CD38/ HLA-DR (C) from 103 dengue patients by fever day. Fever day 0 was regarded as the defervescent day. Days prior to fever day 0 are designed fever day -1 (1 day before defevescence), fever day -2, etc. Days after defervescence day are fever day 1, fever day 2, etc. The number of patients that were evaluated on each day for T cell phenotype is shown below panel C.*

5.6.3 Circulation of surface-activated CD8+ T cells and their relationship to viraemia dynamics

There were 93 patients with a measurable viraemia at enrolment, with DENV-1 the most prevalent serotype detected (49/93 (52%)) (Table 5.1). Staining for intracellular markers of cellular proliferation (nuclear antigen Ki-67) and surface markers of activation ($CD38^+$ and $HLA\text{-}DR^+$), were performed at enrolment and every 2^{nd} day on fresh whole blood samples. Results are shown for DENV-1 and DENV-3 only as the sample size for other serotypes was small. Double-positive $CD8^+$ T cells ($CD38^+HLA\text{-}DR^+$, $CD38^+Ki\text{-}67^+$ or $HLA\text{-}DR^+Ki\text{-}67^+$ were increased in blood only when the DENV-1 (Figure 5.2A, C, E) or DENV-3 (Figure 5.2B, D, F) viraemia had already begun to decline. Indeed, phenotypically activated $CD8^+$ T cells were most prominent after defervescence, a time when most patients were aviraemic. Collectively, these data suggests the commencement of clearance of viraemia, and a sizable fraction of the resolved viraemia, occurs in the absence of measurable peripheral blood T cell activation as defined by the activation markers used here.

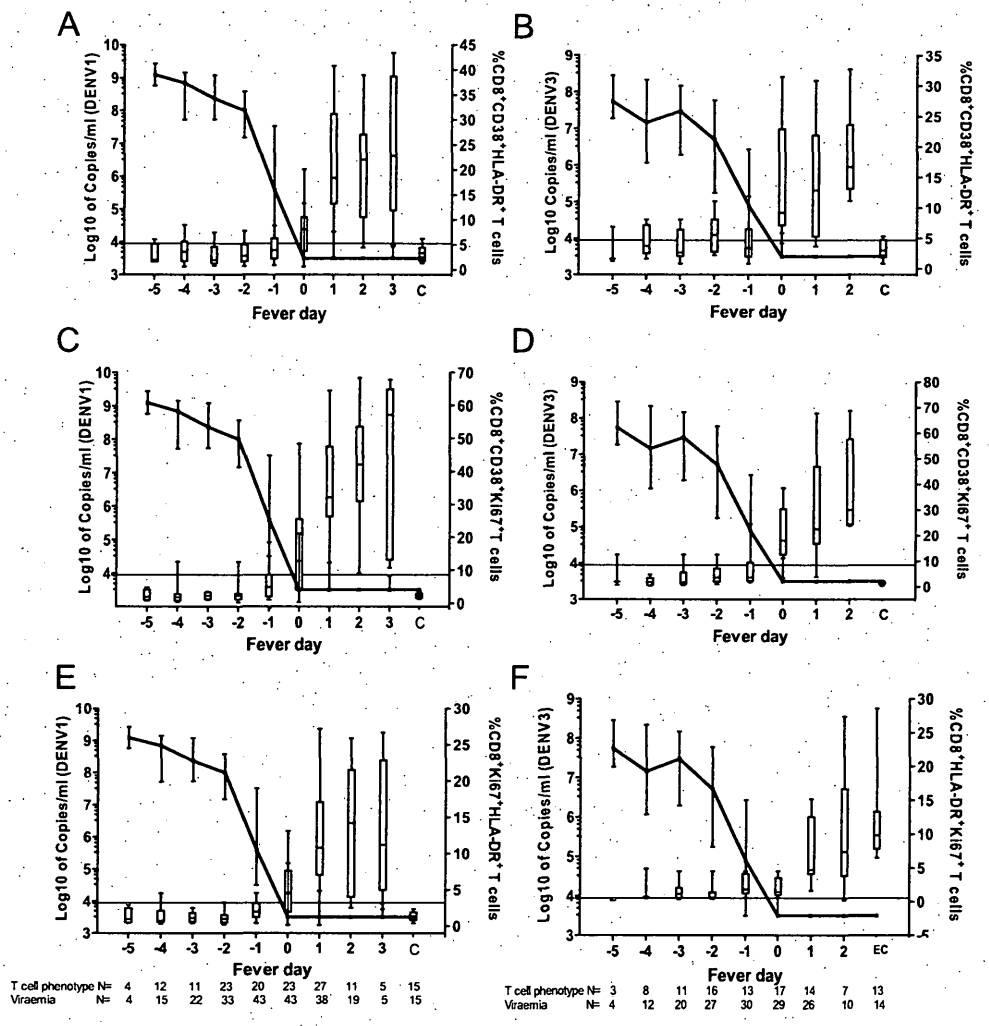

Figure 5.2 Relationship between kinetics of viraemia and the appearance of surface activated CD8+ T cells in peripheral blood. *Shown in each panel are the mean (±95% CI) of DENV-1 (panel A, C and E) or DENV-3 (panel B, D and F) viraemia levels in serial plasma samples from children with DF (DENV-1, n=39 and DENV-3, n=26) or DHF (DENV-1, n=10 and DENV-3, n=4) by fever day. In each panel, the box and whisker plots represent the percentage of CD8$^+$ T cells that were double positive for CD38/ HLA-DR (panel A, B), CD38/Ki-67 (panel C, D) or HLA-DR /Ki-67 (panel E, F). The number of patients that were evaluated on each day are shown below panels E and F.*

5.6.4 $CD8^+$ T cell activation and their relationship to thrombocytopaenia and vascular leakage

Thrombocytopaenia and vascular leakage are two of the most prominent features of DHF. In DHF patients (n=17), haemoconcentration began during the febrile phase and on average peaked 1 day (fever day -1) before defervescence (Fig. 5.3A, B and C). Similarly, thrombocytopaenia was evident during the febrile phase and before increased $CD8^+$ T cell activation in DHF patients (Fig. 5.4A, B and C). In contrast, an increase in the percentage of surface activated $CD8^+$ T cells ($CD38^+HLA\text{-}DR^+$, $CD38^+Ki\text{-}67^+$ or $HLA\text{-}DR^+Ki\text{-}67^+$) did not occur in DF or DHF patients until the day of defervescence (Fig. 5.3 and Fig. 5.4), suggesting haemoconcentration and thrombocytopaenia in DHF patients commenced in the absence of any increase in the percentage of surface activated $CD8^+$ T cells in peripheral blood.

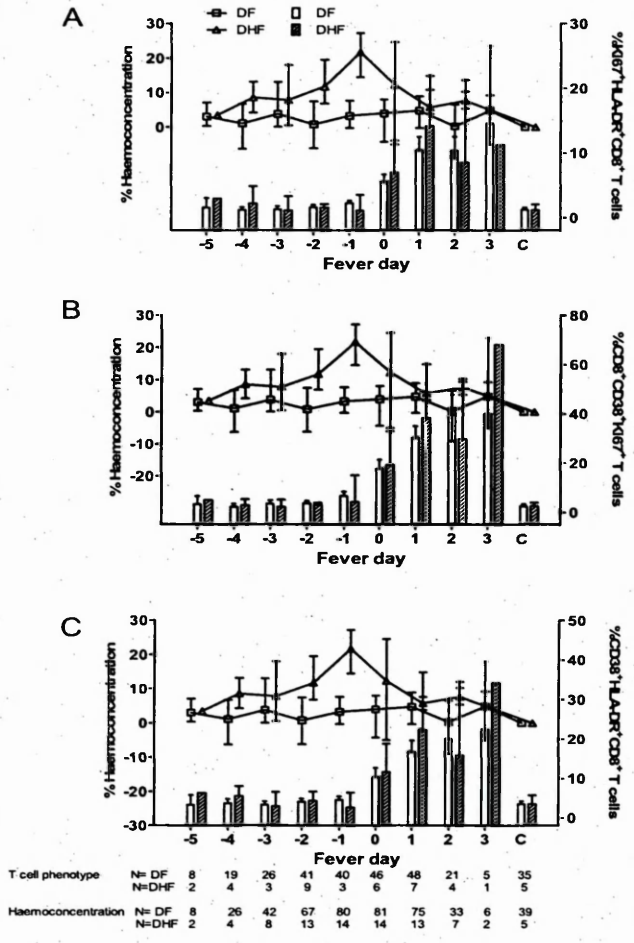

Figure 5.3 Relationship between haemoconcentration and the appearance of surface activated CD8+ T cells in peripheral blood. *Shown in each panel are the mean (±95% CI) percentage levels of haemoconcentration in serial plasma samples from children with DF (n=86) or DHF (n=17) by fever day. Haemoconcentration was measured against autologous convalescent samples for 82% of patients. In each panel, the bars represent the mean (±95% CI) percentage of CD8$^+$ T cells that were double positive for Ki-67 /HLA-DR (panel A), CD38/Ki-67 (panel B) or CD38/ HLA-DR (panel C). The number of patients that were evaluated on each day for either T cell phenotype or level of haemoconcentration is shown below panel C.*

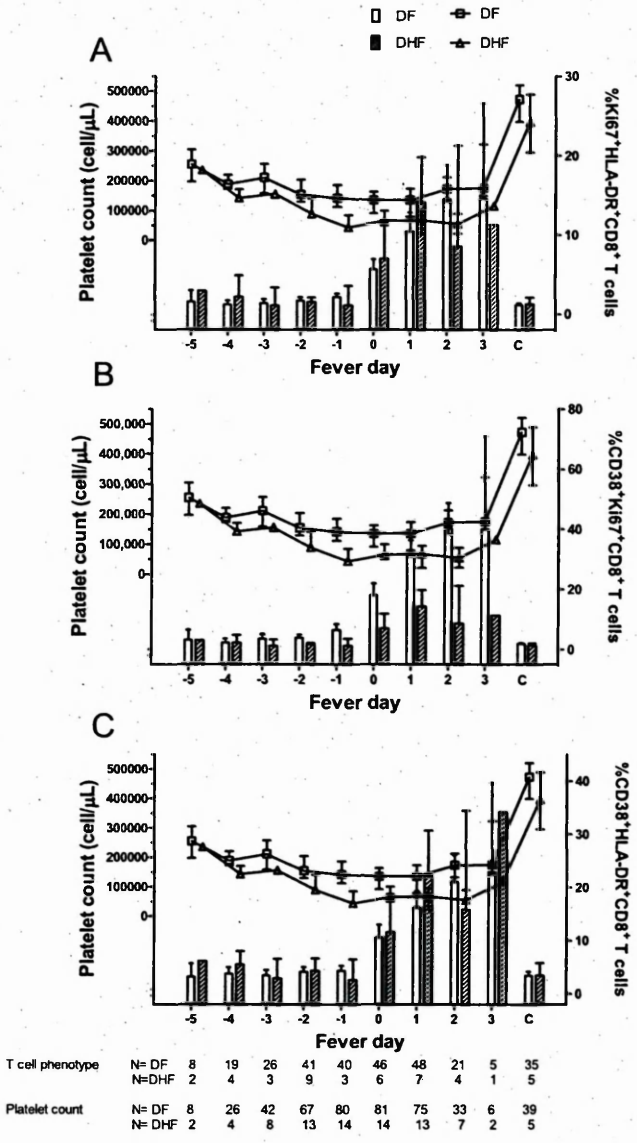

Figure 5.4 Relationship between thrombocytopaenia and the appearance of surface activated CD8+ T cells in peripheral blood. *Shown in each panel are the mean (±95% CI) platelet count in serial plasma samples from children with DF (n=86) or DHF (n=17) by fever day. In each panel, the bars represent the mean (±95% CI) percentage of CD8$^+$ T cells that were double positive for Ki-67 /HLA-DR (panel A), CD38/Ki-67 (panel B) or CD38/ HLA-DR (panel C). The number of patients that were evaluated on each day for either T cell phenotype or platelet count is shown below panel C.*

5.6.5 DENV-specific CD8+ T cell responses and their relationship to vascular leakage

Previous studies have suggested the the envelope and NS3 proteins are frequent targets of immune responses in flavivirus infections, including dengue (Kurane and Ennis 1994), (Mathew, Kurane et al. 1996). Our previous finding is consistent with these findings that have emphasized the importance of NS3 as important T-cell target (chapter 3). The result found a T-cell response to peptide FSPGTSGSPIIDKKG, which covers NS3 residues 130-144, in four of 39 patients. This result are in agreement with Mongkolsapaya et al shown peptide NS3133-142 as a dominant, DENV-specific HLA-A*1101-restricted T cell epitope (Mongkolsapaya, Dejnirattisai et al. 2003). HLA-A*11 is one of the most frequent alleles in Vietnamese population (Loke et al, 2001). To examine DENV-specific responses, whole blood samples (median, 4, range, 2 to 6 samples investigated per patient) from each study participant was stained at enrolment and then every second day with a pool of HLA-class I tetramers carrying the HLA-A*11-restricted NS3133-142 peptide from DENV-1, DENV-2 and DENV-3/4. Of the 103 dengue patients, 33 patients (32%) had at least one sample with a measurable NS3133-142- specific tetramer staining population of CD8+ T cells, including 9 dengue patients with DHF and 24 dengue patients with DF (Figure 5.5). The median fever day on which tetramer positive cells were first detected in the 9 DHF patients was +1 (range -3 to 12) and this was 2 days later than the mean peak in haemoconcentration described for these patients in Fig. 5.3.

Amongst the 33 patients who were ever tetramer positive, NS3133-142-specific T cells were detected during the febrile phase in less than half (13/33, (39%)) the cases. In the early convalescent afebrile phase, measurable NS3133-142-specific CD8+ T cells were present in a significantly greater proportion of patients who were ever positive (23/33 (70%)) ($P = 0.02$). The percentage of circulating NS3133-142- specific CD8+ T cells during the early

convalescence phase was significant higher than during the febrile phase (paired t test, P = 0.037) (Fig. 5.6). These results suggest NS3133-142-specific CD8+ T cells reach measurable frequencies around the time of defervescence and after the commencement of haemoconcentration in DHF patients.

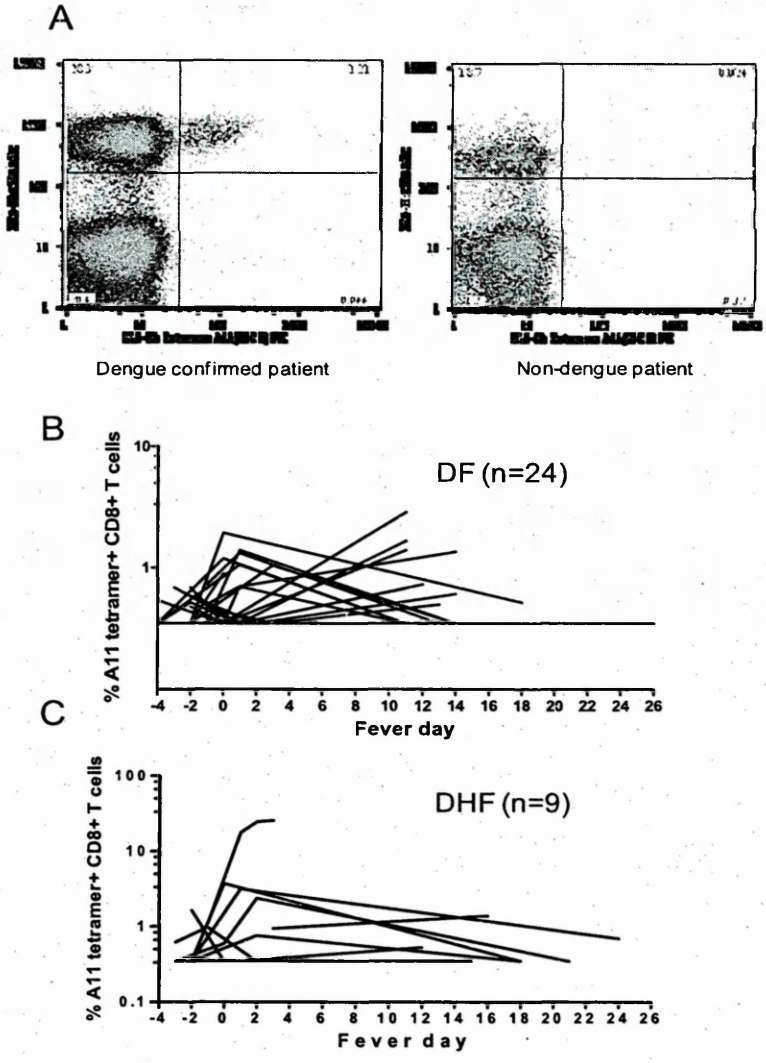

Figure 5.5 DENV-specific CD8+ T cell responses. *Whole blood samples were double-stained with a pool of PE-conjugated HLA-A*11 tetramers carrying the HLA-A*11-restricted $NS3_{133-142}$ peptide from DENV1-4 (Tetramer A11 (GTS) PE) and APC conjugated monoclonal antibody to CD8 (CD8 APC). Panel A shows the specificity of the A11 tetramer. Flow-cytometry plot showing detection of $NS3_{133-142}$-specific CD8$^+$ T cells in whole blood of dengue confirmed patient (left) and non-dengue patient (right). Panel B and C show the frequency of $NS3_{133-142}$-specific CD8$^+$ T cells in whole blood from dengue patients with DF (n=24) and DHF (n=9) by fever day.*

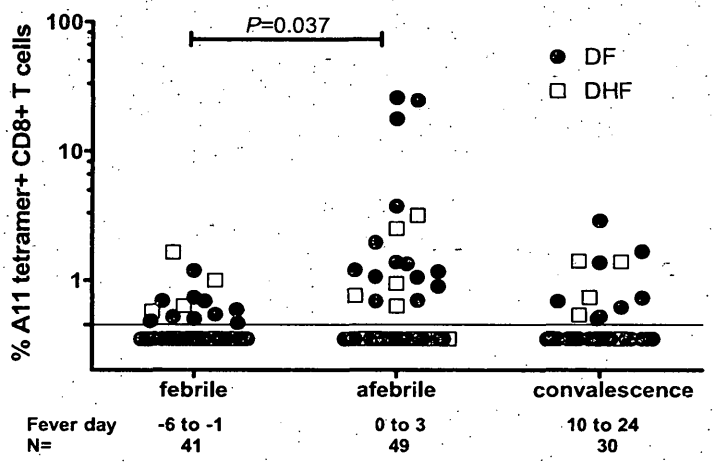

Figure 5.6 . Frequencies of NS3$_{133-142}$-specfic CD8+ T cells during the febrile and afebrile phase. *Serial whole blood samples from 103 children with dengue were stained with a pool of tetramers (DENV-1, DENV-2 and DENV-3/4) specific for NS3$_{133-142}$-specfic CD8$^+$ T cells. The scatterplot shows the frequency of tetramer staining NS3$_{133-142}$-specfic CD8$^+$ T cells in individual patient samples according to whether they were detected during the febrile phase (fever day, FD, -6 to -1), afebrile phase (FD, 0 to 3) or convalescence (FD, 10-24). The frequency of circulating DENV-specific CD8$^+$ T cells at early convalescence phase were significant higher than at febrile phase (P = 0.037, by Mann-Whitney test). The lower limit of detection (solid horizontal line) was defined as the mean plus 3 standard deviations of the frequency of tetramer-positive staining events in blood samples from patients with no evidence of dengue (62 measurements in 23 non-dengue patients).*

5.6.6 Timing of DENV-specific CD8$^+$ T cell responses in relationship to haematological markers

To explore more fully the relationship between the appearance of measurable NS3133-142-specific T cells in blood and haemoconcentration and thrombocytopaenia in a larger patient

population, pooled tetramers were used to stain paired (study enrolment and early convalescent) peripheral blood samples from 422 children hospitalised with suspected dengue, of whom 390 were laboratory confirmed. The characteristics of this patient population are described in Table 5.2. At enrolment, the median illness day was 4 days (range 1-6) and the median number of days prior to defervescence 2 days (range -6 to 0). Amongst the 390 patients with laboratory confirmed dengue, there were 62 (16%) patients (41 with DHF and 21 with DF) with blood samples that were ever positive with the pooled NS3133-142- specific tetramers. All 62 patients were confirmed as being HLA-A*11-positive by SSP-PCR.

Table 5.2 Characteristic of the patient population in whom tetramer staining was performed for NS3$_{133-142}$- specific T cells

	Tetramer positive		Tetramer negative
Variable	DF (n = 21) median (range)/ No. (%)	DHF (n = 41) median (range)/ No. (%)	Dengue (n=328) median (range)/ No. (%)
Male sex, no. (%)	13 (61.9)	25(60.9)	176
Age (years)	10(3-14)	10(2-15)	10(1-15)
Day of illness at enrolment	3(1-4)	3(1-6)	4(2-7)
Fever day at enrolment [a]	-2(-3 to 0)	-2(-5 to 0)	-2(-6 to 0)
Infecting serotype,.			
DENV-1	4 (22.2%)	12 (31.6%)	186(63.9%)
DENV-2	10 (55.6%)	19 (50%)	83(28.5%)
DENV-3	4 (22.2%)	7 (18.4%)	21(7.2%)
DENV-4	0 (0%)	0 (0%)	1(0.3%)
Secondary infection, no. (%)	18(85.7)	34(82.9)	247
Log 10 of viremia, mean (range), cDNA copies/mL[b]	6.82(3.98-9.77)	7.49(3.78-12.21)	6.80(3.03-11.83)
Platelet nadir, cells/μL	61,000 (33,000-124,000)	36,000 (10,000-96,000)	50,000 (1,000-270,000)
Maximum hemoconcentration, %	13.9 (2.2-19.4)	28(10.5-54.1)[c]	22(-22.6-52.78)

NOTE. Data are median (range) values, unless otherwise indicated.

[a] The day of defervescence was regarded as fever day 0.

[b] DENV viraemia measured at enrollment.

[c] one patient without haemoconcentration but with pleural effusion by ultrasound finding

Daily haematocrit measurements indicated haemoconcentration (>20%) was first present a median of 1 day (fever day range: -5 to 2 days) prior to defervescence in the 41 patients with DHF that were ever positive with the pooled NS3133-142-specific tetramers (Fig. 5.7A). Thrombocytopaenia of <100,000 cells/mm3 was also first detected a median of 1 day (fever day range: -5 to 2 days) prior to defervescence in the same patients (Fig. 5.7B). In contrast, NS3133-142-specific CD8+ T cells first became detectable in DHF patients on fever day +1

(fever day range: -3 to 4 days) (Fig. 5.8A), a median of 2 days (fever day range, 0 to 5 days) after thrombocytopaenia (<100,000 cells/mm3) was first detected (comparison of median fever day, P<0.0001) (Fig. 5.8B) and a median of 2 days (fever day range, 0 to 5 days) after haemoconcentration (≥20%) was first detected in each patient (comparison of median fever day, P<0.0001) (Fig. 5.8C). The timing of these events is summarised in Table 5.3. Collectively, these data indicate NS3133-142- specific CD8+ T cells generally become measurable in the peripheral blood only after the commencement of vascular leakage or thrombocytopaenia in patients with DHF.

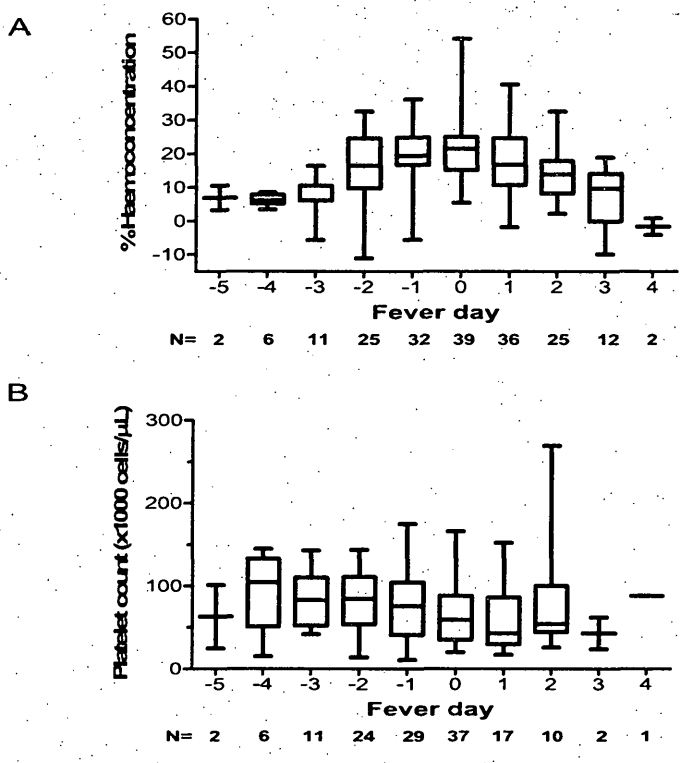

Figure 5.7 Timing of haemoconcentration in children with DHF. *The box and whisker plots represent percentage haemoconcentration (panel A) and platelet counts (panel B) in children with DHF (n=41) by fever day. Levels of haemoconcentration were determined by comparison of daily Hct values in patients against healthy population age and sex stratified mean Hct values (see Table 1). Haemoconcentration of >20% was first detected a median of 1 day before defervesence in these children. The number of children with haematocrit or platelet measurements on each fever day is shown beneath the X-axis.*

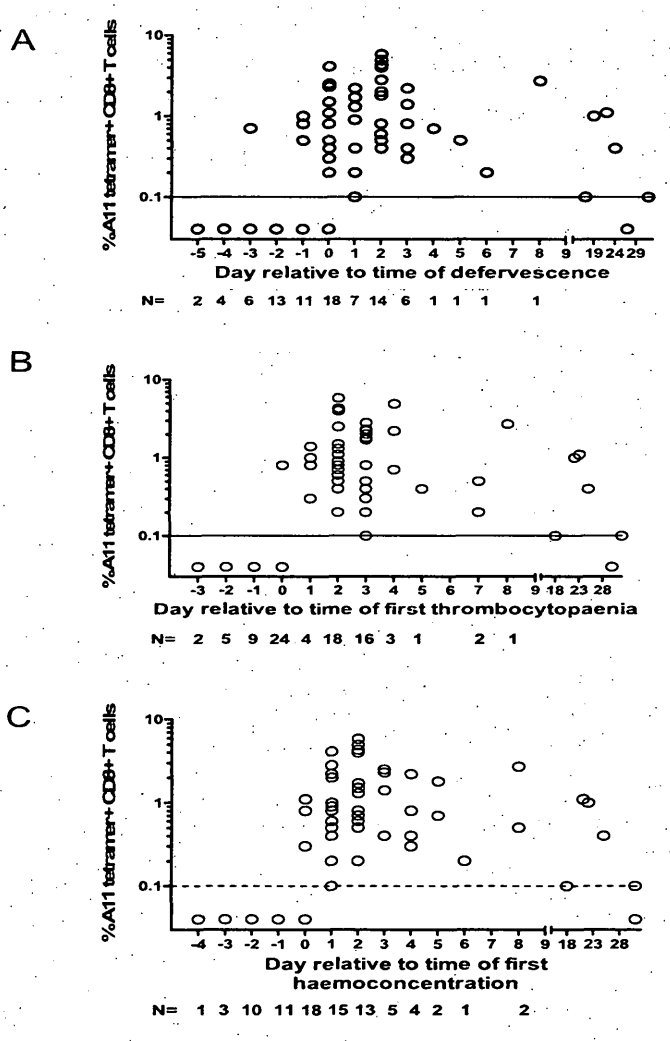

Figure 5.8 Temporal relationship between detection and frequency of HLA-A*1101-restricted NS3$_{133-142}$-specfic CD8+ T cell responses and haemoconcentration, thrombocytopaenia and defervescence in children with dengue. *Shown in each scatterplot is the percentage of NS3$_{133-142}$-specfic CD8$^+$ T cell detected by tetramer staining in individual patient blood samples against a reference timepoint of day of defervescence (day 0 in panel A), day when platelet count of <100,000 cells/μl was first detected in each patient (day 0 in panel B) and day when haemoconcentration of >20% was first detected in each patient (day 0 in panel C). The values below the X-axis are the number of patients evaluated on each day.*

Table 5.3 Timing of haematological events in relation to appearance of $NS3_{133-142}$-specific T cells

Event in DHF patients (n=41)	Median fever day (range)	*P* value
First haemoconconcentration (>20%)	-1(-5 to 2)	
First thrombocytopaenia (<100,000 cells/mm^3)	-1 (-5 to 2)	
First detection of $NS3_{133-142}$-specific T cells	+1 (-3 to 4)	$P<0.0001$[a,b]

[a] Compared to first haemoconcentration (>20%)

[b] Compared to first thrombocytopaenia (<100,000 cells/mm^3)

5.7 Discussion

Secondary heterotypic DENV infections are a risk factor for DHF in children and adults. Anaemestic, cross-reactive T cells responses are a part of the host response to secondary infection and have been suggested to contribute to immunopathogenesis. The major findings in the current study are that surface activated $CD8^+$ T cells and $NS3_{133-142}$ specific $CD8^+$ T cells are generally not measurable in peripheral blood prior to the commencement of haemoconcentration, thrombocytopaenia or resolution of viraemia and therefore it seems unlikely they are critical triggers of these events.

Significant immune activation undoubtedly occurs in patients with DHF and secondary infections. Relatively higher levels of various pro-inflammatory cytokines and their receptors and soluble CD4/8 are found in acute sera of children with DHF compared with children with DF (Kurane, Innis et al. 1991; Green, Vaughn et al. 1999; Libraty, Endy et al. 2002). DHF patients also have a greater percentage of $CD8^+$ T cells bearing the early activation marker $CD69^+$ than DF patients during the febrile phase (Green, Pichyangkul et al. 1999). In the context of virus- specific responses, Mongkolsapaya *et al* detected very low frequencies of $NS3_{133-142}$-specific T cells in the late febrile phase with a subsequent peak in frequency a few weeks after illness onset (Mongkolsapaya, Dejnirattisai et al. 2003). The very low frequency of $NS3_{133-142}$-specific T cells during the febrile phase was suggested to be a consequence of huge proliferation balanced by massive apoptosis (Mongkolsapaya, Dejnirattisai et al. 2003). However these findings were not analysed in relation to the commencement of capillary leakage. Our own previous studies have indicated DENV-epitope specific T cells are difficult to detect during the febrile phase by ELISPOT assay, but that responses are readily measured in early convalescence (Simmons, Dong et al. 2005). Other studies of virus-specific responses demonstrated ELISPOT frequencies of T cells specific for the HLA-B*7 restricted

epitope $NS3_{222-230}$ were higher in the late convalescent phase in patients who had DHF compared to DF; assessments during the febrile phase were not performed (Zivna, Green et al. 2002). Collectively, a limitation of previous studies of T cell responses in dengue is that the temporal relationship (or lack thereof) between measurable T cell immune activation and the commencement of vascular leakage (as opposed to when vascular leakage is at its most severe) has not been directly addressed. This is important for understanding the triggers for capillary leakage.

In two populations of children with DHF we found that haemoconcentration and thrombocytopaenia first occurred prior to the appearance in peripheral blood of $CD8^+$ T cells bearing markers of activation (CD38, HLA-DR) or proliferation (Ki-67). Frequencies of proliferating $CD8^+$ T cells ($Ki\text{-}67^+$) were high in early convalescence and this was consistent with previous studies that have used this marker (Mongkolsapaya, Dejnirattisai et al. 2003). $NS3_{133-142}$-specific $CD8^+$ T cells were also rarely detected during the febrile phase, and significantly, were generally not measurable prior to the detection of haemoconcentration (>20%) or thrombocytopaenia in children with DHF. The temporal mismatch between the first detection of haemoconcentration in particular and measurable $CD8^+$ T cells or $NS3_{133-142}$-specific $CD8^+$ T cells suggests these cells have a negligible role in triggering capillary leakage. Our finding differ from studies on acute viral infection that activated HCMV and EBV specific CD8 was detected during pathological human viral infection (Doisne et al, 2004) (Tuuminen et al, 2007) (Papagno et al, 2004) (Sandalova et al, 2010). In contrast to HCMV and EBV-specific CD8 cells, they observed that CD8 T cells specific for influenza were not activated during the acute phase of heterologous acute viral infection (Sandalova et al, 2010). It is possible that T cells responding to altered peptide ligands in vivo may not express these surface markers or enter cell cycle. An alternative explanation for the very low

frequency of $NS3_{133-142}$-specific T cells during the febrile phase (this study and (Mongkolsapaya, Dejnirattisai et al. 2003), is that they are sequestered in sites of infection or have down-regulated T cell receptors (Dong, Moran et al. 2007). This would imply a massive expansion and distribution of central and/or effector memory $NS3_{133-142}$-specific $CD8^+$ T cells after just a few days of infection and negligible ongoing circulation of $CD8^+$ T cells expressing the relevant, specific T cell receptor. Here, the resurrection from memory of response has rapid mobilized right after viral load falls abruptly. Memory cells have a much lower threshold for activation compared with naive cells (Veiga-Fernandes et al, 2000), however, so there is a risk that clones activated by original antigenic sin may have lower affinity and be less effective at clearing the secondary challenge (Alexander-Miller, et al, 1996) and indeed may promote immunopathology. Original antigenic sin has been shown in T cell response that expansion of T cells with relatively lower affinity for the currently infecting virus and higher affinity for serotypes presumed to be previously encountered (Mongkolsapaya et al, 2003). A second alternative explanation is that they are sequestered and proliferating in lymphoid tissue during the febrile phase but have yet to enter the peripheral circulation. Studies (e.g. fine biopsy collections) to address these issues will assist our understanding of pathogenesis but are of course ethically and practically problematic in children with dengue.

A plausible role for T cells in the immunopathogensis of secondary dengue is that they act to amplify an already established pro-inflammatory cascade mediated by innate responses to a large viral burden. In this two stage model, a large viral antigen mass (plausibly mediated by ADE) might be necessary and sufficient to trigger capillary leakage through innate mechanisms such as complement activation, cytokine secretion by activated macrophages/dendritic cells and/or NS1 mediated pertubations of the vascular endothelium.

Exacerbation of capillary leakage that has already commenced might occur when effector T cells accumulate at sites of infection. Such a model could also explain DHF in infants with primary infections and no DENV-specific memory T cells; the innate response (complement activation, cytokine secretion) may be necessary and sufficient to trigger clinically significant capillary leakage in infants because of their intrinsically poor capacity to compensate for microvascular leakage compared to older children or adults (Bethell, Gamble et al. 2001). Consistent with this, many of the cytokines found to be elevated in children with DHF and secondary infection are also elevated in infants with primary infection (Nguyen, Lei et al. 2004; Tran Nguyen Bich Chau 2008).

There are several limitations to our study. First, our investigations of surface markers on $CD8^+$ T cells was not exhaustive but focused on two well-described markers of activation (HLA-DR and CD38) and one intracellular marker of cellular proliferation (Ki-67). A plausible explanation for the surface activated $CD8^+$T cells ($CD38^+HLA\text{-}DR^+$, $CD38^+Ki\text{-}67^+$ or $HLA\text{-}DR^+Ki\text{-}67^+$) were not measurable in peripheral blood during febrile phase is that, dengue-specific activated CD8 T cells are true memory CD8 cells without any recent encounter to their specific ligand. Memory CD8 cells specific for persistent and non-persistent viruses not only differs in term of phenotypic profile in healthy individuals, but respond differently to the pathological condition triggered by a heterologous acute viral infection (Sandalova et al, 2010). It is possible that T cells responding to altered peptide ligands in vivo may not express these surface markers or enter cell cycle and this should be a focus for future research. Secondly, we investigated $CD8^+$ T cells only when T regulatory and conventional $CD4^+$ T cells could also be important (Mangada, Ennis et al. 2004; Luhn, Simmons et al. 2007; Moran, Simmons et al. 2008). Luhn et al show that the T reg cell population expands in acute dengue infection has the conventional phenotype and

suppressive capacity. Their demonstration that T reg cells suppress the dengue-specific secretion of vasoactive cytokines of effector T cells leads us to suggest that this population plays an active role in dengue infection (Luhn et al, 2007). Third, our analysis of DENV-specific $CD8^+$ T cells was limited to one dominant epitope ($NS3_{133-142}$) restricted through the most common class I HLA allele in the Vietnamese population, HLA-A*11. Reponses to other DENV $CD8^+$ T epitopes might occur with greater rapidity than that described in this study. Fourth, all of the results are presented as percentage of CD8 expressing markers or binding tetramers rather than as absolute counts. Here, CD8 percentage takes in consideration factors which could cause a false high or false low CD8 count. The absolute count can reflect exact number of CD8 cells which contribute to disease progression. The absolute count is the more important measure of immune status and is preferred over the percentage for making treatment decision in infectious diseases (Kelly et al, 2004). Finally, our study was biased toward patients with DENV-1 as this was the most prevalent serotype in circulation at the time.

Nevertheless, this study provides the first description of the temporal mismatch between the $CD8^+$ T cell response to a dominant epitope and commencement of vascular leakage. Future studies of T cell responses in dengue need to consider the timing of responses relative to the commencement of capillary leakage, arguably the most important clinical manifestation of DHF.

Chapter 6

General Discussion

Chapter 6: General Discussion

Cellular immune responses, and particularly responses elicited by altered peptide ligands, have been nominated as being very important to the pathogenesis of severe dengue. This premise stemmed in part from the epidemiological association between secondary dengue and severe disease, and the finding of robust humoral and cellular immune responses in these patients, characteristic of a memory response. Advances to our understanding of T cell responses in the pathogenesis of dengue that are described in this thesis include the importance of NS3 and cross-reactive T cells during acute secondary infection and also the paradoxical finding that the overall breadth and magnitude of the T cell response was not significantly related to clinical parameters. Furthermore, the temporal mismatch between the appearance of surface activated and DENV-specific $CD8^+$T cell suggests other mechanisms are responsible for triggering of vascular leakage.

Mapping and characterization of T cell epitopes in DENV is important for understanding the host response and in the development of dengue vaccines. There are variants of most CD8 T cell epitopes in different DENV serotypes, but the amount of intraserotype diversity in the epitope sequences is small, with most serotypes being represented by one or two sequences. The interaction between TCR and altered peptide ligands can reportedly result in dramatically different phenotypes of the T cells, ranging from inducing selective stimulatory functions to completely turning off their functional capacity (Pfeiffer et al, 1995) (Sloan-Lancaster et al, 1993).

Our study on T-cell responses in patients with secondary dengue identified forty-seven different peptides that evoked significant IFN-γ ELISPOT responses in 39 patients, and of these, 34 peptides contained potentially novel T cell epitopes. NS3, and particularly $NS_{200-324}$,

were important T cell targets. The breadth and magnitude of ELISPOT responses to DENV-2 peptides was independent of the infecting dengue serotype, suggesting cross-reactive T cells dominate the acute response during secondary infection. Acute ELISPOT responses were weakly correlated with the extent of haemoconcentration in individual patients, but not with the nadir of thrombocytopaenia or the overall clinical disease grade. $NS3_{556-564}$ and $Env_{414-422}$ were identified as novel HLA-A*24 and B*07-restricted CD8+ T cell epitopes, respectively. Acute T cell responses to natural variants of $Env_{414-422}$ and $NS3_{556-564}$ were largely cross-reactive and peaked during disease convalescence. Duangchida et al have reported that a number of T-cell epitopes in dengue infection where NS3 responses were the most frequently seen and there was a very strong association between the magnitude of the response and disease severity.

By phenotyping $CD8^+$ T cells ($CD38^+$/HLA-DR^+, $CD38^+$/Ki-67^+ or HLA-DR^+/Ki-67^+) in serial blood samples from children with dengue, we found no evidence of increased $CD8^+$ T cell activation prior to the commencement of resolution of viraemia or haemoconcentration. Investigations with MHC class I tetramers to detect $NS3_{133-142}$- specific $CD8^+$ T cells in two independent cohorts of children suggested the commencement of haemoconcentration and thrombocytopaenia in DHF patients generally begins before the appearance of measurable frequencies of $NS3_{133-142}$- specific $CD8^+$ T cells. The temporal mismatch between the appearance of surface activated and DENV-specific $CD8^+$ T cells suggests other mechanisms are responsible for triggering capillary leakage in children with DHF. The absence or very low frequency of $NS3_{133-142}$-specific T cells during the febrile period can be explained that they are sequestered in sites of infection or have down-regulated T cell receptors (Dong, Moran et al. 2007). This would imply a massive expansion and distribution of central and/or effector memory $NS3_{133-142}$-specific $CD8^+$ T cells after a few days of infection and negligible

ongoing circulation of $CD8^+$ T cells expressing the relevant, specific T cell receptor. A second explanation is that they are sequestered and proliferating in lymphoid tissue during the febrile phase but have yet to enter the peripheral circulation. Studies to address these issues, in either primate animal models or more directly in patients (e.g. fine biopsy collections), will be needed to resolve these questions.

It would be very informative to compare acute CD8+ T cell responses between adults and children with asymptomatic, mild and severe dengue disease, throughout the infection, disease and recovery phases. Results from such study may provide more direct evidence on the exact role of CD8+ T cells in DHF diseases pathogenesis, be it significant or negligible. Further studies, both children and adults, will help determine whether there are differences in the kinetic of responses between children and adults.

Our investigations of surface markers on CD8+ T cells was focused on two well-described markers of activation (HLA-DR and CD38) and one intracellular marker of cellular proliferation (Ki-67). This is one of our limitations because T cells responding to altered peptides ligands in vivo may not express these surface markers or enter cell cycle and this should be a focus for future studies. There are other well-described surface markers such as CD69, CD27, CD28, CD45RO, CD44 and Perforin and these have been described on $CD8^+$ T cells from dengue patients previously (Mongkolsapaya, 2003) (Green, 1999) (Azeredo, 2006). The antigenic peptide-specific T cells showed an activated effector phenotype, with almost all of them expressing CD45RO, CD27, CD38 and HLA-DR during acute phase but CD28 activated marker reducted in the follow-up samples (Mongkolsapaya, 2003). The majority of NK cells from dengue patients display early markers for activation (CD69, HLA-DR, and CD38) and cell adhesion molecules (CD44, CD11a) during the acute phase of disease (Azeredo, 2006). The percent of cells expressing CD69 was also increased on CD8 T

cells and NK cells in children who developed DHF more than in those with DF (Green, 1999).

In this study, we investigated CD8+ T cells only when T regulatory and conventional CD4+ T cells could also be important. Luhn et al previously reported that T regulatory cells of patients with acute dengue had a conventional phenotype with suppressive capacity. T regulatory cells in acute dengue could also suppress secretion of vasoactive cytokines from effector T cells, leading to the suggestion that this population plays an active role regulating the host T cell response in secondary dengue (Luhn, 2007).

This study has found that HLA Class I and II polymorphisms are associated with susceptibility to DSS. In particular, secondary DENV-2 infected children with HLA-B*44 were likely to have DSS, whereas secondary DENV-2 infected children with HLA-Cw*12 and HLA-DQB1*03 were at increased risk of DHF when compared with the controls. HLA-A and HLA-DRB1 polymorphisms were not associated to DSS. So far, HLA class I and II associations with disease severity were detected in Vietnamese patients undergoing secondary infection would imply that HLA class I and II-restricted cross-reactive T cell drive immune responses are contributing to the pathology of DENV.

The significance of HLA class I and II polymorphisms on susceptibility to severe dengue has been suggested by some previous studies (Table 3.1), even though the allelic associations in each study differ. Those studies were carried out at different geographic locations. Therefore, differences in association could be due to naturally occurring differences in HLA frequencies between ethnic groups, or possibly because of differences in circulating viral serotype and epidemiological setting, e.g. primary vs secondary dengue, children vs adults. The resolution of the HLA typing technique could also be important. Studies to confirm the associations

found in this thesis (HLA-B*44, HLA-Cw*12, and HLA-DQB1*03) should be carried out , in a second Vietnamese population of severe dengue cases. This approach of discovery and validation has become the norm for large genetic association studies (but was not the norm when this study was conducted in 2006). However, studies in other populations may provide additional information on the overall influence of HLA on DHF disease susceptibility.

During secondary infections, both the humoral and cellular flavivirus-specific responses can confer protection or be involved in the pathogenic event. In genetically "more resistant" individuals such as those who have an HLA type associated with protection, the interaction with the specific dengue protein epitopes could induce an immune response that would help to clear the infection before disease symptoms appear. The presence of the susceptibility-associated allotypes could, in turn, induce an overly vigorous immune response responsible for the most severe clinical pictures. Many factors, including differences in viral load, intrinsic viral virulence, host immune response, immune enhancement, are also likely to contribute to the final disease expression. Research to try to understand the pathophysiologic mechanisms underlying the various clinical manifestations seen in dengue infections is needed if we are to develop new treatments or vaccines against this disease.

The potential involvement of multiple serotypes of dengue virus, DENV-specific antibodies and T cells in the pathogenesis of severe dengue and the lack of animal models to assess these interactions are important challenges in furthering our knowledge of dengue. Management of dengue currently relies on symptomatic treatment with fluid replacement when necessary that aims to compensate for plasma leakage and swift fluid resuscitation if circulatory failure occurs. The only method currently available to prevent dengue is the control of Aedes aegypti, the mosquito vector. This approach has proved unable to reduce the burden of dengue in most endemic settings because it requires substantial and ongoing

community commitment. Vaccination is the most promising strategy to control the spread of dengue. Dengue vaccines must provide solid and long lasting protection against all four serotypes of dengue viruses. Vaccine-induced DENV-specific antibodies/T cells may decrease or protect against disease severity through their effects to decrease viremia burden. Conversely, it is also feasible that dengue vaccines will increase the risk severe disease when vaccinees are exposed to natural infection.

In recent years there has been considerable progress in the production of a vaccine against dengue with advanced candidates undergoing Phase II testing and positioned to initiate large scale efficacy trials within 1-3 years (Bhamarapravati, 2000) (van Der Most, 2000) (Kochel, 2000) (Guirahkoo, 2002) (Monath, 2007). The aetiology of DHF appears to be multifactor. Cross-reactive immune responses between dengue serotypes and ADE have been proposed to constitute mechanism leading to severe dengue disease. An ideal vaccine should induce both humoral and cellular immunity. There is still a big gap in our knowledge of immune responses in dengue infections, especially cellular immune responses. With the available information, it is not possible to tell whether a T cell-based vaccine, or in fact any of the other dengue candidate vaccine strategies, will actually be beneficial in the long run. For basic research on the immune responses to DENV to advance the development of new treatments and preventive measures (such as vaccines), priorities for future immunologic research on dengue are- (i) more detailed clinical studies of natural DENV infection are needed to confirm the disease associations of different measures of the innate and adaptive immune responses *in vivo*. These studies should optimally involve longitudinal evaluations to evaluate the kinetics of each component of the immune response and should encompass the broadest representation of viral and human genotypes. (ii) further refinement of animal models of DENV infection (including both humanized and non-humanized mice) is needed to capture

the key features of DHF in humans. In addition to the vascular leakage phenomenon, a valid animal model must recapitulate the temporal dissociation between viral burden and vascular permeability as well as the contribution of DENV-specific antibody and T-cell responses. (iii) the anticipated initiation of large-scale (phase IIb/III) vaccine efficacy trials in DENV-endemic populations will present a singular opportunity to conduct the critical studies to identify immunologic correlates of protective and/or pathologic immune responses. Efforts to maximize our understanding of DHF pathogenesis should be given priority as it should assist in rational vaccine development. With this knowledge in hand, we will be on much better ground to develop a truly effective vaccine against dengue.

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Appendix 1: Primer pairs and the HLA class I and II alleles detected

Lane	Sense Primer	Antisense Primer	Amplicon Size (base pairs)	Alleles amplified
1	625 / 292	400	443	A*0101/2/3/4/6/7/8/9, *0301/2/3/4/5/6/7/8/9/10, *1101/2/3/4/5/6/7/8/9/12/13/14, *2417, *3101/2/5/6/7/8, *3201/2/3/4/5/6/7, *3601/2/3/4, *6810/14, *7401/2/3/5/6/7/8, *8001
2	286	431	636	A*0101/2/3/4/6/7/9
3	753	249	123	A*0201/4/6/7/9/10/11/14/15/16/17/18/20/21/25/26/28/29/30/31/32/33/34/35/36/39/40/41/42/43/45/46/48/51/52/53/55/56/57/58/59, *2301/4/5/6/7/8/9, *2413/18/24, *6901
4	3421	420	549	A*0201/2/3/9/11/12/13/16/19/20/22/24/25/26/27/29/30/31/32/34/35/36/37/38/40/42/43/45/46/47/48/49/50/52/53/55/56/58/59, *2503
5	296	302	496	A*0201/2/3/4/5/6/7/8/9/10/11/12/13/14/15/16/17/18/19/20/21/22/25/26/27/29/30/31/32/33/34/35/36/37/38/39/40/41/42/43/44/45/46/47/49/51/52/53/54/56/57/58/59
6	1173	299	205	A*0301/2/4/7/8/9, *2418, *3204, *6819
7	291	299	635	A*0301/2/3/4/7/8/9, *2418, *3204, *3602, *6819
8	290	249	539	A*0234/35/56, *0301/3/4/5/6/7/9, *2424, *2901/2/3/4/6/7, *3001/8/11, *3402/3/4, *6901
9	286	168	637	A*3601/2/3
10	284	302	471	A*0246/48, *2301/2/4/5/6/7/8, *2402/3/4/5/6/7/9/10/11/13/14/15/17/18/19/20/22/23/25/26/27/28/30/32/33/34/35/37
11	284	244	588	A*2301/2/3/5/6/7/8, *2402/4/5/6/7/9/11/13/14/15/17/19/20/21/25/26/27/28/30/32/34/35/37
12	292	249	564	A*0246/48, *2301/3/4/5/6/7/8, *2413/18, *3007, *3108
13	292	170	564	A*2402/3/4/5/7/9/10/11/14/15/17/19/20/21/23/25/26/27/28/29/30/31/32/33/34/35/37
14	208	146	511	A*2402/3/5/7/8/9/10/11/14/15/17/20/21/23/25/26/27/29/30/31/33/34/35/36/37
15	292	1171	519	A*2402
16	1191 / 1168	1189 / 1167	1056	A*2307, *2402/4/5/6/7/8/9/11/13/14/15/17/19/20/21/24/25/26/27/28/29/30/31
17	239	145 / 167	171	A*2501/2/3/4, *2601/3/5/7/8/10/11/12/13/14/15/16/17/18, *4301, *6601/2/3/4
18	193	298	447	A*2501/2/3/4, *2601/2/3/4/5/6/8/9/10/11/12/13/14/15/16/17/18, *3401/2/3/4, *6601/2/3/4
19	294	298	407	A*2501/2/3/4
20	288	298	407	A*2601/2/4/7/8/9/10/11/12/13/14/15/16/17/18, *4301
21	174	298	449	A*4301
22	451 / 239	168	171	A*2609, *3103/4, *3401/2/3/4
23	475 / 290	298	426	A*2502, *2613, *3401/2/3/4, *6601/2/4
24	290	303 / 167	559	A*1101/2/3/4/5/6/7/8/9/10/11/12/13/14, *2502, *2613, *6601/4
25	751	752	188	A*0235, *0301/2/3/4/5/6/7/10, *1101/3/4/5/7/8/9/10/11/12/13, *2407/19/24, *2502, *2613, *2901/2/3/4/5/6/7, *3401/2/3/4, *6601/2/4, *6801/2/6/7/8/9/10/11/12/13/14/16/17/18/19/21/22, *6901
26	174	300	472	A*2901/2/3/4/5/6/7
27	295	301	568	A*3001/2/3/4/6/7/8/9/10/11

Appendix 1: Primer pairs and the HLA class I and II alleles detected (continue)

Lane	Sense Primer	Antisense Primer	Amplicon Size (base pairs)	Alleles amplified
28	754	249	578	A*3001/2/7/8/9/10/12, *3101/2/3/4/5/6/7
29	434	486	199	A*3101/3/4/5/6
30	3421	414	550	A*0103, *0204, *3201/2/3/5/6, *3603, *7401/2/3/4/5/6/7/8
31	3421	234	261	A*2503, *3201/2/3/4/5/6
32	434	429	201	A*3301/3/4/5/6
33	1	399	585	A*3301/4/5
34	493	494	155	A*7401/2/4/5/6/7/8
35	193	302	475	A*0255, *2424, *2610, *6801/2/3/4/5/6/7/8/9/11/12/15/16/17/18/19/20/21/22, *6901
36	290	171	390	A*0234/35/56, *6901
37	367	287	501	A*8001
38	193	221	626	B*0702/3/4/5/6/7/8/9/10/11/12/14/15/16/17/18/20/21/22/23/24/25/26/27/28/29/30/31, *4025, *4806, *8101
39	203	285	412	B*0702/4/7/9/11/12/14/15/17/18/19/20/21/22/23/24/25/26/29/30/31, *5603/9
40	271	517	502	B*0702/3/4/5/6/8/9/10/11/13/15/16/17/19/20/22/23/24/25/26/27/28/29/30/31, *0801/2/3/4/5/6/7/8/10/11/13/14/15, *1405, *1568, *1814, *3903/14, *4201/2, *4801/4/5/6/7, *8101
41	203	425	412	B*0705/6, *4201/2/4, *5504/8, *5605/6, *8101
42	242	220	571	B*0801/4/5/6/7/8/9/10/11/12/13/14/15/16, *4101/2/3/4, *4201/2/4
43	195	212	550	B*0801/2/3/7/8/9/11/12/14/15/16, *3538, *4406, *5108/20
44	195	220	613	B*0801/2/3/6/7/8/9/11/12/13/14/15/16
45	280	225	642	B*2704/6/10/15/18/20/21/24, *4005/15/16/23/26/28/32, *4901/2, *5001/2/4
46	208	215	392	B*4418, *4901/3, *5115, *5901
47	246	215	607	B*4415/18, *4501/3/4/5/6, *4901/2/3, *5001/2/4, *5402
48	207	219	542	B*0720, *1514, *4409, *4501/2/3/5/6, *5002, *8201/2, *8301
49	192	212	549	B*3701/3/4/5, *4101/2/3/4/5/6, *4402/5/8/9/11/14/15/16/17/18/19/20/21/22/23/24/25/27, *4501/2/3/4
50	202 / 272	393 / 285	488	B*1546/53, *1804,*3519, *4003/9/18/20/24/31/32/33/42/44, *4104, B*4402/3/4/4/6/7/9/11/12/13/16/17/19/20/21/22/23/24/25/26/27/28/29/30/32 *5306
51	435	212	500	B*0802, *4402/5/8/12/14/15/16/17/19/20/21/22/23/24/27
52	435	393	373	B*4402/3/4/7/8/12/13/16/19/20/21/22/23/24/26/27/28/29/30/32
53	435	382	500	B*1301/2/3/4/6/7/10, *1536, *1809, *2701, *4403/7/10/13/26/30/31/32, *4704, *4902, *5309, *5607
54	196	227	340	B*0802, *1310, *1809, *3802/4/8, *4402/3/4/5/8/11/12/13/14/16/17/19/21/22/23/24/26/27/29/30/31/32
55	192	220	612	B*4101/2/3/4/5

Appendix 1: Primer pairs and the HLA class I and II alleles detected (continue)

Lane	Sense Primer	Antisense Primer	Amplicon Size (base pairs)	Alleles amplified
56	272	276	573	B*1546/53, *3519, *4002/3/4/5/6/8/9/11/13/14/15/16/18/19/20/24/26/27/28/29/32/35/37/39/40/44, *4101/2/3/4/5/6, *4402/3/4/5/7/9/10/11/12/13/14/15/16/17/18/19/20/21/22/23/24/25/26/27/28/29/30/32, *4501/2/3/4/5/6, *4701/2/3/4, *4901/2, *5001/2/4
57	192	247	472	B*1533, *4001/2/3/4/5/6/9/10/11/12/14/15/16/18/19/20/21/22/23/24/27/29/30/31/32/33/34/36/37/38/39/40/42/43/44, *4101/2/3/4/5/6, *4431, *4801/3/4/5/7
58	272	218	634	B*4001/2/3/4/6/7/8/9/10/11/13/14/16/18/19/20/22/23/24/25/27/29/30/31/32/33/34/35/36/37/38/40/42/43/44, *4416/21/31, *4701/2/3/4
59	280	229	614	B*2724, *4001/7/22/23/25/30/31/33/34/36/38/42/43, *4431
60	243	215	493	B*1301/2/3/6/7/8/9, *1542/73
61	197	127	396	B*1401/2/3/4
62	205	232	183	B*1402/3/5/6, *3526, *3805, *3904
63	207	217	514	B*1548/69, *3535, *3901/2/3/4/5/6/7/8/9/10/11/12/13/14/15/16/17/18/19/22/23/24/25/26, *6701
64	581	217	535	B*1548/69, *3535, *3801/2/4/5/6/7/8/9, *3905/7/8/11/13/20, *4429, *5119
65	206	217	555	B*3910/16/17/20, *6701
66	435 / 208	217	515	B*3801/2/3/4/5/6/7/8/9, *4429, *5119
67	209	217	619	B*1569, *3801/2/3/4/5/8/9, *3901/2/3/4/5/6/7/8/9/10/11/12/13/14/15/16/17/18/20/22/23/24/25/26/27, *6701
68	194	213	381	B*5705, *5801/2/4/5/6
69	194	224	358	B*5701/2/3/4/6/7/8/9
70	187	214	465	B*1801/2/3/4/5/6/7/8/9/10/11/12/13/14/15/17/18
71	209	236	429	B*0809, *2714, *3906, *4204, *5501/2/3/5/9/10/11/12, *5601/5/7/8, *5901, *7301
72	242	215	390	B*1309, *1542/73, *3917, *4501/3/4/5/6, *5001/2/4, *5401/2, *5501/2/3/5/7/9/10/11/12, *5601/2/4/8/10, *8201/2, Cw*0312, *1507
73	203	238	558	B*0720/24, *4506, *5508, *5601/2/3/4/5/6/7/9, *8201/2, *8301
74	395	236	428	B*5401/2, *5507
75	280	281 / 282	150	B*2701/2/3/4/5/6/7/8/9/10/11/13/14/15/17/19/20/21/24/25
76	575	228	444	B*0814, *1517, *2701/2/4/5/8/10/12/13/14/15/16/17/18/19/23/25, *3702, *4701/2/3/4
77	188	212	613	B*3538, *3701/3/4/5, *4406, *5108/20
78	192	392	429	B*1558, *3701/3/4/5, *3803, *3902/8/13/23, *4036, *4502, *4807
79	192	228	421	B*2718, *3702, *4701/2/3/4
80	367	236	296	B*7301
81	203	220	601	B*4201/2/4
82	209	229	574	B*2724, *4012, *4801/3/4/6/7, *8101
83	194	225	523	B*1516/17/67, *5806

Appendix 1: Primer pairs and the HLA class I and II alleles detected (continue)

Lane	Sense Primer	Antisense Primer	Amplicon Size (base pairs)	Alleles amplified
84	240	241	467	B*4601/2, *6702
85	243	226	484	B*1304/10, *1501/2/4/5/6/7/8/11/12/13/14/15/16/19/20/21/24/25/26/27/28/31/32/33/34/35/36/38/39/40/43/44/45/50/55/56/57/60/63/65/66/67/70/71, *4601/2, *5701/6/8
86	243	250	125	B*1309, *1501/2/4/5/6/7/8/11/12/14/15/19/20/21/25/26/27/28/30/31/32/33/34/35/38/39/40/42/44/45/48/50/55/56/58/60/63/65/66/70/71/73, *4021
87	192	214	428	B*1304/10, *1501/3/4/5/6/7/12/14/19/20/24/25/26/27/32/33/34/35/36/38/39/40/43/46/47/49/50/53/54/57/60/61/62/65/68/69/70/71, *1812, *3528, *4003/20/38, *4417, *4802
88	243	223	427	B*1301/6/7, *1502/13/20/21/25/36/44, *4408, *5705
89	271	223	494	B*0712/14/18, *1562, *3525, *4802, *8301. Cw*0311
90	209	214	493	B*0709/11/17, *1503/18/23/29/47/49/51/52/54/61/62/64/68/69/72, *3525/26, *3907, *4802, *5603/9
91	271	238	698	B*0720/24, *1503/9/10/18/23/29/37/51/54/61/62/64/68/72, *3525, *4802, *8201/2, *8301. Cw*0311, *0703
92	189	226	422	B*0710, *1510/18/21/23/37/44/51/52/72, *3526, *3907
93	243	219 / 244	644	B*1512/14/19, *4408, *5707
94	193	377	560	A*2501/2/3, *2601/2/3/4/5/6/9/10/11/12/13/14/15/16/17/18, *3401, *6601/2/3/4, *6801/2/3/4/5/6/7/11/12/15/16/17/18/19/20/21/22. B*1508/11/15, *3514/43/44, *5603
95	193	223	376	B*0712/14/18, *1502/13/21/44, *3501/2/3/4/6/7/8/9/11/12/14/15/18/19/20/21/24/25/26/27/29/33/34/35/36/38/39/40/41/42, *4406/12, *5104, *5301/2/3/4/5/6/8/9, *5609, *8301
96	188	237	129	B*1801/2/3/4/5/6/7/8/10/11/12/13/14/15/17/18, *3501/2/3/4/5/6/7/8/9/10/11/12/13/14/15/16/17/18/20/21/22/23/24/28/29/30/31/32/33/34/35/36/37/38/39/40/41/42/43/44, *3919, *5606, *7801/2/3/4/5
97	195	213 / 277	396	B*3501/2/3/4/5/6/7/8/9/11/14/15/17/18/19/21/23/24/25/27/29/30/32/33/34/35/36/37/38/40/41/42, *5301/2/3/4/5/6/8
98	195	226	423	B*0708, *0807, *1508/29, *1807, *3501/5/7/8/11/14/15/17/19/21/23/24/25/27/29/30/32/34/35/37/39/40/41/42/43, *5301/2/3/5/6/8
99	195	392	430	B*3503/6/33/36/38, *3806/7, *5113, *5304/7
100	207	216	407	B*1501/9, *4026/28, *5605/6, *7801/2/3/4/5
101	208	216	408	B*5101/2/3/4/5/6/7/8/9/11/12/13/14/16/17/18/19/20/21/22/23/26/27/28/29/30/31/32, *5201/2/3/4/5
102	202	236	494	B*4903, *5101/2/3/7/8/9/10/11/12/13/14/16/17/18/19/20/21/22/23/24/26/27/28/29/30/31/32, *5201/2/3, *5605/6, *7801/2/3
103	193	216	458	B*1509, *5101/2/3/4/5/6/7/8/9/11/12/13/14/16/17/18/19/20/21/22/23/26/27/28/29/30/31/32, *5605/6, *7801/2/3/4
104	195	399	595	B*1807, *3521/24, *5101/3/4/6/8/9/11/12/13/14/16/17/18/19/20/21/24/26/27/28/29/30/31/32, *5302/6, *7801/2
105	192	216	447	B*1501, *4026/28, *5201/2/3/4/5, *7805

Appendix 1: Primer pairs and the HLA class I and II alleles detected (continue)

Lane	Sense Primer	Antisense Primer	Amplicon Size (base pairs)	Alleles amplified
106	1719	438	393	B*0727, *0802/3, *1301/2/3/4/6/7/8/10, *1513/16/17/23/24/36/43/67, *1809, *2701/2/3/4/5/6/7/9/10/11/13/14/15/16/17/19/20/21/23/24/25, *3701/2/3/4, *3801/2/3/4/5/6/7/8/9, *4013/19, *4402/3/4/5/6/7/8/10/11/12/13/14/15/16/17/18/19/20/21/22/23/24/25/26/27/28/29/30/31/32, *4701/3/4, *4901/2/3, *5101/2/3/4/5/6/7/8/9/10/11/13/14/15/16/17/18/19/20/21/22/23/24/26/27/28/29/30/31/32, *5201/2/3/4/5, *5301/2/3/4/5/6/7/8/9, *5607, *5701/2/3/4/5/6/7/9, *5801/2/4/5/6, *5901
107	1719	237	376	B*0702/3/4/5/6/7/8/9/10/12/13/14/15/16/17/18/19/20/21/22/23/24/25/26/28/29/30/31, *0801/4/5/7/8/9/10/11/12/13/14/15/16, *1309, *1401/2/3/4/5/6, *1501/2/3/4/5/6/7/8/9/10/11/12/14/15/18/19/20/21/25/26/27/28/29/30/31/32/33/34/35/37/38/39/40/42/44/45/46/47/48/49/50/51/52/53/54/55/56/58/60/61/62/63/64/65/66/68/69/70/71/72/73, *1801/2/3/4/5/6/7/8/10/11/12/13/14/15/17/18, *2708/12/18, *3501/2/3/4/5/6/7/8/9/10/11/12/13/14/15/16/17/18/19/20/21/22/23/24/25/26/28/29/30/31/32/33/34/35/36/37/38/39/40/41/42/43/44, *3901/2/3/4/5/6/7/8/9/10/11/12/13/14/15/16/17/18/19/22/23/24/25/26/27, *4001/2/3/4/5/6/7/8/9/10/11/12/14/15/16/18/20/21/22/23/24/25/26/27/28/29/30/31/32/33/34/35/36/38/39/40/42/43/44, *4101/2/3/4/6, *4201/2/4, *4409, *4501/2/3/4/5/6, *4601/2, *4702/3, *4801/2/3/4/5/6/7, *5001/2/4, *5401/2, *5501/2/3/4/5/7/8/9/10/11, *5601/2/3/4/5/6/8/9/10, *6701/2, *7301, *7801/2/3/4/5, *8101, *8201/2, *8301
108	368	315	347	Cw*0102/3/4/6/7/8/9
109	366	145	529	Cw*0202/3/4/5, *0608, *1701/2/3
110	368	389	571	Cw*0302/3/4/5/6/8/9/10/11/12/13/14
111	366	143	338	Cw*0401/3/4/5/6/7/8/9/10, *1801/2
112	271	143	507	B*8201/2. Cw*0708, *1801/2
113	366	379	571	Cw*0501/3/4/5/6
114	240	818	490	Cw*0501/2/3/4/5/6, *0802/4/5/7
115	367	127	304	Cw*0602/3/4/5/6/7/8
116	4	184	491	Cw*0314, *0701/2/4/5/6/8/10/11/13/14/15/16, *0807
117	313	184	523	Cw*0701/6/7/9/16
118	2242	247	132	Cw*0105, *0702/3/10/13/15/17
119	367	379	543	Cw*0704/11/12
120	165	166 / 317	161	Cw*0801/2/3/4/5/6/7/8/9
121	159	389	530	Cw*0303/11/12/13
122	130 / 160	389	529	Cw*0302/4/5/6/7/8/9/10/14/15
123	369	126	545	Cw*1202/8

Appendix 1: Primer pairs and the HLA class I and II alleles detected (continue)

Lane	Sense Primer	Antisense Primer	Amplicon Size (base pairs)	Alleles amplified
124	368	157	453	Cw*1203/6
125	371	388	548	Cw*1402/3/4
126	366	223	325	Cw*0307, *1502/3/4/5/6/8/9/10/11
127	366	382	509	Cw*0203, *0307/15, *0404/6, *0604, *0707/9, *1502/3/4/5/6/9/10/11, *1701/2/3
128	368	146	520	Cw*1601
129	366	146	510	Cw*1602
130	1605	377	215	Cw*0104/9, *0205, *0602/3/7/8, *1203/4/5/6/7, *1604
131	368	127	342	Cw*1203/6, *1405, *1601/4
132	366	377	509	Cw*0202/4/5, *0602/3/7/8, *1204/5
133	366	157	443	Cw*1204/5
134	36	39	195	DRB1*0101/2/4/5/6/7/8/9
135	36	38	255	DRB1*0102/4/6
136	36	40	196	DRB1*0103
137	41	252	207	DRB1*1501/2/3/4/5/6/7/8/9/11/12/13
138	41	38	257	DRB1*1501/3/4/5/6/7/9/10/12/13
139	41	491	137	DRB1*1507/11, *1601/2/3/4/5/7/8
140	68	255	212	DRB1*0301/2/3/4/5/6/7/8/9/10/11/12/13/14/15/16/17/18/19/20/21/22/23, *1107
141	46	38	217	DRB1*0301/4/6/8/10/11/12/13/15/16/18/19/20/22/23, *1327
142	44	37	189	DRB1*0302/5/14, *1109/20, *1302/5/26/29/31/34/36/39/41, *1402/3/9/19/24/27/30, *1608. DRB3*0108
143	68	491	140	DRB1*0302/3/6, *0820, *1117/37/41, *1303/7/8/12/13/19/26/32/33/36/37/38/40/47/48/49/53, *1401/2/3/5/6/7/8/9/12/13/14/16/18/19/20/22/23/24/25/26/27/29/32/34/36/37/38/40/41/43/44/45
144	3291	3258	178	DRB1*0401/2/3/4/5/6/7/8/9/10/11/12/13/14/15/16/17/18/19/20/21/22/23/24/25/26/27/28/29/30/31/32/33/34/35/36/37/38/39/40/41/42/43
145	47	498	212	DRB1*0401/2/9/13/14/16/21/26/33/35/37/38
146	47	2510	174	DRB1*0401/2/3/4/6/7/8/13/14/18/19/20/21/22/23/25/27/31/32/33/35/36/37/38/39/41/42/43/44
147	47	500	211	DRB1*0403/6/7/11/17/20/27/39/41
148	48	256	204	DRB1*0701/3/4/5/6/7
149	1737	49	184	DRB1*0701/5/6/7
150	50	51 / 102	214	DRB1*0801/2/3/4/5/6/7/8/9/10/11/12/13/15/16/17/18/19/22/23, *1415
151	50	491	131	DRB1*0801/2/3/4/5/6/7/8/9/10/11/12/13/15/16/18/19/22/23/24, *1404/11/15/28/31
152	2518	1635	139	DRB1*0803/10/12/14/15/18/19/23, *1118/19/31, *1312/13/30
153	263	58 / 51	155	DRB1*0809, *1117, *1318, *1401/4/5/7/8/10/11/14/15/23/26/28/33/35/36/39/44/45

Appendix 1: Primer pairs and the HLA class I and II alleles detected (continue)

Lane	Sense Primer	Antisense Primer	Amplicon Size (base pairs)	Alleles amplified
154	46	49	193	DRB1*0901/2
155	53	37	204	DRB1*1001
156	3189 / 273	54	179	DRB1*0308, *1101/2/3/4/5/6/7/8/9/10/11/12/13/14/15/16/17/18/19/20/21/23/24/25/26/27/28/29/32/34/36/37/39/40/41/42/43, *1204, *1411
157	1630	37	119	DRB1*1101/5/8/9/10/11/12/14/15/19/20/22/23/24/26/27/28/29/30/31/32/33/37/39
158	44	54	105	DRB1*0308, *1109/16/20/40
159	50	256	163	DRB1*1201/2/3/5/6/7/8
160	50	55	248	DRB1*0812/22, *1201/2/4/5/6/7/8, *1428
161	68 / 273	259	211	DRB1*1102/3/11/14/16/20/21/36/40/41, *1301/2/4/8/15/16/17/19/20/22/23/24/27/28/29/31/32/34/35/36/38/39/40/41/43/45/48/51/52/53, *1416
162	263	259 / 252	153	DRB1*1116/20/40, *1301/2/8/9/16/20/27/28/29/31/32/34/35/36/39/40/41/43/51/52, *1416/37
163	44	40 / 485	130	DRB1*1116/20, *1301/2/6/10/15/16/27/28/31/32/34/35/36/39/40/41/51/53, *1419/21
164	44	258 / 485	140	DRB1*1310, *1419/21
165	68	485	211	DRB1*1303/10/33/37, *1419/21
166	68	102	171	DRB1*0312, *1303/4/12/13/21/30/32/33/38/48/49, *1413
167	68	104	201	DRB1*0820, *1101/3/4/6/9/10/11/12/15/23/24/25/27/28/29/32/33/35/37/38/39/40/41/43, *1305/7/11/14/18/21/24/26/42/46/47/49/50, *1422/25/27
168	68	2510	174	DRB1*0301/2/3/4/5/6/7/9/11/14/15/16/17/18/19/20/21/22/23, *0820, *1301/2/5/6/7/8/9/10/11/14/15/16/18/19/20/22/23/24/25/27/28/29/34/35/36/37/40/41/42/44/47/50/51/52/53, *1402/3/5/6/9/12/14/17/19/20/21/23/24/27/29/30/33/36/40/41/42
169	68	58	213	DRB1*1117, *1401/5/7/8/14/18/23/26/33/35/36/42/44/45
170	1631	38	123	DRB1*0310, *1343, *1401/4/10/16/26/28/31/32/35/38/39
171	68	107	172	DRB1*0310, *1343/45, *1401/7/16/22/25/26/32/35/38
172	2489	38	190	DRB1*0301/3/6/7/8/10/11/12/13/15/16/18/19/20/22, *1116, *1301/6/9/10/15/18/20/27/28/32/35/40/42/43/51/53, *1406/12/17/18/21/29/33
173	61	492	173	DRB5*0101/2/3/4/5/6/7/8/9/10/11, *0202/3/4/5
174	69	268	222	DRB3*0101/3/4/5/6/8/9/10
175	1674	255	215	DRB3*0102
176	151	76	117	DRB1*0317. DRB3*0107, *0201/2/3/5/6/7/8/11/12/13/14/16/17
177	70	2510	174	DRB3*0201/2/3/4/5/6/10/11/13/14/15/17
178	70	256	172	DRB3*0209, *0301/2/3

Appendix 1: Primer pairs and the HLA class I and II alleles detected (continue)

Lane	Sense Primer	Antisense Primer	Amplicon Size (base pairs)	Alleles amplified
179	60	38	213	DRB4*0101/2/3/4/5/6
180	283	314	151	DRB4*0101/2/3/4/5/6, *0201
181	270	866	151	DRB4*0103
182	77	350	195	DQB1*0305
183	82	112	198	DQB1*0201/2/3
184	77	78	208	DQB1*0401/2
185	79	152	207	DQB1*0501/2/3/4
186	347	111	249	DQB1*0601/6
187	348	350	112	DQB1*0602/3/4/7/8/10/11/13/14/15/16/20
188	349	619	98	DQB1*0604/5/6/9/12/17/18
189	181	112	208	DQB1*0301/4/9/10/13
190	88	350	179	DQB1*0302/3/7/8/11/12, *0602/3/4/5/7/8/9/10/11/12/13/14/15/16/17/18/19/20
191	353	831	122	DQB1*0302/7/8/11
192	1465	1466	131	DQB1*0203, *0303/6/12

Appendix 2: Sense and antisense primer sequences for SSP-PCR

Sense Primer	Sequences	Sense Primer	Sequences
625	ggCCggAgTATTgggACCA	400	CTTgCCgTCgTAggCgTC
292	ggCCggAgTATTgggACgA	431	AgCCCgTCCACgCACCg
286	CgACgCCgCgAgCCAgAA	249	CCTCCAggTAggCTCTCAA
753	AggATTACATCgCCCTgAAA	302	CCAAgAgCgCAggTCCTCT
3421	CCCACTCCATgAggTATTTCTT	299	CACTCCACgCACgTgCCA
296	gTggATAgAgCAggAgggT	299	CACTCCACgCACgTgCCA
1173	ATggCTgCgACgTggggT	249	CCTCCAggTAggCTCTCAA
291	AgCgACgCCgCgAgCCA	168	gAgCCACTCCACgCACgT
290	ACggAATgTgAAggCCCAg	302	CCAAgAgCgCAggTCCTCT
286	CgACgCCgCgAgCCAgAA	244	CAggTATCTgCggAgCCC
284	TATTgggACgAggAgACAg	249	CCTCCAggTAggCTCTCAA
284	TATTgggACgAggAgACAg	170	CCTCCAggTAggCTCTCTg
292	ggCCggAgTATTgggACgA	146	CCCTCCAggTAggCTCTCT
292	ggCCggAgTATTgggACgA	145	gAgCCACTCCACgCACTC
208	ACCgAgAgAACCTgCggAT	167	gAgCCACTCCACgCACCg
292	ggCCggAgTATTgggACgA	298	ATgTAATCCTTgCCgTCgTAA
239	gggTACCAgCAggACgCT	298	ATgTAATCCTTgCCgTCgTAA
193	ggAgTATTgggACCggAAC	298	ATgTAATCCTTgCCgTCgTAA
294	TCACAgACTgACCgAgAgAg	298	ATgTAATCCTTgCCgTCgTAA
288	TCACAgACTgACCgAgCgAA	168	gAgCCACTCCACgCACgT
174	CCggAgTATTgggACCTgC	298	ATgTAATCCTTgCCgTCgTAA
451	gggTACCggCAggACgCT	303	CTCTCTgCTgCTCCgCCg
239	gggTACCAgCAggACgCT	167	gAgCCACTCCACgCACCg
475	ACggAAAgTgAAggCCCAg	300	AgCgCAggTCCTCgTTCAA
290	ACggAATgTgAAggCCCAg	301	CCgTCgTAggCgTgCTgT
290	ACggAATgTgAAggCCCAg	249	CCTCCAggTAggCTCTCAA
174	CCggAgTATTgggACCTgC	486	CTTCACATTCCgTgTCTCCT
295	CCCggCCCggCAgTggA	414	ACgTCgCAgCCATACATCA
754	gATAgAgCAggAgAggCCT	429	gCCTTCACATTCCgTgTgTT
434	CCACTCCATgAggTATTTCAC	399	CCTTCCCgTTCTCCAggTg
3421	CCCACTCCATgAggTATTTCTT	494	gCAgggTCCCCAggTCCA
3421	CCCACTCCATgAggTATTTCTT	302	CCAAgAgCgCAggTCCTCT
434	CCACTCCATgAggTATTTCAC	171	CCgCggAggAAgCgCCA
1	CACggAATgTgAAggCCCAC	287	gAgCCCgTCCACgCACTC
493	CACgCAgTTCgTgCggTTT	221	TACCAgCgCgCTCCAgCT
193	ggAgTATTgggACCggAAC	285	CgTCgTAggCgTACTggTC
290	ACggAATgTgAAggCCCAg	517	ACgTCgCAgCCgTACATg
367	TACTACAACCAgAgCgAggA	425	CgTCgTAggCgTACTggTT
193	ggAgTATTgggACCggAAC	220	CCgCgCgCTCCAgCgTg
203	CAgATCTACAAggCCCAgg	212	CCTCCAggTAggCTCTgTC
271	CCgCggAggAAgCgCCA	220	CCgCgCgCTCCAgCgTg
203	CAgATCTACAAggCCCAgg	225	CTCTCAgCTgCTCCgCCT
242	CgAgAgAgCCTgCggAAC	215	ATCCTTgCCgTCgTAggCT
195	gACCggAACACACAgATCTT	215	ATCCTTgCCgTCgTAggCT

Appendix 2: Sense and antisense primer sequences for SSP-PCR (continue)

Sense Primer	Sequences	Sense Primer	Sequences
195	gACCggAACACACAgATCTT	219	CCAggTATCTgCggAgCg
280	gCTACgTggACgACACgCT	212	CCTCCAggTAggCTCTgTC
208	ACCgAgAgAACCTgCggAT	393	gTCgTAggCgTCCTggTC
246	CCACTCCATgAggTATTTCC	285	CgTCgTAggCgTACTggTC
207	CCgAgAgAgCCTgCggAA	212	CCTCCAggTAggCTCTgTC
192	ACCgggAgACACAgATCTC	393	gTCgTAggCgTCCTggTC
202	ggggAgCCCCgCTTCATT	382	CCTCCAggTAggCTCTCAg
272	CgCCACgAgTCCgAggAA	227	gCCCCACgTCgCAgCCg
435	CCTgCgCACCgCgCTCC	220	CCgCgCgCTCCAgCgTg
435	CCTgCgCACCgCgCTCC	276	TCCCACTTgCgCTgggT
435	CCTgCgCACCgCgCTCC	247	gCggCggTCCAggAgCg
196	TACCgAgAgAACCTgCgC	218	gAgCCACTCCACgCACTC
192	ACCgggAgACACAgATCTC	229	CTCCAACTTgCgCTgggA
272	CgCCACgAgTCCgAggAA	215	ATCCTTgCCgTCgTAggCT
192	ACCgggAgACACAgATCTC	127	ggTCgCAgCCATACATCCA
272	CgCCACgAgTCCgAggAA	217	CgTgCCCTCCAggTAggT
280	gCTACgTggACgACACgCT	217	CgTgCCCTCCAggTAggT
243	CgCgAgTCCgAggATggC	217	CgTgCCCTCCAggTAggT
197	AgCAggAggggCCggAA	217	CgTgCCCTCCAggTAggT
207	CCgAgAgAgCCTgCggAA	217	CgTgCCCTCCAggTAggT
581	CAAgACCAACACACAgACTT	213	gAggAggCgCCCgTCg
206	gACCggAACACACAgATCTA	224	CgTCgCAgCCATACATCAC
435	CCTgCgCACCgCgCTCC	214	CTTgCCgTCgTAggCgg
208	ACCgAgAgAACCTgCggAT	236	CCATACATCgTCTgCCAA
209	CgCCgCgAgTCCgAgAgA	215	ATCCTTgCCgTCgTAggCT
194	AACATgAAggCCTCCgCg	238	gAgCCACTCCACgCACAg
194	AACATgAAggCCTCCgCg	236	CCATACATCgTCTgCCAA
187	gCgCCgTggATAgAgCAA	281	CTCggTCAgTCTgTgCCTT
209	CgCCgCgAgTCCgAgAgA	282	TCTCggTAAgTCTgTgCCTT
242	CgAgAgAgCCTgCggAAC	228	TCgTAggCgTCCTggTgg
203	CAgATCTACAAggCCCAgg	212	CCTCCAggTAggCTCTgTC
395	gCCgCgAgTTCgAgAgg	392	CCTTgCCgTCgTAggCgA
280	gCTACgTggACgACACgCT	228	TCgTAggCgTCCTggTgg
575	gCAggAggggCCggAgT	236	CCATACATCgTCTgCCAA
188	gCCgCgAgTCCgAggAC	220	CCgCgCgCTCCAgCgTg
192	ACCgggAgACACAgATCTC	229	CTCCAACTTgCgCTgggA
192	ACCgggAgACACAgATCTC	225	CTCTCAgCTgCTCCgCCT
367	TACTACAACCAgAgCgAggA	241	gCCgCggTCCAggAgCT
203	CAgATCTACAAggCCCAgg	226	gTCgTAggCggACTggTC
209	CgCCgCgAgTCCgAgAgA	250	gCAggTTCCgCAggCTCT
194	AACATgAAggCCTCCgCg	214	CTTgCCgTCgTAggCgg
240	gAgACACAgAAgTACAAgCg	223	gCCATACATCCTCTggATgA
243	CgCgAgTCCgAggATggC	223	gCCATACATCCTCTggATgA
243	CgCgAgTCCgAggATggC	214	CTTgCCgTCgTAggCgg
192	ACCgggAgACACAgATCTC	238	gAgCCACTCCACgCACAg

Appendix 2: Sense and antisense primer sequences for SSP-PCR (continue)

Sense Primer	Sequences	Sense Primer	Sequences
243	CgCgAgTCCgAggATggC	226	gTCgTAggCggACTggTC
271	CCgCggAggAAgCgCCA	219	CCAggTATCTgCggAgCg
209	CgCCgCgAgTCCgAgAgA	244	CAggTATCTgCggAgCCC
271	CCgCggAggAAgCgCCA	377	CCTCCAggTAggCTCTCCA
189	gCgCCgTggATAgAgCAA	223	gCCATACATCCTCTggATgA
243	CgCgAgTCCgAggATggC	237	gCgCAggTTCCgCAggC
193	ggAgTATTgggACCggAAC	213	gAggAggCgCCCgTCg
193	ggAgTATTgggACCggAAC	277	ggAggAAgCgCCCgTCg
188	gCCgCgAgTCCgAggAC	226	gTCgTAggCggACTggTC
195	gACCggAACACACAgATCTT	392	CCTTgCCgTCgTAggCgA
195	gACCggAACACACAgATCTT	216	CgTTCAgggCgATgTAATCT
195	gACCggAACACACAgATCTT	216	CgTTCAgggCgATgTAATCT
207	CCgAgAgAgCCTgCggAA	236	CCATACATCgTCTgCCAA
208	ACCgAgAgAACCTgCggAT	216	CgTTCAgggCgATgTAATCT
202	ggggAgCCCCgCTTCATT	399	CCTTCCCgTTCTCCAggTg
193	ggAgTATTgggACCggAAC	216	CgTTCAgggCgATgTAATCT
195	gACCggAACACACAgATCTT	237	gCgCAggTTCCgCAggC
192	ACCgggAgACACAgATCTC	315	CCCCAggTCgCAgCCAC
1719	gCgAggggACCgCAggC	145	gAgCCACTCCACgCACTC
1719	gCgAggggACCgCAggC	389	AgCgTCTCCTTCCCATTCTT
368	CACAgACTgACCgAgTgAg	143	gCCCCAggTCgCAgCCAA
366	CCgAgTgAACCTgCggAAA	143	gCCCCAggTCgCAgCCAA
368	CACAgACTgACCgAgTgAg	379	CgCgCgCTgCAgCgTCTT
366	CCgAgTgAACCTgCggAAA	818	gggTgATCTgAgCCgCCT
271	CCgCggAggAAgCgCCA	127	ggTCgCAgCCATACATCCA
366	CCgAgTgAACCTgCggAAA	184	CgCACgggCCgCCTCCA
240	gAgACACAgAAgTACAAgCg	184	CgCACgggCCgCCTCCA
367	TACTACAACCAgAgCgAggA	247	gCggCggTCCAggAgCg
4	CACAggCTgACCgAgTgAg	379	CgCgCgCTgCAgCgTCTT
313	ggACCgggAgACACAgAAC	166	gCgCAggTTCCgCAggC
367	TACTACAACCAgAgCgAggA	317	TCTCAgCTgCTCCgCCgT
165	ACgACACgCAgTTCgTgCA	389	AgCgTCTCCTTCCCATTCTT
159	TACAACCAgAgCgAggCCA	389	AgCgTCTCCTTCCCATTCTT
130	CCgCgggTATgACCAgTC	126	TgAgCCgCCgTgTCCgCA
160	ACAACCAgAgCgAggCCg	157	CCgCCgTgTCCgCggCA
369	AgTCCAAgAggggAgCCg	388	ggTCgCAgCCAAACATCCA
368	CACAgACTgACCgAgTgAg	223	gCCATACATCCTCTggATgA
371	CCACTCCATgAggTATTTCTC	382	CCTCCAggTAggCTCTCAg
366	CCgAgTgAACCTgCggAAA	146	CCCTCCAggTAggCTCTCT
366	CCgAgTgAACCTgCggAAA	146	CCCTCCAggTAggCTCTCT
368	CACAgACTgACCgAgTgAg	377	CCTCCAggTAggCTCTCCA
366	CCgAgTgAACCTgCggAAA	127	ggTCgCAgCCATACATCCA
368	CACAgACTgACCgAgTgAg	377	CCTCCAggTAggCTCTCCA
366	CCgAgTgAACCTgCggAAA	157	CCgCCgTgTCCgCggCA
366	CCgAgTgAACCTgCggAAA	39	CCgCCTCTgCTCCAggAg
36	TTgTggCAgCTTAAgTTTgAAT	38	CTgCACTgTgAAgCTCTCCA

Appendix 2: Sense and antisense primer sequences for SSP-PCR (continue)

Sense Primer	Sequences	Sense Primer	Sequences
36	TTgTggCAgCTTAAgTTTgAAT	252	CCACCgCggCCCgCgC
41	TCCTgTggCAgCCTAAgAg	38	CTgCACTgTgAAgCTCTCCA
41	TCCTgTggCAgCCTAAgAg	491	CTCCgTCACCgCCCggT
41	TCCTgTggCAgCCTAAgAg	255	gTCCACCCggCCCCgCT
68	gTTTCTTggAgTACTCTACgTC	38	CTgCACTgTgAAgCTCTCCA
46	gACggAgCgggTgCggTA	37	CTgCACTgTgAAgCTCTCAC
44	TACTTCCATAACCAggAggAgA	491	CTCCgTCACCgCCCggT
68	gTTTCTTggAgTACTCTACgTC	2510	ggCTgTTCCAgTACTCggCATC
3291	CggTTCCTggACAgATACTTCTATC	256	CTgTTCCAggACTCggCgA
47	gTTTCTTggAgCAggTTAAACA	49	CCCgTAgTTgTgTCTgCACAC
47	gTTTCTTggAgCAggTTAAACA	51	CTgCAgTAggTgTCCACCAg
47	gTTTCTTggAgCAggTTAAACA	102	TgTTCCAgTACTCggCgCT
48	CCTgTggCAgggTAAgTATA	491	CTCCgTCACCgCCCggT
1737	ggTgCAgTTgCTggAAAgACT	1635	CCCgCCTgTCTTCCAggAT
50	AgTACTCTACgggTgAgTgTT	58	TCTgCAATAggTgTCCACCT
50	AgTACTCTACgggTgAgTgTT	51	CTgCAgTAggTgTCCACCAg
2518	CTggACAgATACTTCTATAACCAA	49	CCCgTAgTTgTgTCTgCACAC
263	gTTCCTggACAgATACTTCC	37	CTgCACTgTgAAgCTCTCAC
46	gACggAgCgggTgCggTA	54	CTggCTgTTCCAgTACTCCT
53	CggTTgCTggAAAgACgCg	37	CTgCACTgTgAAgCTCTCAC
273	TTCTTggAgTACTCTACggg	54	CTggCTgTTCCAgTACTCCT
1630	TggggCggCCTgATgAg	256	CTgTTCCAggACTCggCgA
44	TACTTCCATAACCAggAggAgA	55	CACTgTgAAgCTCTCCACAg
50	AgTACTCTACgggTgAgTgTT	259	TCCACCgCggCCCgCTC
50	AgTACTCTACgggTgAgTgTT	259	TCCACCgCggCCCgCTC
68	gTTTCTTggAgTACTCTACgTC	252	CCACCgCggCCCgCgC
273	TTCTTggAgTACTCTACggg	40	CCCgCTCgTCTTCCAggAT
263	gTTCCTggACAgATACTTCC	485	TCCACCgCggCCCgCTT
44	TACTTCCATAACCAggAggAgA	258	ACCgCggCCCgCCTgTC
44	TACTTCCATAACCAggAggAgA	485	TCCACCgCggCCCgCTT
68	gTTTCTTggAgTACTCTACgTC	485	TCCACCgCggCCCgCTT
68	gTTTCTTggAgTACTCTACgTC	102	TgTTCCAgTACTCggCgCT
68	gTTTCTTggAgTACTCTACgTC	104	CCCgCCTgTCTTCCAggAA
68	gTTTCTTggAgTACTCTACgTC	2510	ggCTgTTCCAgTACTCggCATC
68	gTTTCTTggAgTACTCTACgTC	58	TCTgCAATAggTgTCCACCT
1631	gAgCTggggCggCCTgC	38	CTgCACTgTgAAgCTCTCCA
68	gTTTCTTggAgTACTCTACgTC	38	CTgCACTgTgAAgCTCTCCA
2489	ATACTTCCATAACCAggAggAgA	492	gCTgTTCCAgTACTCAgCg
61	gTTTCTTgCAgCAggATAAgTA	268	CTgCAgTAATTgTCCACCCg
69	TTTCTTggAgCTgCgTAAgTC	255	gTCCACCCggCCCCgCT
1674	CACgTTTCTTggAgCTgTg	76	ggAgTACCgggCggTgAg
151	CCgTAgTTgTgTCTgCAgTAA	2510	ggCTgTTCCAgTACTCggCATC
70	gTTTCTTggAgCTgCTTAAgTC	256	CTgTTCCAggACTCggCgA
70	gTTTCTTggAgCTgCTTAAgTC	38	CTgCACTgTgAAgCTCTCCA
60	gAgCgAgTgTggAACCTgA	350	TgCACACCgTgTCCAACTC

Appendix 2: Sense and antisense primer sequences for SSP-PCR (continue)

Sense Primer	Sequences	Sense Primer	Sequences
77	gCTACTTCACCAACgggACC	112	CgTgCggAgCTCCAACTg
82	gTgCgTCTTgTgAgCAgAAg	78	TggTAgTTgTgTCTgCATACg
77	gCTACTTCACCAACgggACC	152	CCCgCggTACgCCACCTC
79	ACggAgCgCgTgCgggg	111	CCgCggAACgCCACCTC
347	TTTCgTgCTCCAgTTTAAggC	350	TgCACACCgTgTCCAACTC
348	gACgTgggggTgTACCgC	619	CCTgCggCgTCACCgCC
349	ggAgCgCgTgCgTCTTgTA	112	CgTgCggAgCTCCAACTg
181	gACggAgCgCgTgCgTTA	350	TgCACACCgTgTCCAACTC
88	gACggAgCgCgTgCgTCT	831	TggCTgTTCCAgTACTCggCgg
353	gTgCgTCTTgTgACCAgATA	1466	gCTgTTCCAgTACTCggCgT
1465	ggACggAgCgCgTgCgTCT		

Appendix 3: Overlapping peptides span on DENV serotype 2 isolate (strain 16681)

Capsid

MNNQRKKAKNTPFNMLKRERNRVSTVQQLTKRFSLGMLQGRGPLKLFMALVAFLRFLTIPPTAGILKRWGTIK

NNQRKKAKNTPFNMLKRERN #221

PFNMLKRERNRVSTVQQLTK #226

RVSTVQQLTKRFSLGMLQGR #222

RFSLGMLQGRGPLKLFMALV #227

GPLKLFMALVAFLRFLTIPP #223

AFLRFLTIPPTAGILKRWGT #228

TAGILKRWGTIK

IK

prM

KSKAINVLRGFRKEIGRMLNILNRRRRSAGMIIMLIPTVMAFHLTTRNGEPHMIVSRQEKGKSLLFKTEDGVN

KSKAINVLR #224

KSKAINVLRGFRKEIGRM #229

RGFRKEIGRMLNILNRRRRS #225

LNILNRRRRSAGMIIMLIFIVMA #230

FHLTTRNGEPHMIVSRQEKG #231

HMIVSRQEKGKSLLFKTEDG #239

KSLLFKTEDGVN

VN

M

MCTLMAMDLGELCEDTITYKCPLLRQNEPEDIDCWCNSTSTWVTYGTCTTMGEHRREKRSVALVPHVGMGLET

MCTLMAMD #232

MCTLMAMDLGELCEDTIT #240

LGELCEDTITYKCPLLRQNE #233

YKCPLLRQNEPEDIDCWCNS #241

PEDIDCWCNSTSTWVTYGTC #234

TSTWVTYGTCTTMGEHRREK #242

TTMGEHRREKRSVALVPHVG #235

RSVALVPHVGMGLET

MGLET

E

RTETWMSSEGAWKHVQRIETWILRHPGFTMMAAILAYTIGTTHFQRALIFILLTAVTPSMTMRCIGMSNRDFV

RTETW #243

RTETWMSSEGAWKHV #236

MSSEGAWKHVQRIETWILRH #244

QRIETWILRHPGFTMMAAIL #237

PGFTMMAAILAYTIGTTHFQ #245

AYTIGTTHFQRALIFILLTA #238

RALIFILLTAVTPSMT #246

MRCIGMSNRDFV

MSNRDFV

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                                                                                                                                FV
EGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYCIEAKLTNTTTESRCPTQGEPSLNEE
EGV #1
EGVSGGSW #34
EGVSGGSWVDIVL #66
   SGGSWVDIVLEHGSC #2
        VDIVLEHGSCVTTMA #35
             EHGSCVTTMAKNKPT #67
                  VTTMAKNKPTLDFEL #3
                       KNKPTLDFELIKTEA #36
                            LDFELIKTEAKQPAT #68
                                 IKTEAKQPATLRKYC #4
                                      KQPATLRKYCIEAKL #37
                                           LRKYCIEAKLTNTTT #69
                                                IEAKLTNTTTESRCP #5
                                                     TNTTTESRCPTQGEP #38
                                                          ESRCPTQGEPSLNEE #70
                                                               TQGEPSLNEE
                                                                    SLNEE
QDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCAMFRCKKNMEGKVVQPENLEYTIVITPHSGEEHAVGNDTGKH
QDKRF #6
QDKRFVCKHS #39
QDKRFVCKHSMVDRG #71
     VCKHSMVDRGWGNGC #7
          MVDRGWGNGCGLFGK #40
               WGNGCGLFGKGGIVT #72
                    GLFGKGGIVTCAMFR #8
                         GGIVTCAMFRCKKNM #41
                              CAMFRCKKNMEGKVV #73
                                   CKKNMEGKVVQPENL #9
                                        EGKVVQPENLEYTIV #42
                                             QPENLEYTIVITPHS #74
                                                  EYTIVITPHSGEEHA #10
                                                       ITPHSGEEHAVGNDT #43
                                                            GEEHAVGNDTGKH
                                                                 VGNDTGKH
                                                                      GKH
GKEIKITPQSSITEAELTGYGTVTMECSPRTGLDFNEMVLLQMENKAWLVHRQWFLDLPLPWLPGADTQGSN
GK #75
GKEIKIT #11
GKEIKITPQSST #44
  EIKITPQSSTTEAEL #76
       PQSSTTEAELTGYGT #12
            TEAELTGYGTVTMEC #45
                 TGYGTVTMECSPRTG #77
                      VTMECSPRTGLDFNE #13
                           SPRTGLDFNEMVLLQ #46
                                LDFNEMVLLQMENKA #78
                                     MVLLQMENKAWLVHR #14
                                          MENKAWLVHRQWFLD #47
                                               WLVHRQWFLDLPLPW #79
                                                    QWFLDLPLPWLPGAD #15
                                                         LPLPWLPGADTQGSN #48
                                                              LPGADTQGSN
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                                                                    TQGSN
WIQKETLVTFKNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSGNLLFTGHLKCRLRMDKLQLKGMSYSMCTG
WIQKE #80
WIQKETLVTF #16
WIQKETLVTFKNPHA #49
     TLVTFKNPHAKKQDV #81
          KNPHAKKQDVVVLGS #17
               KKQDVVVLGSQEGAM #50
                    VVLGSQEGAMHTALT #82
                         QEGAMHTALTGATEI #18
                              HTALTGATEIQMSSG #51
                                   GATEIQMSSGNLLFT #83
                                        QMSSGNLLFTGHLKC #19
                                             NLLFTGHLKCRLRMD #52
                                                  GHLKCRLRMDKLQLK #84
                                                       RLRMDKLQLKGMSYS #20
                                                            KLQLKGMSYSMCTG
                                                                 GMSYSMCTG
                                                                      MCTG
KFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMDLEKRHVLGRLITVNPIVTEKDSPVNIEAEPPFGDSYK
#53
KFKVVK #85
KFKVVKEIAET #21
 FKVVKEIAETQHGTI #54
      EIAETQHGTIVIRVQ #86
           QHGTIVIRVQYEGDG #22
                VIRVQYEGDGSPCKI #55
                     YEGDGSPCKIPFEIM #87
                          SPCKIPFEIMDLEKR #23
                               PFEIMDLEKRHVLGR #56
                                    DLEKRHVLGRLITVN #88
                                         HVLGRLITVNPIVTE #24
                                              LITVNPIVTEKDSPV #57
                                                   PIVTEKDSPVNIEAE #89
                                                        KDSPVNIEAEPPFGD #25
                                                             NIEAEPPFGDSY
                                                                  PPFGDSY
                                                                       SY
IIIGVEPGQLKLNWFKKGSSIGQMFETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSG
III#58
IIIGVEPG #90
IIIGVEPGQLKLN #26
   GVEPGQLKLNWFKKG #59
        QLKLNWFKKGSSIGQ #91
             WFKKGSSIGQMFETT #27
                  SSIGQMFETTMRGAK # 60
                       MFETTMRGAKRMAIL #92
                            MRGAKRMAILGDTAW #28
                                 RMAILGDTAWDFGSL #61
                                      GDTAWDFGSLGGVFT #93
                                           DFGSLGGVFTSIGKA #29
                                                GGVFTSIGKALHQVFGA #62
                                                     SIGKALHQVFGAIYG #94
                                                          LHQVFGAIYGAAFSG #30
                                                                 IYGAAFSG
                                                                    AAFSG
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NS1

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VSWTMKILI #63
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VSWTMKILIGVIITW #31
GVIITWIGMNSRSTSLS #64
VIITWGMNSRSTSLS #96
IGMNSRSTSLSVTLV #32
VTLVLVGIVTLVLGVMV #65
VTLVLVGIVTLYLGVMV #97
LVGIVTLYLGVMV #33

TEQYKFQPESPSKLASAIQKAHEEGICGIRSVTRLENLMWKQITPELNHILSENEVKLTIMTGDIKGIMQAGKRS
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CDSKLMSAAIKDNRAVHADMGYWIESALNDTWKIEKASFIEVKNCHWPKSHTLWSNGVLESEMIIPKNLAGPVSQ
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NS2A

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PETILELTDALALGMMVLKMVRNMEKYQLAVTIMAILCVPNAVILQNAWKVSCTILAVVSVSPLLLTSSQQ

NS2B

KTDWIPLALTIKGLNPTAIFLTTLSRTSKKRSWPLNEAIMAVGMVSILASSLLKNDIPMTGPLVAGGLLTVCYVL
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NS3

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WDVPSPPPMGKAELE #139
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KAELEDGAYRIKQKG #99
DGAYRIKQKGILGYS #140
IKQKGILGYSQIGAG #181
ILGYSQIGAGVYKEG #100
QIGAGVYKEGTFHTM #141
VYKEGTFHTMWHVTR #182
TFHTMWHVTRGAVLM #101
WHVTRGAVLM
GAVLM

HKGKRIEPSWADVKKDLISYGGGWKLEGEWKEGEEVQVLALEPGKNPRAVQTKPGLFKTNAGTIGAVSLDFS

HKGKR #142
HKGKRIEPSW #183
HKGKRIEPSWADVKK #102
IEPSWADVKKDLISY #143
ADVKKDLISYGGGWK #184
DLISYGGGWKLEGEW #103
GGGWKLEGEWKEGEE #144
LEGEWKEGEEVQVLA #185
KEGEEVQVLALEPGK #104
VQVLALEPGKNPRAV #145
LEPGKNPRAVQTKPG #186
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QTKPGLFKTNAGTIG #146
LFKTNAGTIGAVSLD #187
AGTIGAVSLDFS

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                                                                       FS
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PGT #106
PGTSGSPI #147
PGTSGSPIIDKKG #188
   SGSPIIDKKGKVVGL #107
       IDKKGKVVGLYGNGV #148
            KVVGLYGNGVVTRSG #189
                 YGNGVVTRSGAYVSA #108
                      VTRSGAYVSAIAQTE #149
                           AYVSAIAQTEKSIED #190
                                IAQTEKSIEDNPEIE #109
                                     KSIEDNPEIEDDIFR #150
                                          NPEIEDDIFRKRRLT #191
                                               DDIFRKRRLTIMDLH #110
                                                    KRRLTIMDLHPGAGK #151
                                                         IMDLHPGAGKTKRYL #192
                                                              PGAGKTKRYL
                                                                 TKRYL
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PAIVR #111
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     EAIKRGLRTLILAPT #112
          GLRTLIAPTRVVAA #153
               ILAPTRVVAAEMEEA #194
                    RVVAAEMEEALRGLP #113
                         EMEEALRGLPIRYQT #154
                              LRGLPIRYQTPAIRA #195
                                   IRYQTPAIRAEHTGR #114
                                        PAIRAEHTGREIVDL #155
                                             EHTGREIVDLMCHAT #196
                                                  EIVDLMCHATFTMRL #115
                                                       MCHATFTMRLLSPVR #156
                                                            FTMRLLSPVRVPN
                                                                 LSPVRVPN
                                                                    VPN
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YN #197
YNLIIMD #116
YNLIIMDEAHFT #157
  LIIMDEAHFTDPASI #198
       EAHFTDPASIAARGY #117
            DPASIAARGYISTRV #158
              AARGYISTRVEMGEA #199
                   ISTRVEMGEAAGIFM #118
                        EMGEAAGIFMTATPP #159
                             AGIFMTATPPGSRDP #200
                                  TATPPGSRDPFPQSN #119
                                       GSRDPFPQSNAPIID #160
                                            FPQSNAPIIDEEREI #201
                                                 APIIDEEREIPERSW #120
                                                      EEREIPERSWNSGHE #161
                                                           PERSWNSGHE
                                                                NSGHE
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WVTDFKGKTVWFVPSIKAGNDIAACLRKNGKKVIQLSRKTFDSEYVKTRTNDWDFVVTTDISEMGANFKAERVID
WVTDF #202
WVTDFKGKTV #121
WVTDFKGKTVWFVPS #162
     KGKTVWFVPSIKAGN #203
          WFVPSIKAGNDIAAC #122
               IKAGNDIAACLSKNG #163
                    DIAACLSKNGKKVIQ #204
                         LSKNGKKVIQLSRKT #123
                              KKVIQLSRKTFDSEY #164
                                   SRKTFDSEYAKTRT #205
                                        FDSEYAKTRTNDWDF #124
                                             AKTRTNDWDFVVTTD #165
                                                  NDWDFVVTTDISEMG #206
                                                       VVTTDISEMGANFKA #125
                                                            ISEMGANFKAERVID#166
                                                                 ANFKAERVID
                                                                      ERVID
PRRCMKPVILTDGEERVILAGPMPVTHSSAAQRRGRIGRNPKNENDQYIYMGEPLENDEDCAHWKEAKMLLD
PRRCM #207
PRRCMKPVIL #126
PRRCMKPVILTDGEE #167
     KPVILTDGEERVILA #208
          TDGEERVILAGPMPV #127
               RVILAGPMPVTHSSA #168
                    GPMPVTHSSAAQRRG #209
                         THSSAAQRRGRIGRN #128
                              AQRRGRIGRNPKNEN #169
                                   RIGRNPKNENDQYIY #210
                                        PKNENDQYIYMGEPL #129
                                             DQYIYMGEPLENDED #170
                                                  MGEPLENDEDCAHWK #211
                                                       ENDEDCAHWKEAKML #130
                                                            CAHWKEAKMLLD
                                                                 EAKMLLD
                                                                      LD
NINTPEGIIPSMFEPEREKVDAIDGEYRLRGEARKTFVDLMRRGDLPVWLAYRVAAEGINYADRRWCFDGVKN
NIN #171
NINTPEGI #212
NINTPEGIIPSMF #131
   TPEGIIPSMFEPERE #172
        IPSMFEPEREKVDAI #213
               EPEREKVDAIDGEYR #132
                    KVDAIDGEYRLRGEA #173
                         DGEYRLRGEARTTFV #214
                              LRGEARTTFVDLMRR #133
                                   RTTFVDLMRRGDLPV #174
                                        DLMRRGDLPVWLAYR #215
                                             GDLPVWLAYRVAAEG #134
                                                  WLAYRVAAEGINYAD #175
                                                       VAAEGINYADRRWCF #216
                                                            INYADRRWCFDGVKN #135
                                                                 RRWCFDGVKN
                                                                      DGVKN
```

NS4A

NQILEENVEVEIWTKEGERKKLKPRWLDARIYSDPLALKEFKEFAAGRKSLTLNLITEMGRLPTFMTQKARD

NQILE #176
NQILEENVEV #217
NQILEENVEVEIWTK #136
ENVEVEIWTKEGERK #177
EIWTKEGERKKLKPR #218
EGERKKLKPRWLDAIY #137
KLKPRWLDARIYSDP #178
WLDARIYSDPLALKE #219
SDPLALKEFKEFAAGRK #138
LALKEFKEFAAGRK #179
FKEFAAGRK #220

SLTLNLITEMGRLPTFMTQK #247
GRLPTFMTQKARD
ARD

ALDNLAVLHTAEAGGRAYNHALSELPETLETLLLLTLLATVTGGIFLFLMSGRGIGKMTLGMCCIITASILL

ALDNLAV #254
ALDNLAVLHTAEAGGRA #248
LHTAEAGGRAYNHALSELPE #255
YNHALSELPETLETLLLLTL #249
TLETLLLLTLLATVTGGIFL #256
LATVTGGIFLFLMSGRGIGK #250
FLMSGRGIGKMTLGMCCIIT #257
MTLGMCCIITASILL
ASILL

WYAQIQPHWIAASIILEFFLIVLLIPEPEKQRTPQDNQLTYVVIAILTVVAATMA

WYAQI #251
WYAQIQPHWIAASII #258
QPHWIAASIILEFFLIVLLI #252
LEFFLIVLLIPEPEKQRTPQ #259
PEPEKQRTPQDNQLTYVVIA #253
DNQLTYVVIAILTVVAATMA #260

NS4B

NEMGFLEKTKKDLGLGSIATQQPESNILDIDLRPASAWTLYAVATTFVTPMLRHSIENSSVNVSLTAIANQATVL
MGLGKGWPLSKMDIGVPLLAIGCYSQVNPITLTAALFLLVAHYAIIGPGLQAKATREAQKRAAAGIMKNPTVDGI
TVIDLDPIPYDPKFEKQLGQVMLLVLCVTQVLMMRTTWALCEALTLATGPISTLWEGNPGRFWNTTIAVSMANIF
RGSYLAGAGLLF

NS5

SIMKNTTNTRRGTGNIGETLGEKWKSRLNALGKSEFQIYKKSGIQEVDRTLAKEGIKRGETDHHAVSRGSAKLRW
FVERNMVTPEGKVVDLGCGRGGWSYYCGGLKNVREVKGLTKGGPGHEEPIPMSTYGWNLVRLQSGVDVFFIPPEK
CDTLLCDIGESSPNPTVEAGRTLRVLNLVENWLNNNTQFCIKVLNPYMPSVIEKMEALQRKYGGALVRNPLSRNS
THEMYWVSNASGNIVSSVNMISRMLINRFTMRYKKATYEPDVDLGSGTRNIGIESEIPNLDIIGKRIEKIKQEHE

TSWHYDQDHPYKTWAYHGSYETKQTGSASSMVNGVVRLLTKPWDVVPMVTQMAMTDTTPFGQQRVFKEKVDTRTQ
EPKEGTKKLMKITAEWLWKELGKKKTPRMCTREEFTRKVRSNAALGAIFTDENKWKSAREAVEDSRFWELVDKER
NLHLEGKCETCVYNMMGKREKKLGEFGKAKGSRAIWYMWLGARFLEFEALGFLNEDHWFSRENSLSGVEGEGLHK
LGYILRDVSKKEGGAMYADDTAGWDTRITLEDLKNEEMVTNHMEGEHKKLAEAIFKLTYQNKVVRVQRPTPRGTV
MDIISRRDQRGSGQVGTYGLNTFTNMEAQLIRQMEGEGVFKSIQHLTITEEIAVQNWLARVGRERLSRMAISGDD
CVVKPLDDRFASALTALNDMGKIRKDIQQWEPSRGWNDWTQVPFCSHHFHELIMKDGRVLVVPCRNQDELIGRAR
ISQGAGWSLRETACLGKSYAQMWSLMYFHRRDLRLAANAICSAVPSHWVPTSRTTWSIHAKHEWMTTEDMLTVWN
RVWIQENPWMEDKTPVESWEEIPYLGKREDQWCGSLIGLTSRATWAKNIQAAINQVRSLIGNEEYTDYMPSMKRF
RREEEEAGVLW

Appendix 4: Matrix of peptide pools

Pool	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
3	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
4	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64
5	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
6	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96
7	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112
8	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128
9	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144
10	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160
11	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176
12	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192
13	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208
14	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224
15	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240
16	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256
													257	258	259	260

Pool peptide

Peptide

Appendix 5: The breadth and magnitude of responses from all patients

Study	number	Peptide(s) recognised	Sequence	SFU/million PBMC	mean SFU/million per patients
BC	309	154	EMEEALRGLPIRYQT	260	260
BC	310	224	TAGILKRWGTIKKSKAINVL	100	100
BC	311	101	TFHTMWHVTRGAVLM	150	130
	311	189	KVVGLYGNGVVTRSG	140	
	311	216	VAAEGINYADRRWCF	100	
BC	313	117	EAHFTDPASIAARGY	160	162.5
	313	143	IEPSWADVKKDLISY	160	
	313	216	VAAEGINYADRRWCF	260	
	313	222	RVSTVQQLTKRFSLGMLQGR	70	
BC	314	229	IKKSKAINVLRGFRKEIGRM	40	34
	314	233	LGELCEDTITYKCPLLRQNE	28	
BC	315	81	TLVTFKNPHAKKQDV	66	177
	315	194	ILAPTRVVAAEMEEA	288	
BC	316	61	RMAILGDTAWDFGSL	128	172
	316	216	VAAEGINYADRRWCF	216	
BC	317	225	RGFRKEIGRMLNILNRRRRS	60	60
BC	318	101	TFHTMWHVTRGAVLM	60	120
	318	153	GLRTLIAPTRVVAA	100	
	318	154	EMEEALRGLPIRYQT	260	
	318	206	NDWDFVVTTDISEMG	100	
	318	200	AGIFMTATPPGSRDP	80	
BC	320	224	TAGILKRWGTIKKSKAINVL	130	130
BC	322	61	RMAILGDTAWDFGSL	60	58
	322	134	GDLPVWLAYRVAAEG	60	
	322	159	EMGEAAGIFMTATPP	70	
	322	194	ILAPTRVVAAEMEEA	40	
	322	224	TAGILKRWGTIKKSKAINVL	60	
BC	323	188	FSPGTSGSPIIDKKG	160	150
	323	189	KVVGLYGNGVVTRSG	140	
BC	324	2	SGGSWVDIVLEHGSC	60	110
	324	61	RMAILGDTAWDFGSL	140	
	324	69	LRKYCIEAKLTNTTT	150	
	324	101	TFHTMWHVTRGAVLM	100	
	324	194	ILAPTRVVAAEMEEA	90	
	324	225	RGFRKEIGRMLNILNRRRRS	70	
	324	229	IKKSKAINVLRGFRKEIGRM	120	
	324	13	VTMECSPRTGLDFNE	120	

Study	number	Peptide(s) recognised	Sequence	SFU/million PBMC	mean SFU/million per patients
	324	237	QRIETWILRHPGFTMMAAIL	120	
	324	244	MSSEGAWKHVQRIETWILRH	130	
BC	325	147	AVSLDFSPGTSGSPI	140	125
	325	157	VPNYNLIIMDEAHFT	190	
	325	195	LRGLPIRYQTPAIRA	45	
BC	327	114	IRYQTPAIRAEHTGR	100	196.25
	327	189	KVVGLYGNGVVTRSG	140	
	327	116	LSPVRVPNYNLIIMD	200	
	327	117	EAHFTDPASIAARGY	200	
	327	131	LDNINTPEGIIPSMF	230	
	327	157	VPNYNLIIMDEAHFT	230	
	327	172	TPEGIIPSMFEPERE	240	
	327	188	FSPGTSGSPIIDKKG	230	
BC	331	114	IRYQTPAIRAEHTGR	210	212.8571429
	331	115	EIVDLMCHATFTMRL	200	
	331	116	LSPVRVPNYNLIIMD	230	
	331	117	EAHFTDPASIAARGY	200	
	331	157	VPNYNLIIMDEAHFT	220	
	331	172	TPEGIIPSMFEPERE	190	
	331	188	FSPGTSGSPIIDKKG	240	
BC	332	198	LIIMDEAHFTDPASI	120	120
BC	333	135	INYADRRWCFDGVKN	310	310
	333	198	LIIMDEAHFTDPASI	310	
	333	216	VAAEGINYADRRWCF	310	
BC	334	47	MENKAWLVHRQWFLD	80	80
BC	336	50	KKQDVVVLGSQEGAM	117	87.5
	336	250	LATVTGGIFLFLMSGRGIGK	58	
BC	337	101	TFHTMWHVTRGAVLM	390	291.25
	337	153	GLRTLIAPTRVVAA	75	
	337	196	EHTGREIVDLMCHAT	200	
	337	233	LGELCEDTITYKCPLLRQNE	500	
BC	338	50	KKQDVVVLGSQEGAM	75	48.33333333
	338	225	RGFRKEIGRMLNILNRRRRS	40	
	338	229	IKKSKAINVLRGFRKEIGRM	30	
BC	343	61	RMAILGDTAWDFGSL	138	107.5
	343	194	ILAPTRVVAAEMEEA	77	
BC	347	178	KLKPRWLDARIYSDP	85	106.3333333
	347	152	TKRYLPAIVREAIKR	85	
	347	184	ADVKKDLISYGGGWK	97	
	347	137	EGERKKLKPRWLDAIY	97	

Study	number	Peptide(s) recognised	Sequence	SFU/million PBMC	mean SFU/million per patients
	347	233	LGELCEDTITYKCPLLRQNE	80	
	347	143	IEPSWADVKKDLISY	194	
BC	348	114	IRYQTPAIRAEHTGR	27	49.33333333
	348	137	EGERKKLKPRWLDAIY	38	
	348	200	AGIFMTATPPGSRDP	83	
BC	349	225	RGFRKEIGRMLNILNRRRRS	80	63.33333333
	349	229	IKKSKAINVLRGFRKEIGRM	30	
	349	69	LRKYCIEAKLTNTTT	80	
BC	350	143	IEPSWADVKKDLISY	100	100
BC	352	61	RMAILGDTAWDFGSL	300	152
	352	194	ILAPTRVVAAEMEEA	116	
	352	225	RGFRKEIGRMLNILNRRRRS	40	
BC	353	114	IRYQTPAIRAEHTGR	100	68
	353	152	TKRYLPAIVREAIKR	64	
	353	244	MSSEGAWKHVQRIETWILRH	60	
	353	195	LRGLPIRYQTPAIRA	48	
BC	354	164	KKVIQLSRKTFDSEY	118	117.6666667
	354	188	FSPGTSGSPIIDKKG	150	
	354	198	LIIMDEAHFTDPASI	85	
BC	500	101	TFHTMWHVTRGAVLM	55	55
BC	501	188	FSPGTSGSPIIDKKG	53	53
BC	502	61	RMAILGDTAWDFGSL	60	60
BC	503	61	RMAILGDTAWDFGSL	110	99
	503	194	ILAPTRVVAAEMEEA	88	
BC	504	61	RMAILGDTAWDFGSL	200	217.5
	504	194	ILAPTRVVAAEMEEA	350	
	504	219	WLDARIYSDPLALKE	250	
	504	229	IKKSKAINVLRGFRKEIGRM	70	
BC	507	154	EMEEALRGLPIRYQT	120	120
BC	508	152	TKRYLPAIVREAIKR	40	98.25
	508	153	GLRTLIAPTRVVAA	100	
	508	200	AGIFMTATPPGSRDP	153	
	508	226	PFNMLKRERNRVSTVQQLTK	100	
BC	509	115	EIVDLMCHATFTMRL	100	84
	509	116	LSPVRVPNYNLIIMD	90	
	509	117	EAHFTDPASIAARGY	80	
	509	131	LDNINTPEGIIPSMF	80	
	509	172	TPEGIIPSMFEPERE	70	
BC	510	2	SGGSWVDIVLEHGSC	400	450
	510	66	FVEGVSGGSWVDIVL	500	