

Generation and characterisation of anti-dengue monoclonal antibodies in a case of severe primary dengue infection

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Abstract

Introduction

Dengue is one of the most important emerging infections with 50% of the world's population living in dengue endemic countries. The clinical spectrum of disease can range from asymptomatic to a severe haemorrhagic illness. Those with secondary infection with a second serotype are most at risk of severe disease. This has led to the theory that a primed immune response ineffectively clears a secondary infection and enhances infection. Teasing out immunology to dengue has become important in aiding effective and safe vaccine design. In this project, I investigated the antibody response of a case of acute severe primary dengue.

Methods

Activated B cells were isolated from a patient with acute severe dengue virus. Recombinant monoclonal antibodies (mAbs) were produced and investigated. The mAbs were assessed for: their ability to recognise dengue; epitopes to which they bind; which amino acids of the structural proteins they bind to.

Results

88 IgH-Ig λ and 44 IgH-Ig κ recombinant IgG antibodies were produced. 44 mAbs were investigated further. Seven of the mAbs showed reactivity to dengue, 3 reacting to dengue 1 alone, 3 had cross reactivity to all four serotypes and JE. Western blot revealed that no antibodies bound to prM or E. A viral like protein (VLP) library was created for further assessment of epitope binding.

Discussion

IgG was produced to dengue despite the early time point. Interestingly 43% (n=3) of antibodies were specific to the infecting dengue serotype, but a further 43% were reactive to all four dengue serotypes. The epitopes for these mAbs were not demonstrated. This may be due to changes to the E and prM proteins during the Western blot that mAbs recognise other epitopes or else the mAbs react to tertiary

structure epitopes. Use of the VLP library to identify the amino acid residues necessary for binding would help in understanding the epitopes.

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Contents

Abstract	2
Acknowledgments	3
Abbreviations	10
Introduction	12
Historical perspective.....	12
Dengue virus and transmission.....	12
Epidemiology and Public Health Significance.....	13
Dengue virus, its transmission and cell cycle.....	16
Dengue virus in the cell.....	20
Clinical Disease.....	26
Risk factors for severe disease	
Host determinants.....	29
Viral determinants.....	31
Immune response to dengue	
Innate immune response.....	32
Subversion of the cellular antiviral response by Dengue virus.....	34
RNAi.....	35
IFN α/β pathway abrogation.....	35
NK cell response.....	37
T cell immune response.....	38

Humoral Immune response.....	39
Monoclonal antibodies and what they had taught us.....	41
Summary.....	43
Hypothesis.....	50
Aims.....	50
Materials and Methods.....	51
Materials.....	51
Primers and vectors.....	51
Media and Buffers.....	52
Methods summary.....	54
Patient.....	54
Isolation of cells.....	54
Activated B cells are then sorted by flow cytometry	55
RT-PCR for immunoglobulin chains (heavy, lambda and kappa).....	56
Nested PCR for amplifying heavy and light chain V genes.....	56
PCR purification.....	56
Kappa chain sequencing.....	57
Cloning PCR for kappa chains.....	57
Expression vector cloning.....	57
Antibody expression by 293T cells.....	59
Anti-human IgG ELISA.....	61
DENV dot blot.....	61
DENV western blot.....	62

VLP alanine scan library production.....	63
Primer design and site directed mutagenesis.....	64
VLP expression by 293T cells.....	65
Concentration ELISA.....	65
Dot blot of VLP	66
Results	68
Isolation of activated B cells.....	68
Production of heavy and light immunoglobulin chains.....	68
Antibody expression by HEK293T cells and concentration measurement.	71
Identification of dengue specific antibodies.....	73
Dot blot of Recombinant antibodies.....	73
Western blot of dengue specific recombinant antibodies.....	75
VLP alanine scan library production.....	76
Discussion	81
Acute phase antibody response to dengue.....	82
Convalescent sera.....	83
Isolation of anti-dengue antibodies.....	83
Assessment of epitope binding of antibodies.....	84
prME VLP library and alanine scan.....	85
Summary.....	86
Further proposed work.....	86
References	88
Appendices	
Appendix 1. Dengue E protein amino acid reference sequences	95
Appendix 2. Papers published by C Roberts during MPhil.....	97

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Figures

Fig. 1. Average annual number of dengue fever and dengue haemorrhagic fever cases as reported to the WHO 1955-2007. Showing the precipitous increase in cases [1]	14
Fig. 2. Global evidence of risk and burden of dengue disease in 2010 [2].	15
Fig. 3. Phylogenetic tree of all 4 strains of DENV derived from the complete open reading frame from GenBank.....	17
Fig 4. Schematic representation of dengue genome.....	18
Fig. 5. Structure of the soluble E dimer and the mature virus particle [3].	20
Fig. 6 Structure of the 'bumpy' virus by cryo-EM [4].....	20
Fig. 7. Dengue trimer structure [3].....	22
Fig.8 Processing of dengue virus in the cell [5].....	25
Fig. 9 Association of prM and E proteins in the immature and mature viral particles [6].....	26
Fig. 10 Clinical disease course of dengue fever [1].....	27
Fig. 11 Suggested dengue case classification and warning signs [1].....	28

Fig. 12. Dengue virus lifecycle and the subversion of cellular antiviral response [7]	36
Fig. 13 Summary of mAb production. Adapted from [8]	60
Fig. 14. Diagram of VLP production. Adapted from [9]	63
Fig 15. Immunoglobulin chain nested PCR. 1% agarose gels demonstrating immunoglobulin chains isolated and amplified from single sorted antibody secreting cells.....	69
Fig 16. Vector ligation gel.	70
Fig 17. Recombinant human IgG concentration ELISA.....	71
Fig 18. Anti-dengue activity and serotype specificity of 53 recombinant antibodies.	74
Fig 19. Western blot assessing specific epitopes of recombinant human IgG.....	75
Fig 20. 3D structure of E protein dimer highlighting the C10 epitope (blue).....	76
Fig. 21. Southern blot confirming presence of VLP products produced by site direct mutagenesis.	77
Fig 22. Representative sample of sequencing results showing presence of alanine substitution.	78
Fig. 23 Expression of VLP prME constructs assessed by dot blot.....	81

Tables

Table 1. Summary of studies investigating monoclonal antibodies.....	45
Table 2. Nested PCR primer sequences.....	51
Table 3. Media recipes.....	52
Table 4. Primary and secondary antibodies.....	52

Table 5. Dengue virus strains used.....	53
Table 6. Dengue patient details.....	54
Table 7. In house anti- E and prM antibodies.....	66
Table 8. Recombinant human IgG concentration ELISA.....	72
Table 9. Anti-dengue activity and serotype specificity of 53 recombinant antibodies.	74
Table 10. Summary of VLP prME constructs and their expression by 293T cells as confirmed by dot blot.	79

Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
ADE	Antibody dependent enhancement
ASC	Antibody secreting cells
bnAbs	Broadly neutralising antibodies
C	Capsid protein
D1-4	Dengue 1-4
DENV1-4	Dengue 1-4
DF	Dengue fever
DHF	Dengue haemorrhagic fever
E	Envelope protein
EBV	Epstein Barr Virus
EDE	Envelope dimer epitope
FLE	Fusion loop epitope
H	Human
HCMV	Human cytomegalovirus
JEV	Japanese encephalitis virus
M	Mouse
mAb	Monoclonal antibody
MAVS	Mitochondrial antiviral signalling
MDA	Melanoma differentiation-associated gene
NK cell	Natural killer cell
NS 1-5	Non-structural proteins
NT	Not tested
ORF	Open Reading Frame

PBL	Peripheral blood leukocytes
PrM	Pre-membrane protein
RC	Replication complex
RdRp	RNA-dependent RNA-polymerase
RER	Rough endoplasmic reticulum
RIG	Retinoic-acid-inducible gene
SL	Stem loop
Ss	single stranded
TBEV	Tick-borne encephalitis virus
TGN	Trans-golgi network
TLR	Toll-like receptor
UTR	Untranslated region
VLP	Virus like particle
WNV	West Nile Virus
WT	Wild type
YFV	Yellow fever virus

Introduction

Historical Perspective

Dengue is a flavivirus transmitted by *Aedes* mosquitoes and is endemic throughout tropical and sub-tropical regions of the world. The first record of dengue is from a Chinese medical encyclopaedia originally published in the Qin Dynasty (265-420AD). It was referred to as 'water poison' and was associated with flying insects [10]. Historical epidemics resembling dengue have been documented in the 17th century in the West Indies and Central America. [11] [12] By the 1800s, the disease was widespread throughout urban tropical coastal cities due to the shipping trade [11]. World War II resulted in rapid global expansion in dengue transmission due to population dispersal, troop movements and social disarray. [13] Later in South America, the yellow fever disease control campaign, which saw widespread DDT spraying, resulted in near elimination of dengue. However, once the campaign was discontinued in the 1970s, dengue saw a resurgence and by 1995, had returned to pre-campaign levels.

Dengue virus and transmission

Dengue is an arbovirus and a member of the flavivirus genus. Other closely related members of the flavivirus family include Japanese encephalitis (JEV), yellow fever virus (YFV), West Nile virus (WNV) and tick borne encephalitis virus (TBEV).

Humans are the main amplifying host for dengue virus, although non-human primates may rarely be infected. The virus is transmitted from viraemic humans to female *Aedes* mosquitoes during a blood meal. The principal mosquito is *A. aegypti*, however *A. albopictus* may also transmit dengue. These are day biting mosquitoes that breed in both clean and stagnant water and are readily adapted to urban environments. They are able to breed in small pools of water in domestic settings, such as house plants, guttering or open water butts. Female mosquitoes also preferentially feed and rest indoors further improving their disease transmission efficiency. [14]

Once the mosquito is infected from a blood meal, the virus infects the midgut of the mosquito, then spreads systemically over 8-12 days, at which point it infects the salivary glands (and remains there for the duration of the mosquitoes' life) and becomes infectious to humans. [1]

Epidemiology and Public Health Significance

There has been a precipitous increase in dengue incidence over the last 50 years and dengue is now one of the most important emerging global diseases [1] [Fig. 1]. It has expanded geographically and approximately 50% of the world's population are living in dengue endemic countries [11]. There are approximately 390 million dengue infections worldwide annually, with 96 million that are clinically manifest [2] [Fig. 2].

Approximately 75% of the world's dengue burden is in South Eastern Asia and Western Pacific regions. [1] However, 2012 saw large outbreaks throughout South America. A number of small outbreaks over recent years have occurred in sub-tropical regions,

namely Madeira 2012, Florida 2013 and Tokyo 2014. [15] [16] [17] [18] Concern over global warming has suggested that Aedes mosquitoes may be able to survive in new regions raising concerns that spread to sub-tropical areas may be permanent. [19]

Fig. 1. Average annual number of dengue fever and dengue haemorrhagic fever cases as reported to the WHO 1955-2007. Showing the precipitous increase in cases [1].

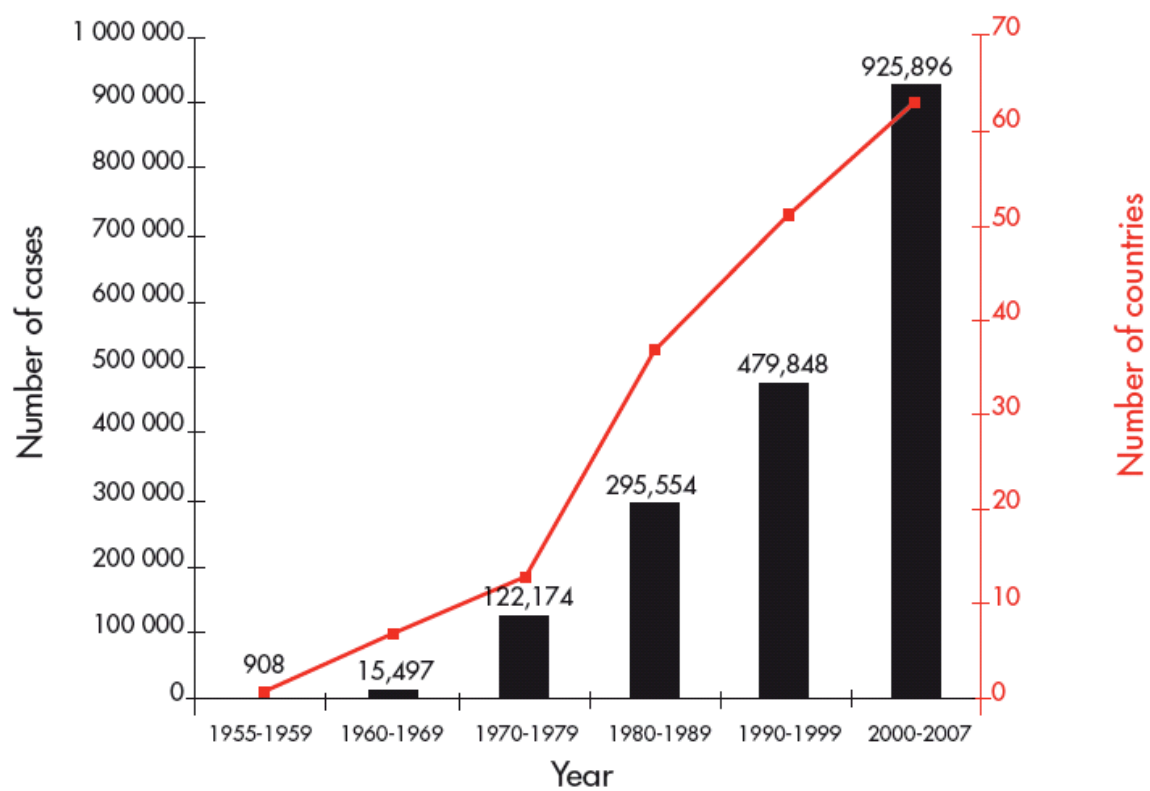
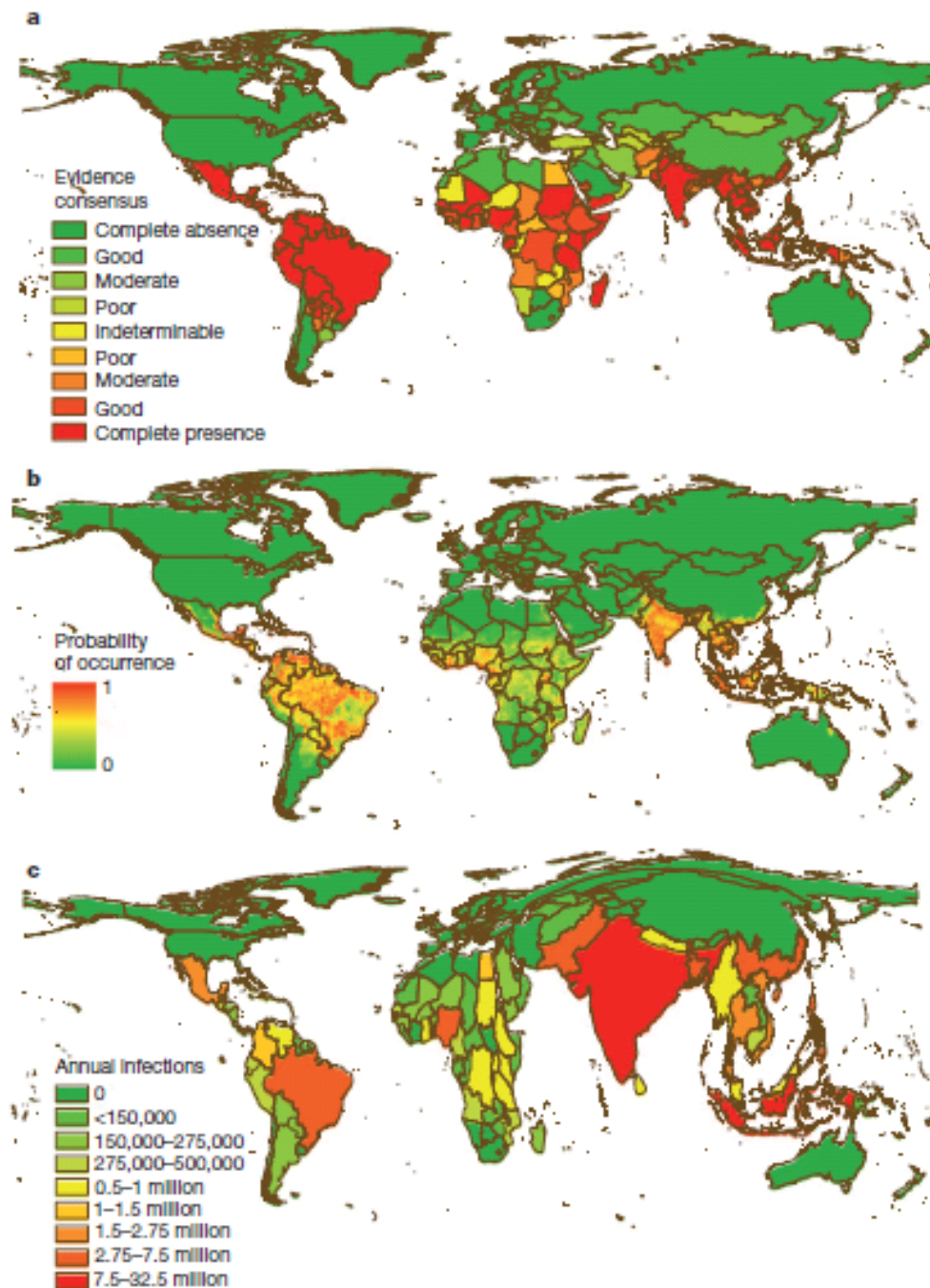


Fig. 2. Global evidence of risk and burden of dengue disease in 2010 [2]. A. national and subnational consensus on data available for dengue infections. B. Assessment of probability of dengue occurrence at 5km x 5km spatial resolution. C. cartogram of annual number of infections for all ages as a proportion of national or subnational geographic data.



Dengue virus, its transmission and cell cycle

Dengue virus (DENV) is a small icosahedral enveloped virus with a single stranded (ss) positive sense RNA genome. There are four serotypes 1-4, each serotype being composed of 3-6 genotypes. [20] The serotypes possess 67-73% identity at the nucleotide level, and 69-78% identity at the amino acid level. Phylogenetic studies suggest that dengue may have emerged from a sylvatic virus. Serotype diversity may have occurred as populations became isolated and the viruses genetically diversified.

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Fig. 3 Phylogenetic tree of all 4 strains of DENV derived from the complete open reading frame from GenBank. [21]

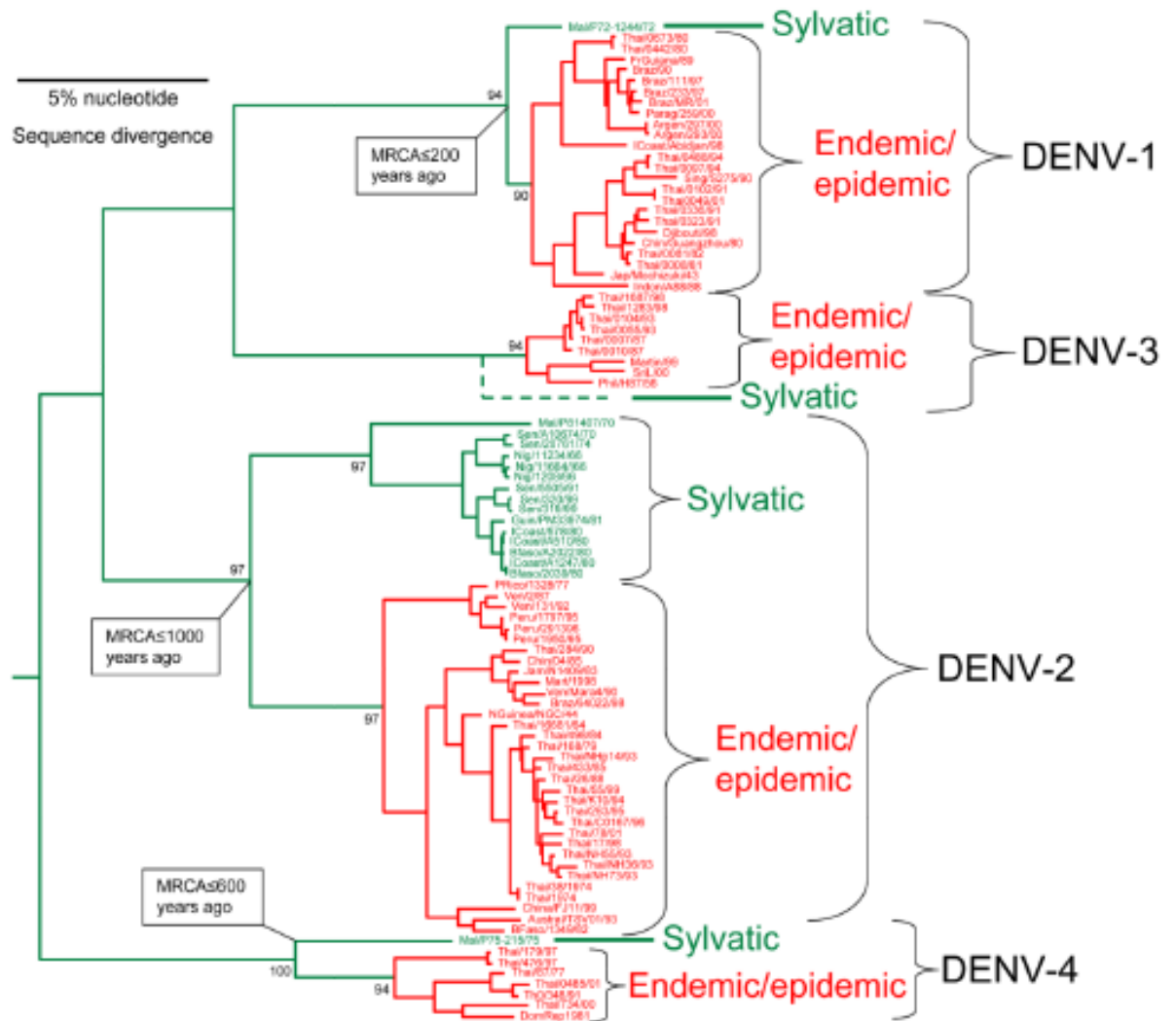
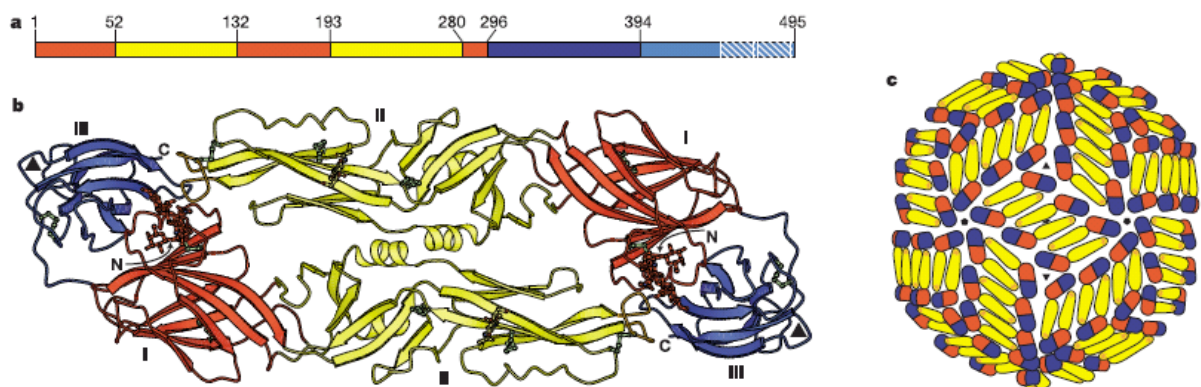


Fig. 4. Schematic representation of dengue genome with proposed functions of the proteins

Structural				Non-structural					
C	prM	E	NS1	NS2A	NS2B	NS3	NS4A	NS4B	NS5
C	Capsid								
	Signal peptides for translocation to ER and nuclear localisation								
prM	E protein chaperone during exocytosis								
	Prevention of premature fusion								
E	Receptor binding and fusion activity								
	Major target of neutralising antibodies								
NS1	May define pathogenesis								
	Possibly interacts with NS4A								
NS2A	Involved in interferon signalling (with NS4A and NS4B)								
NS2B	Cofactor of NS3								
NS3	Protease								
	Helicase								
NS4A	Probably involved in RNA replication								
	Induced membrane rearrangements								
NS4B	Blocks IFN-induced signal transduction cascade								
	Interacts with NS3 and dissociates it from ssRNA								
NS5	Methylation of viral RNA cap								
	Possesses nuclear export signal								
	Reduces expression of STAT2								

The structure of the mature dengue virus particle propagated at 30°C has been shown to be an icosahedral structure made up of 90 E protein dimers in a herringbone pattern. This creates a smooth surface. Each E protein is 495 amino acids long and is made up of a C terminal transmembrane region and an ectodomain of three regions, DI, DII, DIII. DIII has an immunoglobulin like structure and protrudes from the surface, whereas DII is key in membrane fusion [3] [Fig. 4]

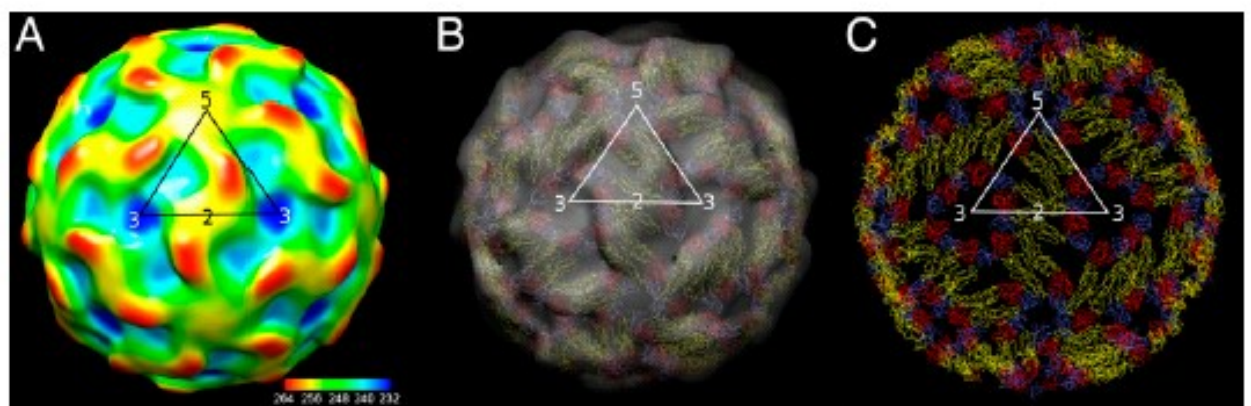
Fig. 5. Structure of the soluble E dimer and the mature virus particle [3]. A. shows the structure of the E protein with Domain I as red, domain II yellow and domain III as blue. B. Shows the conformation of the E protein in the mature virus particle. C. shows the packing of the E protein on the surface of mature virus particle.



The prM protein consists of an N terminal pr region, a furin cleavage site followed by the M protein. The M protein is made up of α helix stem and two transmembrane regions.

The immature viral particle is larger at 600A, as compared to the mature size of 500A, remains an icosahedral shape, but is spiky. There are 60 spikes, which are made up of quasi-trimers of 2 E proteins and an uncleaved precursor-Membrane (prM) protein [23]. Recent studies have found that mature viral particles propagated at human physiological temperature, 37°C (and indeed temperatures higher than 33°C) are 'bumpy' with the E protein rotated 60° [24] [Fig. 5].

Fig. 6 Structure of the 'bumpy' virus by cryo-EM [25]. The structure of the high temperature bumpy virus. A. cryo-EM of the bumpy virus coloured by the radial distance from the centre of the virus. B. E dimers fitted into the electron density map. C. The structure of the bumpy virus particles, DI red, DI yellow, DII blue. Compare with figure 4 where the particle is fully mature.



Dengue virus in the cell

In humans, the initial target cells are thought to be immature dendritic cells [26]. Other cells thought to be infected are monocytes, macrophages, and possibly Kupffer cells, hepatocytes and lymphocytes [27, 28]. It is thought that numerous negatively charged glycoaminoglycans such as heparan sulphate form low affinity attachments with

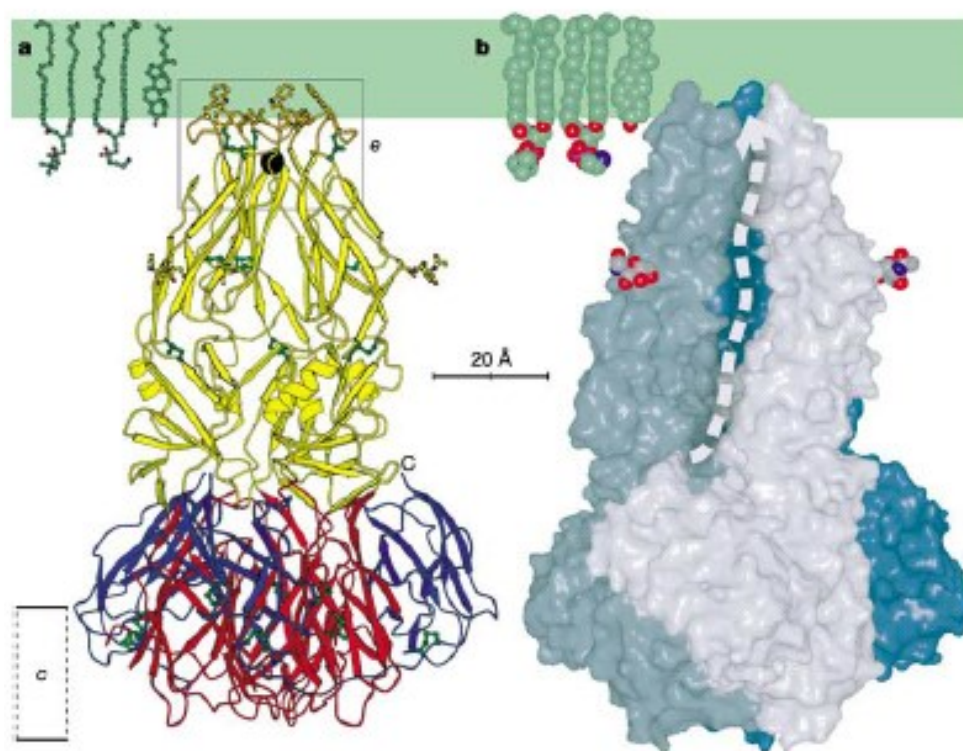
domain III of the E protein. Other receptors implicated in virus attachment are heat shock proteins 70 and 90, neolactotetrasylceramide, CD14, GRP78/BiP, 37kDA/67kDA laminin, and C-type lectins such as DC-SIGN, the mannose receptor, and C-type lectin domain family 5 [29].

Dengue virus particles have been shown through single track analysis to diffuse along the cell surface to clathrin coated pits. It is through the clathrin pits that the particles are endocytosed. The pits bud and invaginate, developing into a clathrin coated vesicle closed by membrane scission by dynamin. Alternative methods of cell entry have been documented in mammalian cells, via lipid rafts and caveolae. The virus containing endocytic vesicles then lose their clathrin and are delivered to early endosomes. These mature into late endosomes. It is from the late endosomes that virus is delivered into the cytosol by membrane fusion. Membrane fusion occurs at 10-13 minutes after cell infection [29].

There are a number of mechanisms by which membrane fusion occurs, low-pH induced conformational change of the E protein, high cholesterol levels, and use of negatively charged lipids. Endosomes have a low-pH; at pH 6.2-6.4 a conformational change occurs in the E protein. Protonation of histidine residues are thought to destabilise the DI-DII interface. This causes dissociation of E protein rafts and the dimers separate into monomers. DII projects outwards exposing its fusion loop to the target membrane. Three E proteins form trimers which once stabilised through DI interactions, allows DII to fold against the trimer into a hairpin [Fig.6]. The energy released through this process causes hemifusion of the outer membrane monolayers with inner membranes intact. Following this a fusion pore is formed and the viral capsid is released into the

cytosol. It appears that the fusion process is promoted by high cholesterol endosomal membranes, by contributing to the low pH environment, but also it seems that DENV can interact with a number of lipids. Anionic lipids have been shown to improve the efficiency of membrane fusion [29].

Fig. 7. Dengue trimer structure [3] a. Ribbon diagram showing domain I in red, domain II in yellow and domain III in blue. Surface representation of the trimer, with the dashed grey line indicating the most likely position for the stem.



Once released into the cytosol, the nucleocapsid disassembles and the genome is transported to the rough endoplasmic reticulum (RER). The precise mechanisms of how

this is achieved has not been elucidated. The liberated viral genome has three interdependent functions: translation, acting as a mRNA for synthesis of the viral polyprotein; replication, acting as a template for the synthesis of further RNA strands; and encapsidation, incorporation into new viral particles. Translation commences at the 5' AUG codon with the viral cap recruiting the required eukaryotic initiation factors and ribosomes. However, capped mRNA is not critical for translation, which suggests that cap independent mechanisms of translation may facilitate viral amplication in divergent hosts (i.e. mosquitoes and humans). The 3' stem loop (SL) is able to stimulate translation in the absence of viral proteins. Thus it is key to promoting the first round of translation. The details of further translation have been poorly established. Following translation, a complex of NS3 and its co-factor NS2B act as viral serine protease, processing other NS proteins required for replication [23].

Following translation, the viral NS proteins interact with host factors to form the Replication Complex (RC). This results in the production of complementary negatively stranded RNA, resulting in an intermediate double-stranded RNA, and finally new (+)RNA. This (+)RNA then goes on to function both as mRNA and genomic RNA for the viral progeny. Circularization of viral RNA allows coupling of translation and replication and occurs on induced cell membranes derived from the ER. Of the NS proteins, NS2A seems to direct the RCs to membrane organelles where replication takes place. NS3 and NS5 form a complex. These act as an ATPase/helicase and an RTPase, they unwind and dephosphorylate the genomic material prior to 5' capping. NS5 acts as the RNA-dependent RNA-polymerase (RdRp). RNA synthesis is regulated by sequences within the 5' and 3' UTRs, the 5'-3'CS and 5'-3'UAR: UAR, CS and cHP are involved with

circularization and promotion of RNA synthesis by acting on RdRp. The functions of other NS proteins are still being elucidated. Their proposed functions are detailed in fig. 3 [30].

Following polyprotein synthesis and genome replication, encapsidation occurs. ER derived organelles contain viral RNA-C protein complex and the structural proteins E and prM. In immature particles, E and prM proteins are complexed in trimers. During virion egress, the particles mature and the E protein forms homodimers. During egress the particles pass through the trans-golgi network (TGN) where the prM proteins are cleaved by the proteinase furin, stimulated by a low pH environment. pr remains associated with the particle until cell release via exocytosis as this prevents premature binding of the E protein to the exosomal membrane of the TGN during transit [Fig. 8]. A mature virion is composed of one copy of RNA, 90 homodimers of E protein, and 180 copies of M protein. Cleavage of the prM in the TGN causes the transition from immature virus (spiky particles) to infectious mature virus (smooth particles). However, cleavage of prM is inefficient and a large number of immature particles are produced [29].

Fig.8 Processing of dengue virus in the cell [31]. 1. Fully mature and some partially mature infectious viral particles attach directly to a diverse array of host receptors. 2. Virus:immune-complexes may also attach via Fc receptors. 3. Receptor mediated endocytosis. 4. Acidification of the endosomal vesicle induces conformational change in the virion and trimerisation of the E protein resulting in release of the nucleocapsid into the cytoplasm. 5. Viral RNA is translated into a single polyprotein at the ER. 6. RNA synthesis of anti-sense viral RNA. 7. Newly synthesised RNA is packaged by the capsid © protein forming the nucleocapsid. 8. The nucleocapsid buds into the ER forming a non-infectious immature particle. 9. Immature viral particles are transported through the Golgi where acidification causes exposure of the furin cleavage site. Host furin cleaves the pr and M proteins. The now mature viral particle is then exported.

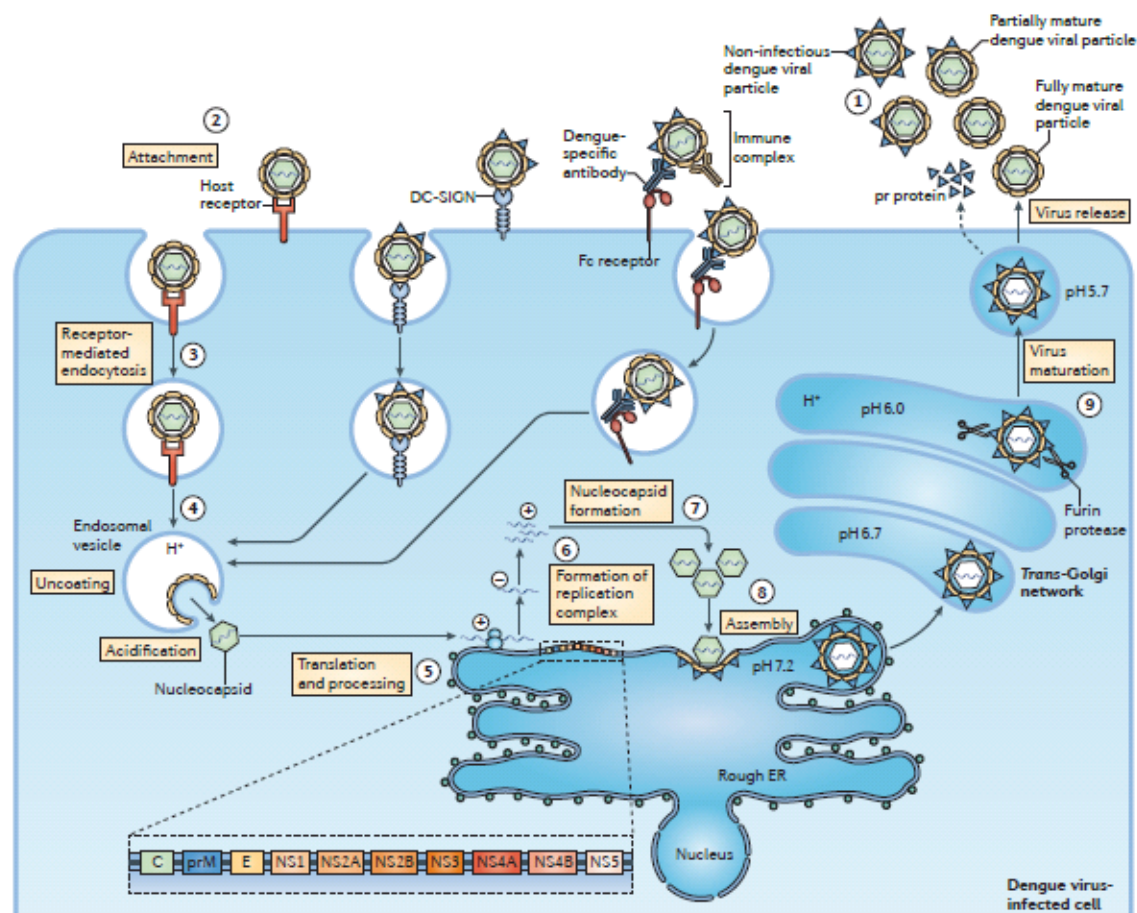
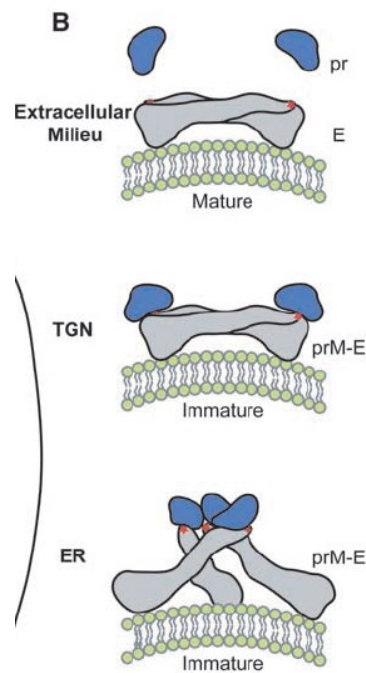


Fig. 9 Association of prM and E proteins in the immature and mature viral particles [6].

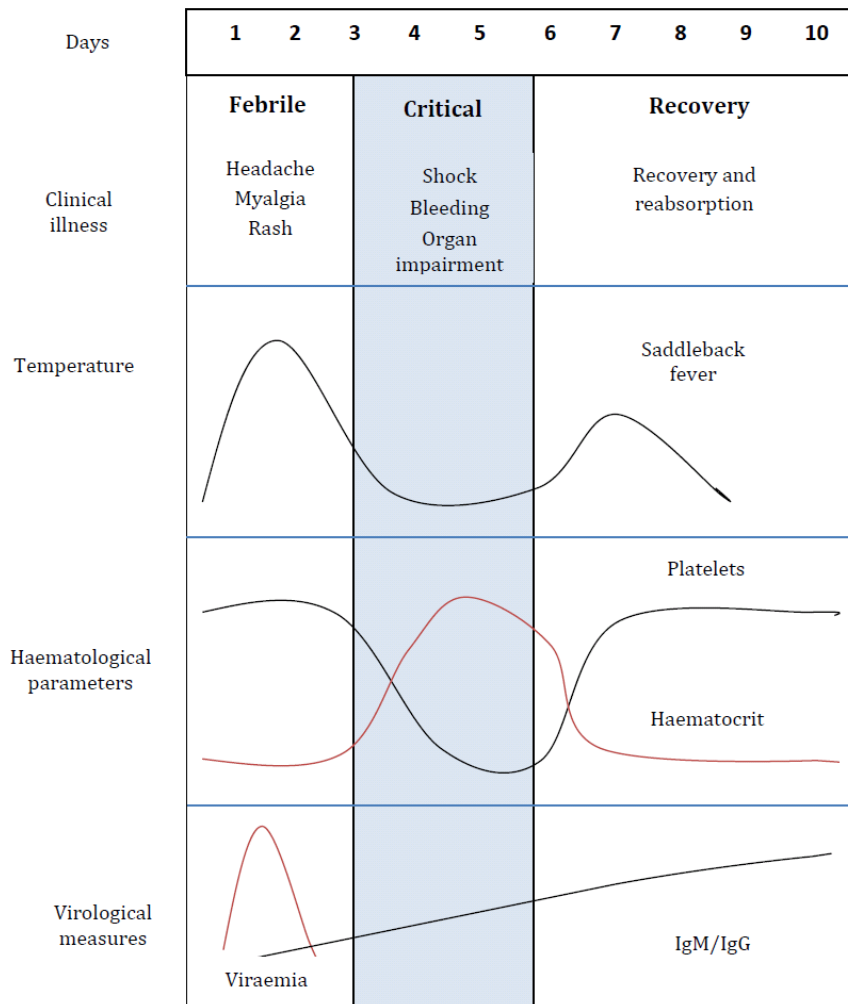
Configuration of the surface viral proteins during maturation of the viral particle. The fusion loops are indicated by red stars.



Clinical Disease

The clinical spectrum of dengue is wide, with most infections being asymptomatic (approximately 75%) [19]. Approximately 5% of infected patients develop severe disease, predominantly affecting children and young adults. After an incubation of 4-10 days, severe disease has three phases, febrile, critical and recovery [fig. 9] [1].

Fig. 10 Clinical disease course of dengue fever [1]. The diagram shows how the viraemic phase of the clinical illness is accompanied by fever, but severe illness develops after the viraemia is cleared.

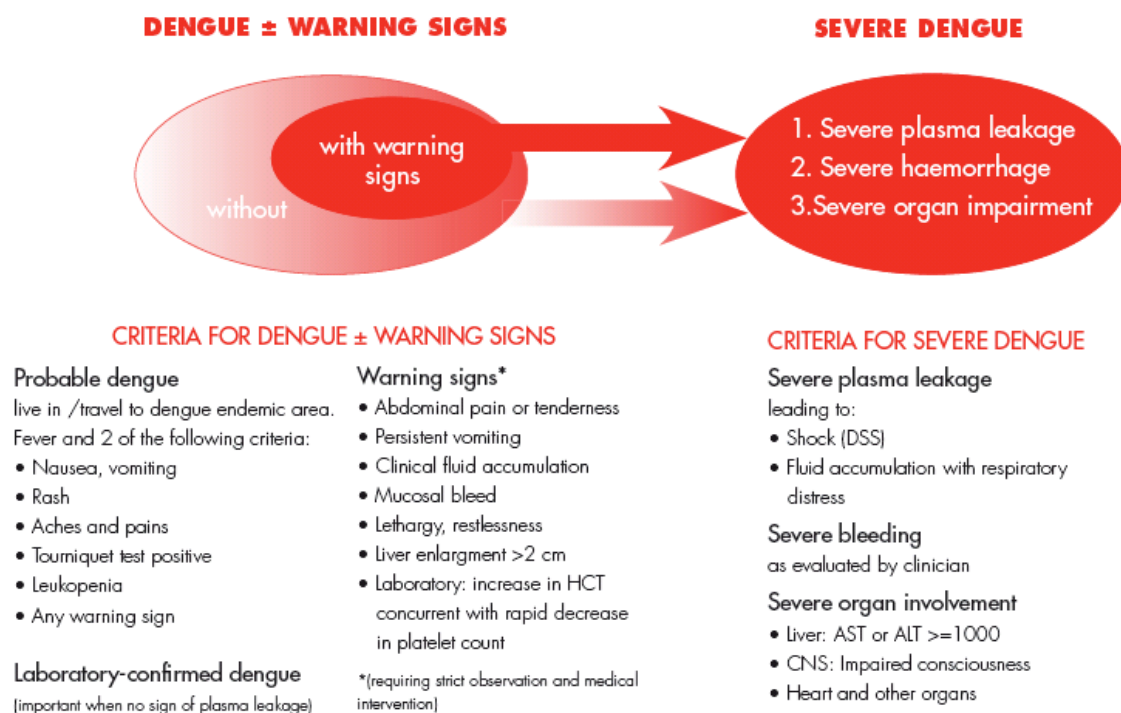


Adapted from WHO guidelines [WHO (WHO, 2009a)] page25

The acute febrile phase, which constitutes the majority of clinical cases, usually lasts 2-7 days and is accompanied by headache, myalgia, arthralgia, retro-orbital pain, and a generalised maculopapular/ petechial rash. At this stage clinical features are indistinguishable between severe and non-severe forms of the disease. Patients may experience hepatomegaly with raised transaminases, leukopenia and

thrombocytopenia. For those who develop severe disease, the critical phase usually occurs between days 3 and 7 and coincides with the normalisation of fever. The onset of severe disease is usually marked by an increasing haematocrit and rapidly falling platelet count. This phase is characterised by a 24-48 hour period of significant vascular leak resulting in shock. Once shock is established, the patient may experience severe blood loss with a concomitant fall in haematocrit, hypothermia, organ impairment and metabolic acidosis. Other manifestations may include hepatitis, encephalitis and myocarditis. Severe dengue is defined by one or more of i) plasma leak that may lead to shock and/or fluid accumulation with or without respiratory distress, ii) severe bleeding, iii) severe organ impairment. If the patient survives the critical phase, gradual re-absorption of the extra vascular fluid occurs over the next few days [1].

Fig. 11 Dengue case classification and warning signs [1]



Risk factors for severe disease

Dengue pathogenesis is the result of a complex interplay of viral and host factors.

Development of severe disease is modulated by a range of factors including preceding dengue infection, age of the host, genetic background of the host, and viral serotype and genotype.

Host determinants

Experiments conducted in healthy volunteers suggest that infection with one serotype confers immunity to that serotype for up to 18 months, but is anticipated to be life-long. However, cross-protective immunity to other serotypes is transient, lasting less than 2 months. Indeed secondary infection is the single most important risk factor for severe disease, suggesting that this transient heterologous immunity not only deteriorates, but also contributes to disease pathogenesis. From epidemiological studies, we know that the incidence of severe disease is much higher in secondary infection with second serotype than primary infection. In a Cuban study, of all the cases of DHS/DSS, 95% were from secondary infection and 5% were from primary infection. This study also showed that infants within the first 6 months of life are at higher risk of severe infection than children older than 6 months of age [32]. It is postulated that maternal antibodies present in infants creates a scenario where infantile infection mimics the phenotype of secondary infection. Antibody responses therefore seem to play an important role. This shall be discussed further in the section on humoral immune response.

In Cuba, distinct epidemic waves have been carefully documented. It was shown that those of African lineage were of decreased risk for severe disease than those of European ancestry [33]. This has been corroborated in a number of other Caribbean settings [34] [35]. A number of genetic studies have highlighted associations with severe disease. Genes identified include: HLA molecules, cell receptors (type 1 interferons, vitamin D receptor (VDR), intracellular adhesion molecule 3, ICAM3, DCSIGN), and blood antigens (platelet antigens and ABO) [36] [37] [38] [39] [40] [41]. Of the HLA polymorphisms identified, few have been verified across different populations. However, HLA-24 has been associated with severe disease in both Vietnam and Malaysia [42] [43]. It is felt that the HLA polymorphisms are associated with differing T cell induced immunopathology. DCSIGN polymorphisms may have a role in disease phenotype as this molecule is a major DENV receptor on dendritic cells. A single-nucleotide polymorphism, (SNP) rs4804803, with GG/AG (as opposed to AA) was associated with DHF, increased DCSIGN expression and increased cytokine production. However counter-intuitively, it was also associated with lower DENV2 viral replication. This was reversed by addition of anti-IP10 antibody suggesting that cytokine production may interfere with viral replication [40].

In a study from Vietnam, SNPs at two loci were found to have genome wide associations with severe dengue disease. These are MICB, Major Histocompatibility Complex (MHC) class I polypeptide sequence B and PLCE1, phospholipase C epsilon 1. The MICB gene encodes an inducible activating ligand for the NKG2D type II receptor on NK cells and CD8 T cells. The authors postulate that this SNP might be associated with a dysfunctional response of NK and or CD8 T cells leading to a higher viral burden and an

increased chance of severe disease. Mutations in the PLCE1 gene are also seen in nephrotic syndrome, where changes to the glomerular basement membrane of the kidney result in proteinuria. It is suggested that perhaps a SNP in the PLCE1 gene may cause changes to the vascular endothelium and thus result in plasma leak [44].

A number of studies have identified other possible risk factors for the development of severe disease. These include extremes of age, female gender, ethnicity and co morbidities such as diabetes and hypertension [45].

Viral determinants

In many parts of the world there is hyper-endemic transmission of dengue with all four serotypes co-circulating. Consequently secondary infection, particularly in these countries, probably occurs in excess of documented severe disease. Epidemiological studies have shown that severe disease may occur in individuals with primary infection [32]. Tertiary and quaternary infections have been shown to be responsible for severe disease, in Thailand 0.08-0.8% of cases [46], where as in Cuba they account for 17.5% of severe cases [47].

There are a number of viral determinants, which are felt to contribute to severe disease. Dengue serotypes 2 and 3 are more often linked with severe disease than 1 and 4 [48]. Particular genotypes have been shown to cause severe outbreaks. The most notable example is of the serotype replacement in Cuba of the benign Latin American DEN2 strain with the Asian strain in 1981 and a subsequent increase in the number of severe

cases. This correlates with a number of laboratory findings: the Asian strains replicate to higher viral titres in dendritic cells [49], they also infect and disseminate to the head of *A. Egypti* mosquitoes more rapidly and in greater proportion than the American strains [50]. Genetic analysis of the 2 Asian strains has revealed 6 amino acid changes within the prM, E, NS4 and NS5 genes [51]. Mutations within E and NS3 can alter viral virulence, perhaps by improving host cell binding, therefore improving viral replication efficiency [52]. In Nicaragua, the replacement of DEN2 clade N1 with clade N2, saw an increase in the number of severe cases. The new clade was demonstrated to have greater replication capacity *in vitro*, and higher viraemia in patients' sera. Interestingly, waning immunity to DENV1 predisposed to severe disease in those infected with N1 clade DENV2, whereas waning immunity to DENV3 predisposed to severe disease in N2 clade DENV2 [53].

Immune response to dengue

Innate immune response

Dermal Langerhans cells (Dendritic cells, DC) are thought to be the initial target cell after inoculation by an infected mosquito. The DCs are then activated and migrate to regional lymph nodes [54] [30] [55] [26] [56]. The activated DCs then elicit a $\text{IFN}\alpha/\beta$, a $\text{TNF}\alpha$ and a pro-inflammatory response. In the regional lymph nodes, viral replication continues and spreads to as yet undefined cells of the macrophage/ monocyte lineage where it amplifies [57] [58] [59] [60] [61] [62]. Viraemia follows which may facilitate spread to secondary organs such as liver, spleen and bone marrow.

Much of the first line of defence against dengue comes from the pattern recognition receptors such as Toll-like receptors (TLRs), notably TLR-3, TLR-7, TLR-8, and intracellular sensors (e.g. DExE/H box RNA helicases) [63]. These recognise dsRNA, ssRNA and modified RNA. TLR-3 is the dominant TLR responder and recognises DNV RNA after endosomal acidification. It induces strong IL-8 and interferon α/β (IFN α/β) responses [64]. Cytoplasmic helicases, such as retinoic-acid-inducible gene 1(RIG-1) and melanoma differentiation-associated gene 5(MDA5), sense dsRNA in the cytoplasm [65] [66]. These sensors are able to differentiate between viral and host RNA (as ribose 2'-O methylation is only present on host RNA), then signal through the mitochondrial antiviral signalling (MAVS) protein on mitochondria and eventually induces the transcription of IFN α/β [67, 68] [69]. RIG-1 and MDA5 synergise with TLR-3 to limit DENV replication *in vitro* [66].

IFN α/β are able to act in an autocrine manner causing induction of cellular antiviral responses, as well as acting on neighbouring cells to warn them of the infection. IFN α/β acting through IFNAR (IFN α/β receptor) activate the JAK/STAT pathway resulting in production of numerous antiviral proteins and proinflammatory cytokines [70] [71]. The IFNAR signalling also induces alternative signalling pathways such as mitogen activated protein kinase p38 cascade and the phosphatidylinositol 3 kinase cascade that cause the production of proinflammatory cytokines and chemokines [71].

TLR-3 and cytoplasmic helicases can also induce NF κ B, which activates the inflammasome causing production of interleukins 1 and 18. These cytokines recruit immune cells to the site of infection [72].

Another method of intracellular defence is via small RNAs (e.g. miRNAs). miRNAs interact with proteins in the RNA interference (RNAi) pathway and regulate as well as being induced by TLRs and IL-1 signalling [73].

The cell has an array of defense mechanisms it uses against dengue virus which includes TLR induction of IFN α/β , IFN α/β induction of the JAK/STAT pathway, induction of the inflammasome and RNA interference.

Subversion of the cellular antiviral response by Dengue virus

DENV is able to evade the hosts innate immune response by sequestration and by active subversion of the immune response. Dengue virus replicates within the endoplasmic reticulum (ER) of cells, whilst there, it is able to rearrange and expand the ER through the action of NS2B/3 on X-box binding protein 1 [74]. DENV is also able to reduce the unfolded protein response (UPR) to stress, which may prevent or delay host cell death during viral replication [74]. It appears that dengue is also able to subvert autophagy and lipid metabolism. Autophagy is the process by which cytoplasmic contents are degraded. Cytosolic double-membrane vesicles, autophagosomes, engulf cytosolic elements and transport them to lysosomes where they are degraded. Dengue, along with many other positive-stranded RNA viruses is able to hijack autophagosomes and use the machinery to enhance viral replication [75]. It seems that DENV NS3 protein is able to increase fatty acid synthesis [76], the lipid droplets then appear to be used by C protein as a scaffold for infectious viral particle formation [77].

RNAi

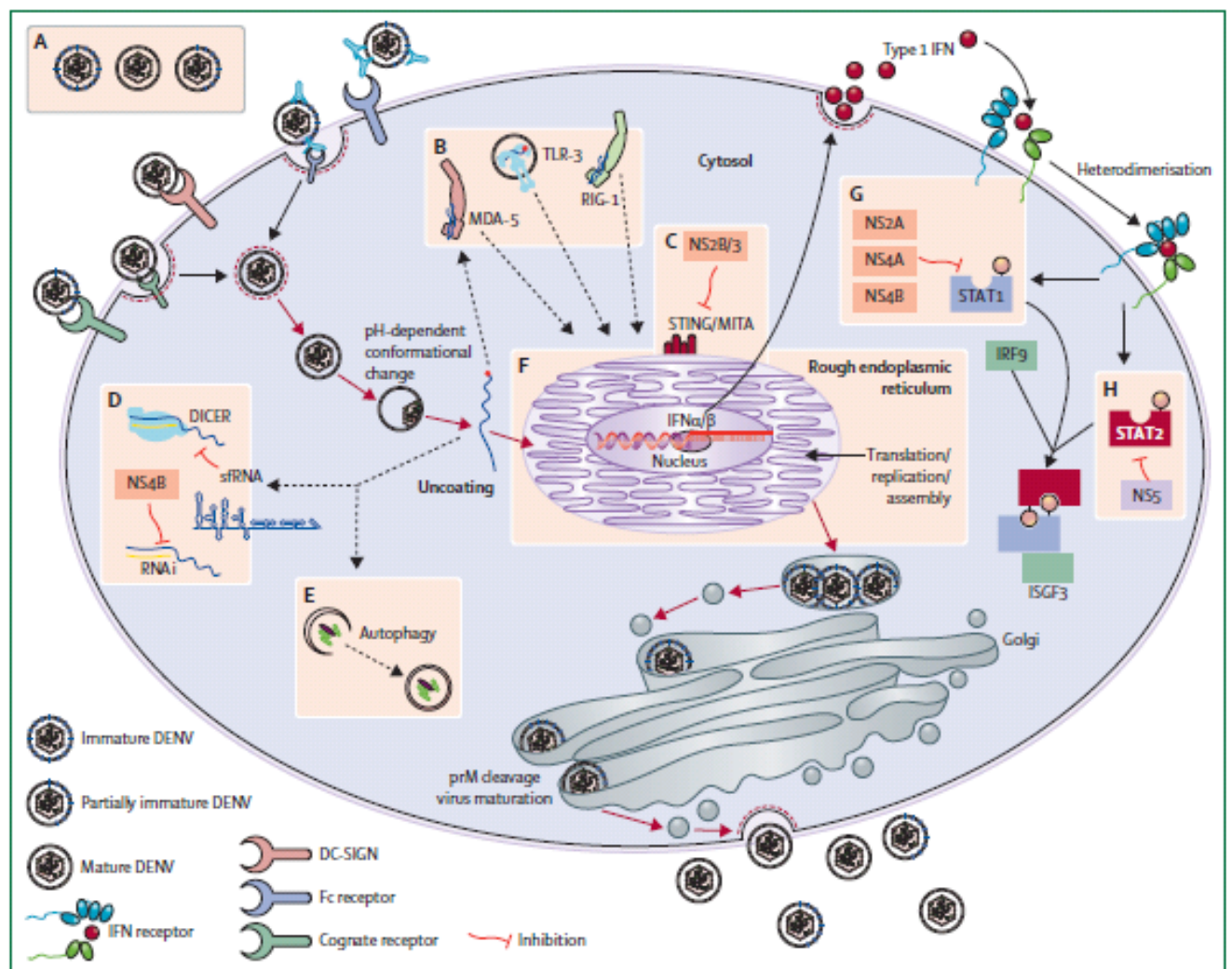
DENV is able to interfere with RNAi pathways through two mechanisms. NS4B directly interferes with Dicer's ability to process small RNAs *in vitro*. [73] In addition, sub-genomic flavivirus RNA (sfRNA), produced from the viral 3' UTR, is able to inhibit cleavage of dsRNA by Dicer by binding to and saturating the RNase [78].

IFN α/β pathway abrogation

Dengue is able to act in multiple ways to interfere with the induction and signalling of IFN α/β . Firstly it is able to evade MDA5 recognition of the viral mRNA cap by its 2'-O methylation [79] [80] [81]. Next DENV is able to interfere with the downstream signalling from RIG-1 and MDA5, thus downregulating the antiviral response through NS2B/3 cleaving MITA/STING [82] [83] [84]. DENV has several ways to interfere with the JAK/STAT pathway. NS2A, NS4A and NS4B inhibit STAT1 phosphorylation and nuclear translocation [85]. DENV is also able to inhibit IFN α/β signalling by triggering the degradation of STAT2 [86]. Indeed, inhibition of IFN α/β and JAK/STAT pathway results in increased viraemia in animal models. This is corroborated by clinical studies showing that a decrease in the number of plasmacytoid dendritic cells (that are a major source of type 1 IFNs), thus mimicking the blockade of IFN production, is associated with an increase in disease severity.

Studies into the overall effect of dengue virus on DCs show that infected DCs undergo apoptosis, express a less mature phenotype than surrounding uninfected DCs, produce IL-10 (down regulates inflammatory cytokines), and have reduced T cell stimulatory capacity [87]. Quite what the cellular mechanisms of this are is not yet known.

Fig. 12. Dengue virus lifecycle and the subversion of cellular antiviral response [7]. Red arrows indicate the dengue virus lifecycle. Tan squares show the subversion of the cellular antiviral response. A. Viral maturity can affect infectivity and antibody binding. B. In ADE, infection can disrupt TLR, RIG-1, and MDA signalling cascades leading suppression of IFN α/β antiviral responses. C. NS 2B/3 cleaves MITA within interferon pathway to downregulate antiviral responses. D. Subgenomic RNA and NS4B interfere with RNAi response. E. Dengue induced autophagy might enhance viral replication. F. Dengue induces ER hypertrophy. G. NS2A, NS4A and NS4B expressed together can inhibit STAT1 phosphorylation and translocation into the nucleus. H. NS5 interferes with IFN α/β signalling by mediating STAT2 degradation via the proteasome.



NK cell response

NK cells seem to play a role early in infection to limit dengue replication. In adults with mild disease, high number of NK cells along with increased expression of activation markers (CD69, CD38, HLA-DR), adhesion molecules (CD11a, CD44), and markers of cytotoxic granules (TIA-1) were seen. This contrasts to those with severe disease, who had reduced number of NK cells [88]. However, in children, it was shown that NK cytotoxicity was increased in patients with severe disease in comparison to those with mild disease [89] [90]. In the immunocompetent mouse model A/J, early activation of NK and B cells was associated with control of viral load and prevention of disease [91] [92]. In experiments in C57BL/6 mice, it was shown that NK cells have site specific viral clearance [93]. In particular they showed that early recruitment of NK cells to the liver was needed for viral clearance [94]. However, this came at the expense of hepatic cell death [95].

Natural killer cells have also been implicated in antibody-dependent cell-mediated cytotoxicity (ADCC). ADCC activity in plasma prior to secondary infection (with either DEN-2 or -3) correlated with serotype specific neutralising antibody titres, anti-DENV IgG1 levels, and a multitypic PRNT₅₀ pattern [96]. However, this did not necessarily correlate clinically. Although high ADCC preceding secondary DENV-3 was associated with lower viraemia, this was not seen with secondary DENV-2.

These studies suggest that NK cells are involved with early control of viraemia, may be involved with cell death related to this control, and may have a role in ADCC.

T cell immune response

The role of T cells in the immunopathology of dengue remains controversial. Severe dengue occurs as the virus load falls and cytokine levels increase. This has led to the hypothesis that T cells may drive the immunopathology of severe dengue. T cell responses have been identified in both mice and humans. T cell epitopes have been investigated and it has been shown that the predominant CD8 T cell epitopes are NS3 and NS5. These are the largest proteins encoded by the dengue genome and are located in the cytoplasm. This may lead to preferential processing and presentation [97].

In acute dengue infection, CD8 response is primarily directed against NS1 and NS5 dengue proteins, whereas CD4 T cells mainly recognise NS1, E and C proteins [98] [99] [100] [101]. Despite this evidence, studies into the kinetics of CD8 responses to acute infection showed that levels were absent or very low during the critical phase of the illness and peak 2 weeks after the critical phase has passed [102]. Massive T cell apoptosis occurs in acute dengue and appears to be greatest in those who have severe disease [103] [104]. However, T cells may be sequestered in tissues during the acute phase of infection.

T cell function varies considerably between mild and severe dengue. In mild dengue, CD8 T cells have more degranulation and lower production of inflammatory cytokines. However in severe dengue many CD8 cells produce the cytokines $\text{TNF}\alpha$ and $\text{IFN}\gamma$, but fail to display degranulation markers. T cell function studies show that the proliferation capacity, cytokine production and gene expression show that these functions are impaired during severe disease and recover during convalescence. [105, 106] [107].

The specific role of T cells in the immune response has yet to be established. The intense and skewed initial response to infection could be responsible for driving severe disease. However, T cells may control viral replication through protective HLA alleles, but this evidence is far from conclusive.

Humoral Immune response

The make-up of the antibody response has been studied. In acute primary infection, IgM response initially dominates, appearing 4-5 days after onset of the illness and lasting for approximately 3 months. IgG is produced from about 1 week after onset of illness, peaking after several weeks, then declining to low levels but persisting for decades.

Dengue mainly induces IgG1 and IgG3 responses (which are the subclasses with highest affinity to FcR). The antibody response in secondary infection is derived from naïve and memory B cells generated in primary infection. In secondary infection, there is a rapid IgG response, which commences on the first day of illness. The magnitude of antibody response is much greater in secondary infection than in primary. In secondary cases the IgM response is variable [108].

Following the epidemiological studies, it was postulated that the humoral immune response might be responsible for severe dengue disease. Ordinarily the interaction of virus with antibody leads to viral neutralization. However, it seems that the infectivity of some antibody-coated viruses is enhanced if susceptible cells bear Fc receptors. Early studies by Halstead in 1977 demonstrated that non-neutralizing anti-dengue antibodies

were able to render non-permissive peripheral blood leukocytes (PBLs) permissive to dengue infection. They also showed that homotypic anti-dengue antibodies were neutralizing at high concentrations, but enhanced at lower concentrations [109]. These observations led to the theory of antibody dependent enhancement (ADE). According to ADE theory, pre-existing heterologous antibodies, developed during primary infection, fail to neutralize virus either because of insufficient avidity or concentration. Instead the antibody-dengue immune complexes are then targeted for uptake into cells through Fc receptors [110]. ADE has been subdivided into intrinsic and extrinsic phases. The extrinsic phase is when there is an increase in the infection of FcR bearing cells and the intrinsic phase, where the infection of FcR cells drives an increase in viral production through FcR ligation [110]. However, in vivo experiments suggest that ADE does not fully explain severe disease pathogenesis. A prospective study showed that pre-dengue sera did not correlate with the clinical severity or viral burden of disease [111].

Fc γ R comprise a multigene family of membrane glycoproteins that aggregate with complexed IgG to cause either activatory or inhibitory effects on cellular function. There are two FcR associated with dengue, Fc γ RIA and Fc γ RIIA. Fc γ RIA is found exclusively on cells of macrophage and dendritic cell lineage and has high affinity for monomeric IgG. Fc γ RIIA is broadly distributed through myelogenous cells and has high affinity for multimeric IgG. Rodrigo studied the interaction of dengue with Fc receptors and found that antibody bound dengue has enhancing activity broadly through its interactions with Fc γ RIIA. However, it didn't appear to need intracellular signal competency to cause its enhancing effects, suggesting that dengue has an as yet undefined alternative intracellular mechanism. Interestingly, enhancing effects through Fc γ RIA were

dependent on standard intracellular signal pathways [112]. High concentrations of weakly neutralizing antibodies form large antibody aggregates that bind to inhibitory FcγRIIA. However, potently neutralizing antibodies did not form aggregates and did not bind FcγRIIA [24].

Monoclonal antibodies and what they have taught us

Monoclonal antibody work was initially performed using mouse models. In these models, mice were immunized with either whole virus or a proportion of the structural proteins. Antibodies made to dengue virus were then identified and characterized. In mice, a significant proportion of the antibodies produced are to E protein and specifically the fusion loop epitope (FLE) and domain III of the E protein. These results however, may be skewed by the immunization regimen, which often includes a boost with DIII protein. Anti-EDIII antibodies are some of the most potent anti-dengue antibodies and achieve 50% neutralization (NT50) in the low picomolar range. Some of these antibodies have been used in disease models and have shown efficacy in both prophylaxis and treatment of dengue disease [113] [114] [115] [116]. A good example of EDIII antibodies is E16, which shows neutralization of West Nile virus in both *in vitro* and *in vivo* settings and being efficacious at both prophylaxis and treatment [117]. However, therapeutic potential of these antibodies is often limited by their serotype specificity. Antibodies to the fusion loop appear to bind in a more complex manner. They bind more effectively to dengue virus when in the presence of prM. It appears that the presence of prM changes the conformation of the virion allowing better access of the FLE antibodies to the epitope. As a result, binding and neutralization of dengue virus by these antibodies is limited by the concentration of prM, with neutralization titres failing to exceed 60-80% even at high concentrations [118].

Recent advances in *in vitro* techniques have meant that human monoclonal antibody response has been studied. Three main techniques have been used. Firstly the use of EBV to immortalize memory B cells; secondly single cell sorting, cDNA cloning and antibody expression from plasmablast of acutely infected individuals; and thirdly the optimization of traditional hybridoma technologies to generate mAbs from memory B cells [119].

In humans, the memory B cell response appears to be dominated by antibodies to prM. In a study by Dejnirattisai, seven patients with secondary dengue infection had their B cells screened for reactivity to dengue; monoclonal antibodies from these cells were produced. The response was predominantly to prM, 60% of western blot positive antibodies, compared to 40% against E [120]. The prM antibodies show poor neutralization even at high concentrations, peaking at 30-50%. As prM is not exposed on the fully mature virion, these antibodies will only bind to partially mature virions, many of which do not have a high enough content of prM to allow neutralization. However, they may be sufficient to promote ADE [121].

Recent studies have found a number of potentially neutralising monoclonal antibodies. These antibodies share the characteristic of binding across more than one E protein, to conformational and quaternary structures. These include 5J7 (DEN3), CR4354 (to West Nile) and HM14c10 (to DEN 1) [122] [123] [124]. Interestingly, 5J7 is strongly neutralising, but specific to dengue 3. Analysis of its binding suggests that this serotype specificity derives from the charges of the amino acids in its binding sites. This highlights the difficulty in designing effective vaccines against dengue; that changes in

amino acids between serotypes results in quite different epitope binding site, and many of these conformational antibodies are serotype specific.

Studies from Dejnirattisai and Rouvinski have found a novel epitope, envelope dimer epitope (EDE). The antibodies bind to the basic repeating envelope dimers making up the virion surface. X-ray crystallography has identified two distinct epitopes, EDE1 and EDE2. These epitopes map to the binding site between prM and E proteins and is highly conserved. Neutralising antibodies binding at this site are broadly neutralising (bnAbs) across serotypes. However, antibodies cannot bind to the EDE if the virus is 100% immature. This is because the EDE is only formed as the viral particle is maturing. The bnAbs identified in these studies have such a high affinity they are able to outcompete uncleaved prM on partially immature virions from their interaction with E dimers [125] [118]. If vaccine design was able to incorporate the EDE as a target, it could potentially allow a broadly neutralising antibody response across serotypes that also generates antibodies that may out compete enhancing antibodies. The following table (Table 1) outlines the studies of mAbs produced to dengue.

Summary

The immune response to dengue is complex and varies according to not only the stage of infection, but also whether the infection is primary or secondary. Understanding the immune response to dengue is key not only to our understanding of the disease, but also to vaccine design. Vaccine studies to date have proved that finding a safe and efficacious vaccine to all four serotypes simultaneously is difficult. If we can understand

the drivers of productive and deleterious immune responses, this can inform further vaccine work.

Table 1. Summary of studies investigating monoclonal antibodies

Author	Year	Mouse human	1'/2' Acute/ convalescent/ mouse	DEN subtype	mAb Neutralising?	ADE?	Therapeutic studies
Studies assessing breadth of antibody response							
(Setthapramote , Sasaki et al. 2012)	2012	H	4 acute secondary 5 early convalescent	2 ?St	Acute: E 81%, prM 6.6% NS1 3.3%; cross react all 4 st Conv: E 13.3%, prM 13.3%, NS1 53.3%	NT	NT
(Beltramello, Williams et al.)	2010	H	Late convalescent 3pt 1' 2pt 2'	2,3,4 1 & 2	EDIII most potent neut, neut 3-4 DEN St, less abundant in H repertoire than M EDI/DII less potent neut, more St cross react than DIII prM low neut	prM high ADE even at high conc	3mAb with potent neut that use 2 epitopes to 4St engineered with LALA variant to prevent FCyR binding, protected against lethal infection in mice
(de Alwis, Smith et al.)	2012	H	1' Convalescent	1pt D2, 1pt D3	most mAbs broadly St cross reactive, weakly neut and recognised prM	NT	NT
(Friberg, Jaiswal et al.)	2012	H	Late convalescent 1' (1Pt)	3	50% rE or NS1	NT	NT
(Shrestha, Brien et al.)	2010	M	M vaccinate with D1	D1 Gt2	79mAb 16 strong neut Gt2 all bind EDIII 2 (E105 E106) strong neut all 5Gt of D1(bound BC loop) Sequence variation did not fully explain neut	NT	NT

mAbs to E protein							
Dejnirattisai, Wongwiwat et al [118]	2015	H	Acute 2' infection	?	145 mAbs generated, 83 bound E, 46 bound to fusion loop epitope. mAbs also bound across E dimer at EDE 1 & 2. EDE 1&2 mAbs neutralised DENV2 to >80% of DC and C6/36 derived virus	ADE 4-8x less with EDE mAb vs FLE mAb	NT
Smith, de Alwis et al [116]	2013	H	Late convalescent 2' infection	?	1C19 to DII fusion loop (aa 93-97) bc region. Ultrahigh potency, neut all 4 serotypes, competes for binding with other mAbs	NT	Reduced viraemia in mice
Sasaki, Setthapramote et al [126]	2013	H	Acute secondary infection 2pt (1DF, 1DHF) Early secondary convalescence (2wk)(5pt)	2	Acute samples: 17 mAbs strong neut to all 4st 13/17 against DII (53-132) E conva: no mAb to neut all 4 serotype	No diff ADE between DF and DHF	mAb protected mice from lethal infection
Costin, Zaitseva et al [115]	2013	H	Early conv 1' Convalescent 1' Early conv 2'	?	3mAbs: 4.8A, DIIC, 1.6D (1 each pt) high neut E protein (DI/II98-109). Inhibit virus endosome fusion	All mediate ADE at low conc	NT
Schieffelin, Costin et al [127]	2010	H	2yr conv	?	3 IgG1 mAb to E All bound 2/4 st, 1 mAb neut 3 st	All cause ADE	NT

De Alwis, Smith et al [128]	2012	H	8pt late conv ? 1'/2'	2/3	IF4, 5J7, 2D22 neut single st Bind quaternary structure. 2D22 by blocking fusion [fibrinisah 2015]	NT	NT
Saokaew, Pongpair et al [129]	2014	H	Manufactured H single chain variable fragment (Scvf) to EDIII	-	15A: Neut D1,2,4 not 3	NT	NT
Williams, Sukopolvi-Petty et al [130]	2013	H/M chimer a	E60 MmAb to WNV E prot 87.1 human Mab modified with LALA		E60 N297Q & 87.1 LALA neut	Protective following Ab enhance infection	? compete with enhancing Ab in mouse model protecting against lethal disease
Yamanaka, Kotaki et al [131]	2013	M	D1 Mochizuki strain imm mice, 26 mAb		MAb 7F4 to DII: strong neut 3H12	7F4: No ADE 3H12: enhancing	NT
Li, Liao et al [132]	2012	M	17 Mmab to E	D2	Humanised DB32-6 bind EDIII at K310 Strong neut	No enhancement	NT
Midgley, Flanagan et al [133]	2012	M	EDIII imm mouse	D2	2H12 cross reactive D1-4 binds AB loop DIII	No enhancement	NT
Cockburn, Navarro et al [134]	2012	M	?		Fab 4EII neut D1-4 binds DIII	NT	NT
Zhou, Austin et al [135]	2013	M	?	3	mAb 8A1 binds EDIII variable neut to D3	NT	NT

Leon-Juarez, Garcia-Cordero et al [136]	2013	M	D2 E prot imm mice	No neut observed	NT	NT
Deng, Dai et al [137]	2011	M	D2 imm mice	2A10G6 neut 1-4, YFV, WNV binds to tip of fusion loop (98DRXW101)	NT	NT
Sukupolvi-Petty, Austin et al [138]	2010	M	D2 imm mice	Protective mAb generated to DI dimer interface, DII lat ridge & fusion loop, DII lat ridge & A strand	NT	In vitro neut correlated with in vivo
Rajamanonmani, Nkenfou et al [139]	2009	M	rDIII imm mice	ScFv 9F12 prevents viral entry. No neut tests	NT	NT
Puttikhunt, Keelapang et al [140]	2008	M	D2 imm mice	2E11 detects conformational prM-E heterodimer	NT	NT

mAbs to prM protein						
Luo, Feng et al [141]	2013	H	From hybridoma	4D10 poor neut D1-4	Potent enhance (aa14-18 prM)	NT
Dejnirattisai, Jumnainsong et al [120]	2010	H	1pt D1, 4Pt D2, 1Pt D4 early convalescent	Anti-prM dominant mAb (60% vs E 40%). Poor neut	enhance	NT
Wang, Li et al [142]	2013	M	Hybridoma	2H2 inhibits maturation virus at high conc (3Fab to each spike) low conc (2Fab to each spike)	NT	NT

Key

Conv= convalescent, D1= dengue 1, D2= dengue 2, D3= dengue 3, D4= Dengue 4, DF= dengue fever, DHF= dengue haemorrhagic fever, DC= dendritic cell, EDE= Edimer dependent epitope, FLE= Fusion loop epitope, H= human, M= Mouse, neut= neutralising, NT= not tested, St= serotype.

Hypothesis

Severe dengue disease may be augmented by naturally occurring antibodies. These antibodies may be produced in primary disease as well as secondary disease.

Aims

Although the majority of cases of severe dengue occur in patients with secondary disease, severe disease may occur in those with primary disease. In these cases, the immune system has not been primed to dengue. Therefore a primed immune response cannot be the full explanation of the cause of severe disease. Understanding the immune response to severe primary disease will shed light not only on the mechanisms of severe disease, but will be instructive in safe the development of vaccines. In this study, I will investigate the IgG response of a patient with acute severe primary dengue.

1. I will assess the development of IgG antibodies at the early time point of day -1 pre-defervescence (timepoint at which the fever normalises).
2. I will assess the nature of the antibodies.
 - a. Whether the antibodies are serotype specific or cross reactive
 - b.** Which epitopes the antibodies react against

Materials and Methods

Materials

Primers and vectors

Primers used for the nested PCR and outlined in table. Vector sequences for pIgy1, pIgx and pIgl λ can be found at NCBI GenBank (accession numbers: FJ475055, FJ475056, FJ475057).

Table 2. Nested PCR primer sequences

Name	5' → 3' sequence
Nested PCR for the Heavv Chain	
5' AgeI VH1/5	CTGCAACCGGTGTACATTCCGAGGTGCAGCTGGTGCAG
5' AgeI VH3	CTGCAACCGGTGTACATTCTGAGGTGCAGCTGGTGGAG
5' AgeI VH4	CTGCAACCGGTGTACATTCCCAGGTGCAGCTGCAGGAG
5' AgeI VH3-23	CTGCAACCGGTGTACATTCTGAGGTGCAGCTGTTGGAG
5' AgeI VH4-34	CTGCAACCGGTGTACATTCCCAGGTGCAGCTACAGCAGTG
5' AgeI VH 1-18	CTGCAACCGGTGTACATTCCCAGGTTTCAGCTGGTGCAG
5' AgeI VH 1-24	CTGCAACCGGTGTACATTCCCAGGTCCAGCTGGTACAG
5' AgeI VH 3-9/30/33	CTGCAACCGGTGTACATTCTGAAGTGCAGCTGGTGGAG
5' AgeI VH 6-1	CTGCAACCGGTGTACATTCCCAGGTACAGCTGCAGCAG
5' AgeI VH4-39	CTGCAACCGGTGTACATTCCCAGCTGCAGCTGCAGGAG
5' AgeI VH1	CTGCAACCGGTGTACATTCCCAGGTGCAGCTGGTGCAG
5' AgeI VH3-33	CTGCAACCGGTGTACATTCTCAGGTGCAGCTGGTGGAG
3' SalI JH1/2/4/5	TGCGAAGTCGACGCTGAGGAGACGGTGACCAG
3' SalI JH3	TGCGAAGTCGACGCTGAAGAGACGGTGACCATTG
3' SalI JH6	TGCGAAGTCGACGCTGAGGAGACGGTGACCGTG
Nested PCR for the Kappa Light Chain	
5' Pan VK	ATGACCCAGWCTCCABYCWCCCTG
3' CK 494	GTGCTGTCCTTGCTGTCCTGCTC
5' AgeI VK1-5	CTGCAACCGGTGTACATTCTGACATCCAGATGACCCAGTC
5' AgeI VK1-9	TTGTGCTGCAACCGGTGTACATTTCAGACATCCAGTTGACCCAGTCT
5' AgeI VK1D-43	CTGCAACCGGTGTACATTGTGCCATCCGGATGACCCAGTC
5' AgeI VK2-24	CTGCAACCGGTGTACATGGGGATATTGTGATGACCCAGAC
5' AgeI VK2-28	CTGCAACCGGTGTACATGGGGATATTGTGATGACTCAGTC

5' AgeI VK3-11	TTGTGCTGCAACCGGTGTACATTTCAGAAATTGTGTTGACACAGTC
5' AgeI VK3-15	CTGCAACCGGTGTACATTTCAGAAATAGTGATGACGCAGTC
5' AgeI VK3-20	TTGTGCTGCAACCGGTGTACATTTCAGAAATTGTGTTGACGCAGTCT
5' AgeI VK4-1	CTGCAACCGGTGTACATTTCGGACATCGTGATGACCCAGTC
3' BsiWI JK1/2/4	GCCACCGTACGTTTGATYTCCACCTTGGTC
3' BsiWI JK3	GCCACCGTACGTTTGATATCCACTTTGGTC
3' BsiWI JK5	GCCACCGTACGTTTAATCTCCAGTCGTGTC

Nested PCR for the Lambda Light Chain

5' AgeI Vλ1	CTGCTACCGGTTCTGGGCCAGTCTGTGCTGACKCAG
5' AgeI Vλ2	CTGCTACCGGTTCTGGGCCAGTCTGCCCTGACTCAG
5' AgeI Vλ3	CTGCTACCGGTTCTGTGACCTCCTATGAGCTGACWCAG
5' AgeI Vλ4/5	CTGCTACCGGTTCTCTCTCSCAGCYTGTGCTGACTCA
5' AgeI Vλ6	CTGCTACCGGTTCTTGGGCCAATTTTATGCTGACTCAG
5' AgeI Vλ7/8	CTGCTACCGGTTCCAATTCYCAGRCTGTGGTGACYCAG
3' XhoI Cλ	CTCCTCACTCGAGGGYGGGAACAGAGTG

Media and Buffers

Table 3. Media recipes

Medium or buffer	Recipe
Blocking Buffer	5% (w/v) milk in 1x PBS
D-10 medium	DMEM; 10 % FCS; 100 units/ml penicillin, 100 µg/ml streptomycin; 2 mM L-Glutamine
PBST	0.1% Tween-20 in 1x PBS
1 x PBS	1 mM KH ₂ PO ₄ ; 10 mM Na ₂ HPO ₄ ; 137 mM NaCl; 2.7 mM KCl; pH 7.4; Sterilised by autoclaving.
Carbonate Buffer	1 tablet of Carbonate-Bicarbonate Buffer (Cat. no. C3041) dissolve in 100 ml distilled water
PNPP solution	p-Nitrophenylphosphate (Biorad, Cat 172-1063)
1 x Running Buffer	25 mM Tris; 250 mM Glycine; 0.05 % (w/v) SDS
1 x Transfer Buffer	20 % Methanol; 47.9 mM Tris; 38.6 mM Glycine; 1.28 mM SDS

Table 4. Primary and secondary antibodies

Antibody	Organism	Origin	Method	Dilution
Primary Antibodies				
anti-human IgG	mouse	Sigma	ELISA	1:10 000
anti-prM 1H10	mouse	in-house isolation	ELISA/Dot blot	5 µg/ml / supernatant from transfected cells
anti-E 4G2	mouse	in-house isolation	Dot blot	Supernatant from transfected cells
Secondary Antibodies				
anti-human IgG (Fc) alkaline phosphatase conjugate	goat	Sigma	ELISA	1:10 000
anti-human IgG HRP	rabbit	Dako	Dot blot/Western Blot	1:1000 / 1:1000
anti-mouse IgG HRP	goat	Dako	Dot blot	1:1000

Table 5. Dengue virus strains used

DENV serotype	DENV strain
Serotype 1	Hawaii
Serotype 2	16681
Serotype 3	H87
Serotype 4	H241

Methods summary

Activated B cells were isolated from a patient with acute dengue virus. Recombinant monoclonal antibodies were then produced from the activated B cells. These mAbs were then assessed for: their ability to recognise dengue and Japanese encephalitis virus; which epitopes on the dengue virus they recognised; which amino acids of the structural proteins they bind to; and whether they inhibit or enhance infection.

Patient

Acute dengue infection was diagnosed clinically and confirmed by identification of the virus by RT-PCR. Disease severity was assessed by WHO criteria [1]. Ethical approval was provided by Siriraj, Khon Kaen and Songkhla hospitals, Thailand. The sample was taken at day -1, defined by defervescence of fever, i.e. the sample was taken the day before the fever resolved.

Table 6. Dengue patient details

Patient ID	Clinical Diagnosis	Type of infection	Serotype	Time of sample
1-0623-3	DHF-1	Primary	DENV 1	Day -1

Isolation of cells

Whole blood is collected from the patient at defervescence (day -1). Peripheral blood mononuclear cells (PBMCs) are isolated using standard protocol. 8ml of blood was mixed with 16µl heparin. The blood was centrifuged at 3000g for 10 minutes; following this the plasma layer was removed. RPMI (PAA) was then added to the remaining blood to be made up to 15ml. This was layered over 15ml Lymphoprep (Axis-Shield). The samples were then centrifuged at 800g for 30minutes. The PBMC layer was removed and diluted in 50ml RPMI then centrifuged at 800g for a further 10 minutes. The supernatant was discarded and the cells resuspended in 50ml RPMI, centrifuged at 360g for 10 minutes and then this was repeated for a further spin at 600g for 5 minutes. [119] [119, 143]

Activated B cells are then sorted by flow cytometry

1 million cells were resuspended in 1ml of 10% FCS in RPMI (R-10); these cells were used for sorting. As controls, 0.5×10^6 cells were aliquotted for each fluorophore to be tested and one aliquot remained unstained. Centrifuge at 2000rpm for 5 minutes.

Activated B cells were selected by adding for the following antibodies (for staining 1×10^6 cells): 4µl anti-CD3 PerCP (245766, BD); 4µl anti-CD27 FITC (555440, BD); 4µl anti-CD38 APC (555462, BD); 4µl anti-CD20 PerCP (245794, BD); 1µl anti-CD19 PE (R 0808, Dako) in 50µl FACS buffer. Controls were prepared by adding each of the fluorophore-conjugated antibodies to each control aliquot

Cells were incubated for 30 minutes on ice, then washed with 2ml FACS buffer and centrifuged at 2000rpm for 5mins. The cells were resuspended in 300-500µl of FACS buffer.

The cells were gated CD19⁺/CD20⁻/CD3⁻/CD27^{high}/CD38^{high}

Antibody secreting cells (ASC) are then separated as single cells in a 96 well plate coated with 10µl of RNase inhibitor buffer (5ml RNase-free water, 50µl 1MTris pH8 and 125µl RNasin). Cells were sorted and frozen immediately on dry ice to -80°C [119] [8].

RT-PCR for immunoglobulin chains (heavy, lambda and kappa)

The PBMCs were thawed and 15µl RT-PCR master mix was added directly to them. Master mix was made from One-step PCR kit (Qiagen) and was made up as follows for 1 reaction: 5µl buffer, 1µl of forward primer (from stock containing 10µl each of 100µM L-VH1, L-VH3, L-VH4/6, L-VH5, L-VK1/2, L-VK 3, LVK4, L-Vλ1, L-Vλ2, L-Vλ3, L-Vλ4/5, L-Vλ6, L-Vλ7, L-Vλ8), 1µl of reverse primer (from stock containing 10µl each CγCH1, CK543, Cλ plus 70µl water), 1µl dNTP, 6.5µl water, 0.5µl *enzyme mix*. Primers were designed to cover all variable genes. Master mix was added to empty wells for negative controls and to 0.7µg RNA from the B cell clone 3-147 for the positive control. [119] [8] Cycling conditions were: 30 min 50°C; 15mins 95°C; 50 cycles of 30 secs 94°C, 30 secs 58°C and 1 min 72°C; followed by 10mins 72°C.

Nested PCR for amplifying heavy and light chain V genes

Master mix was made using HotStarTaq kit (Qiagen): 4µl HotstarTaq buffer, 1µl forward primer (10µM Heavy chain: 5'AgeI VH mix, Lambda chain: 5'AgeI Vλ mix, Kappa chain: PanVκ) 1µl reverse primer (10M Heavy chain: 3'Sall JH mix, Lambda chain: CλXhoI,

Kappa chain: Ck494), 0.5µl dNTP (25µM), 0.25µl HotstarTaq enzyme and make up to 40µl with water. Master mix was then aliquotted, 37µl, and 3µl RT-PCR product [119] [8]. PCR cycling conditions were: 15mins 95°C; 50 cycles of 30 secs 94°C, 30 secs 58°C, 45 secs 72°C; then 10min 72°C.

PCR purification

Gel loading dye was added to each PCR product and then these were loaded onto a 1% agarose gel in TBE buffer for electrophoresis. And run for 30mins at 100V. Products were analysed under UV light. Products were approximately 380bp heavy chain, 400bp lambda chain, 540bp kappa chain. DNA bands were excised from the gel and purified using QIAquick gel extraction kit (Qiagen) [119] [8].

Kappa chain sequencing

Kappa chain PCR product was sequenced directly using the reverse primer Ck494 by the in-house sequencing service using single primer extension. Sequence data analysed to determine which cloning PCR primer to use from the 5' Agel V_K spec and 3' BsiWI J_K spec group.

Cloning PCR for kappa chains

Master mix was made using HotStarTaq kit (Qiagen): 4µl HotstarTaq buffer, 1µl forward primer, 1µl reverse primer, 0.5µl dNTP (25µM), 0.25µl HotstarTaq enzyme and 40µl water. Master mix was then aliquotted, 37µl, and 3µl RT-PCR product. Forward and reverse primers as determined by sequencing.

PCR cycling conditions were: 15mins 95°C; 50 cycles of 30 secs 94°C, 30 secs 58°C, 45 secs 72°C; then 10min 72°C.

Gel loading dye was added to each PCR product and then these were loaded onto a 1% agarose gel in TBE buffer for electrophoresis. And run for 30mins at 100V. Products were analysed under UV light. Products were approximately 350bp [119] [8].

Expression vector cloning

Purified PCR products of heavy and light chains were digested with matching restriction endonucleases and buffers as recommended by the supplier (NEB). Heavy chain: 45µl nested PCR product, 0.125µl Agel-HF, 2µl Sal1-HF, 5µl NE Buffer 4, 0.5µl BSA. Digest for 6 hours 37°C. Lambda chain: 45µl nested PCR product, 0.125µl Agel-HF, 0.5µl XhoI, 5µl NE Buffer 4, 0.5µl BSA. Digest for 6 hours 37°C. Kappa chains: 45µl 2nd round, cloning PCR product, 0.125µl Agel, 5µl Ne Buffer 4, 0.5µl BSA. Digest for 3 hours at 37°C. The add 0.5µl BsiWI. Digest for 3 hours at 55°C. Reactions were purified using QIAquick kit (Qiagen).

Human expression vectors pIgγ1, pIgκ and pIgλ were used. The vectors contained a multiple cloning site, HCMV promoter, amp resistance gene, leader sequence and constant region sequence for either IgG1 Cγ, Cκ, Cλ.

The vector was digested by matching restriction enzymes as above: vector 3mcg, 7µl buffer, 7µl BSA, 1µl enzyme 1, 1µl enzyme 2, plus SDW to make up. This was incubated overnight at 37°C. The restriction enzymes were heat inactivated at 65°C for 20mins. Vector DNA was dephosphorylated with 1 unit CIP (calf intestinal phosphatase) (NEB) per 1mcg vector and incubated for 1 hour at 37°C.

Vectors were purified by running on a 1.5% agarose gel in TBE buffer and run for 30mins at 100V. DNA bands were excised from the gel and purified using QIAquick gel extraction kit (Qiagen).

Digested PCR products were inserted into the expression vector. Vector and insert were added at a 1:3 molar ratio, 2µl ligase buffer, 0.5µl T4 ligase (200 units) (NEB), reaction volume of 20µl at 16°C for 16 hours.

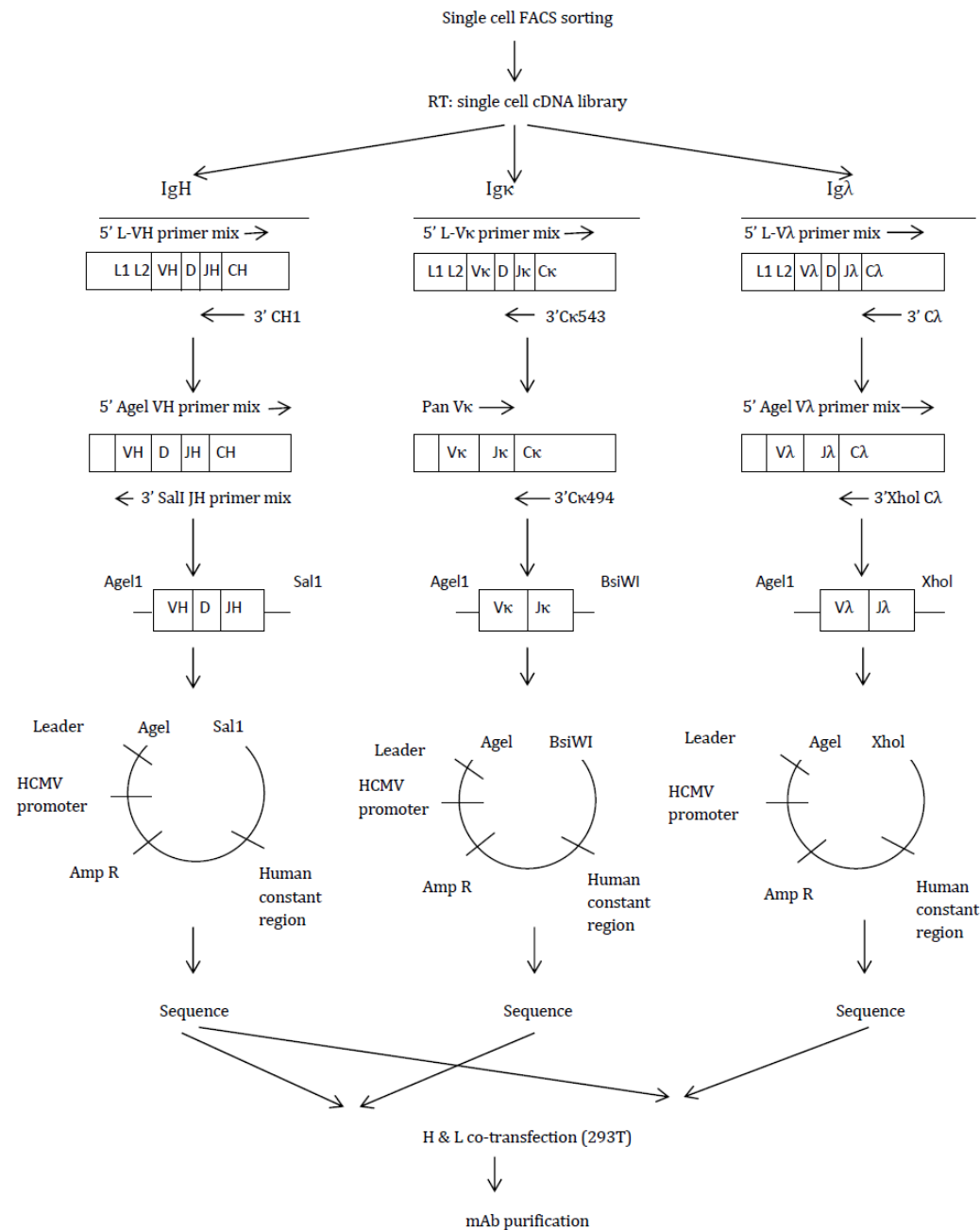
10µl of the ligated vector was used to transform 100µl competent DH5α *E. coli*. Vector plus insert were added to the DH5α *E. coli* on ice and incubated for 30mins, they were then incubated at 42°C for 30secs and returned to ice for 3mins. 1ml of LB broth was added to each aliquot and incubated for 1 hour at 37°C 220rpm. The transformed *E. coli* was pelleted, resuspended and was plated onto LB agar plus 50µg/ml ampicillin and grown overnight at 37°C. 3 small colonies were picked for each chain, and inoculated into LB broth and incubated at 37°C overnight. DNA was extracted using QIAprep spin kit (Qiagen). DNA was sequenced using single primer extension sequencing. The primer IgGint anneals within the C region of the vector allowing reading of the sequence of the vector and insert [119] [8].

Antibody expression by 293T cells

293T cells were seeded at 2×10^5 cells per well (24 well plate) and grown to 50-80% confluency in D-10 at 37 °C, 5% CO₂. 60µl DMEM was aliquotted. To this 1µl of heavy chain DNA, 1µl of light chain DNA and 3µl PEI was added. This was incubated at room temperature for 15 minutes then added to the 293T cells. The 293T cells were incubated at 37 °C, 5% CO₂ for 24 hours, washed and media changed to Ultra-DOMA protein-free media (Biowhittaker) with 50units/ml penicillin and 50µg/ml streptomycin. Cells were then incubated for 4 days at 37°C, 5% CO₂ and the antibody supernatant was harvested. The supernatant was pelleted at 2500rpm for 5 mins and stored in the fridge [119].

Fig. 13 Summary of mAb production. Adapted from [8] Heavy and light chains are produced by RT PCR and then amplified using a nested PCR. The DNA produced is then ligated into an expression vector. The vector was used to transform competent *E. coli* ,

which then produced large volumes of DNA. The DNA from heavy and light chains was co-transfected into 293T cells, which then produced the immunoglobulin chains.



Anti-human IgG ELISA

Concentration ELISAs were performed. 96 well ELISA plates were coated with mouse anti-human IgG at a dilution of 1:10 000, 50μl per well and incubated overnight at 4°C.

The plates were washed 3 times with 200µl PBS-T (PBS plus 0.05% tween). Plates were blocked with 200µl BSA blocking solution (PBS plus 3% BSA) for 1 hour at room temperature. The plates were washed 3 times with 200µl PBS-T. Antibody supernatant from transfection was then added to the wells at concentration of neat, 1:150, 1:600, and incubated for 1 hour at 37°C. As a standard serial 2-fold dilutions of IgG from human serum was used starting at 1mg/ml. Negative controls were blocking buffer alone. The plates were washed 3 times with 200µl PBS-T. 50µl of 1:10 000 secondary antibody (anti-human IgG AP (Fc specific)) was added per well. The plates were incubated for 1 hour at 37°C, then 50µl of PNPP was added to each well and incubated at room temperature in the dark. The absorbance was read at 405nm on a Biorad ELISA plate reader after 30 and 60 minutes incubation. A standard curve was produced using the known concentrations of IgG from serum. Concentrations of antibodies were calculated from the trend line equation.

DENV dot blot

A dot blot was performed to assess whether the recombinant antibodies produced bound to DENV1-4 and/or JE [144] [145].

Supernatant from virus-infected C6/36 cells was inactivated with UV. The supernatant of DENV 1,2,3,4 and JE and non-infected mock were dotted onto nitrocellulose membranes. Membranes were allowed to dry and then added to PBS with 5% milk to block for 60 mins at room temperature. Membranes were washed 3 times with PBS-T. The primary antibody samples were cell supernatant from antibody transfection was diluted in 5% skimmed milk, with controls being anti-prM and anti-influenza. 100µl of primary antibody was added to each membrane strip and incubated at 4°C overnight. The membranes were washed with 3 times with PBS-T. Secondary antibody, anti-human rabbit IgG HRP (P0214) at a dilution of 1:1000 in 5% skimmed milk was added,

100µl per membrane strip. The membranes were incubated at room temperature in the dark for 1 hour. The membranes were washed with 3 times with PBS-T and membranes were developed using luminata Classico Western HRP substrate 80µl per membrane and exposed on photographic film (Kodak).

DENV Western blot

Western blot was performed to assess which structural dengue protein the recombinant antibodies bound to [144] [145].

Lysate from DENV infected C6/36 cells was treated with 1% TritonX-100 in PBS and UV inactivated. Negative control was prepared as above but from non-infected C6/36 cells. The lysate was centrifuged at 8000g for 10minutes and the supernatant was added to non-reducing loading dye. This was loaded onto a 12% SDS gel and run at 100v for 5-10 minutes, then 120v for 90 minutes. The gel was then blotted onto a nitrocellulose membrane in transfer buffer at 0.05A/gel for 1 hour. The membrane was washed twice for 10 minutes in PBS and air dried. The membrane was cut into strips and probed with the recombinant antibody and controls. Recombinant antibody was prepared in a 1:2 dilution in 5% skimmed milk. Controls were: positive control sample, NS1, prM and negative; anti-influenza. 60µl of antibody was added to each membrane strip and incubated at 4°C overnight. The membranes were washed 3 times with PBS-T and then probed with 65µl of the secondary antibody, anti-human rabbit IgG HRP (P0214) at a dilution of 1:1000 in 5% skimmed milk. The membranes were incubated for an hour at room temperature and then washed 3 times with PBS-T. The substrate, luminata Classico Western HRP (WBLUCO100), was added at 65µl per membrane and incubated for 3 minutes at room temperature, then exposed on photographic film (Kodak).

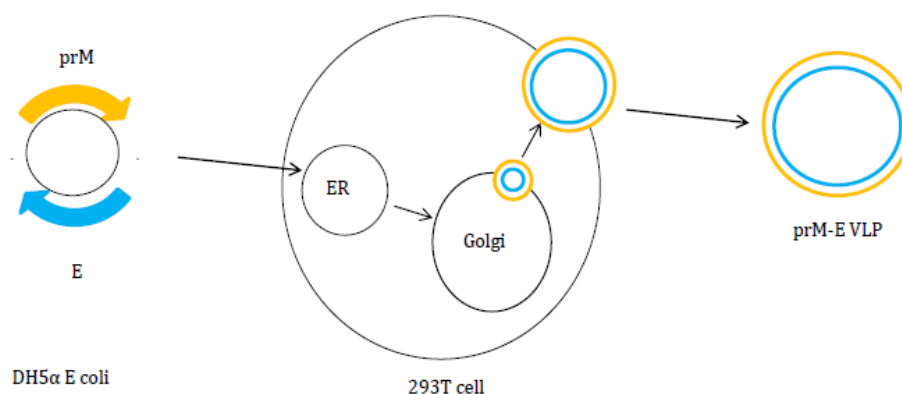
VLP alanine scan library production

Virus-like particles (VLPs) are shell like particles made up only of the structural proteins. Dengue constructs have been made with a combination of the three structural

proteins (E, prM and C). Using VLPs allows changes to be made to the structural proteins easily and outside of the category 3 laboratory. We use constructs from E and prM as: prM is necessary for post-translational modification of E protein; immature viral particles are frequently produced with prM still visible on the external viral particle [146] [9].

An alanine scan was performed to assess key binding residues of the mAbs. Site-directed mutagenesis was performed such that each amino acid of the E and prM proteins is replaced with an alanine amino acid. Alanine is chosen as it is small and chemically inert, so eliminates side chain interactions, but is able to mimic secondary structure preferences of many amino acids [147]. However, some of the mutations introduced may affect production or excretion of the VLP.

Fig. 14. Diagram of VLP production. Adapted from [9] prM and E DNA sequences are ligated into competent *E. coli*. The DNA produced is then used to transfect 293T cells. The 293T cells produce prM and E proteins packaged as VLPs.



Primer design and site directed mutagenesis

Primers to include the alanine substitutions were designed using Quikchange primer design software (<http://www.genomics.agilent.com/primerDesignProgram.jsp>). The primers were then diluted 1:10 with SDW to create a stock. A cloning PCR was performed. Primers were diluted a further 1:10. Master mix: 5µl buffer, 0.5µl forward primer, 0.5µl reverse primer, 2µl dNTP (10mM), 0.5µl magnesium sulphate, 7.5µl 10x enzyme, 0.25µl Pfx, 8.3µl SDW, 0.5µl template (DEN1 prME wild type). Master mix was then aliquotted, 25µl per sample. PCR was performed for 16 cycles at 94°C for 1 min, 57°C for 1 min and 68°C for 7 min.

PCR products were run on a gel to confirm the PCR product. Gel loading dye was added to 5µl of each PCR product and then these were loaded onto a 1.5% agarose gel in TBE buffer for electrophoresis. And run for 30mins at 100V. Products were analysed under UV light. The remainder of the PCR product was purified using QIAquick kit (Qiagen).

Dpn1 digestion was then performed to eliminate template DNA: 32µl PCR product, 3.4µl 10x buffer 4, 0.5µl Dpn1. This was incubated overnight at 37°C. This was followed by heat inactivation of Dpn1 for 20 minutes at 65°C, and then cooled on ice. The product was then used to transform competent DH5α *E. coli* and plated out as described above. Three small colonies were picked from each plate and inoculated into 1.2ml of Terrific broth with ampicillin 75 µg/ml and incubated overnight at 37°C 220rpm. DNA was extracted using Quicklyse (Qiagen). Product DNA was sent for sequencing and then analysed (using http://www.ebi.ac.uk/Tools/st/emboss_transeq/) to assess alanine insertion. If any samples had failed, new colonies were picked from the plates and assessed.

E. coli colonies with the correct alanine substitution inserted, were regrown in 10mls LB broth overnight at 37°C 220rpm. Glycerol stocks of the *E. coli* were prepared using 250µl 50% glycerol and 583µl *E. coli*, placed on ice, and then frozen to -80°C. The remainder of

the E coli was centrifuged at 3000rpm for 10minutes, broth discarded and DNA extracted using miniprep (Qiagen).

VLP expression by 293T cells

293T cells were seeded at 2×10^5 cells per well (24 well plate) and grown to 50-80% confluency in D-10 at 37 °c, 5% CO₂. 60µl DMEM was aliquotted. To this 1µl of VLP DNA and 3µl PEI was added. This was incubated at room temperature for 15 minutes then added to the 293T cells. The 293T cells were incubated at 37 °c, 5% CO₂ for 6 hours, washed and media changed to Ultra-DOMA protein-free media (Biowhittaker) with 50units/ml penicillin and 50µg/ml streptomycin and glutamine. Cells were then incubated for 5 days at 37°C, 5% CO₂ and the antibody supernatant was harvested. The supernatant was pelleted at 2500rpm for 5 mins and stored in the fridge.

Concentration ELISA

An ELISA to determine to concentration of the VLP produced relative to WT was performed.

96 well ELISA plates were coated with anti-prM IgG (1H10) 5µg/ml in 50µl carbonate buffer and incubated overnight at 4°C. The plate was washed 3 times with 200µl PBS-T and blocked with 200µl blocking solution (3% BSA in 1x PBS). The plates were incubated for 1 hour at room temperature and washed 3 times with PBS-T as before. 50µl of each VLP mutant was added to wells at concentration of neat, 1:4, 1:16 and 1:32 and incubated for 1 hour at 37°C. The plates were washed as before. 50µl of mixed antibody [table 7] (designed to bind to either E or prM) were added to each well and incubated for 1 hour at 37°C plates were washed again as previously and 50µl of secondary antibody was added, IgG AP goat anti-human (A9544) at a dilution of 1:10 000 in BSA blocking solution. The plates were incubated for a further hour at 37°C,

washed with PBS-T and 50µl PNPP added to each well and incubated at room temperature in the dark for 30/45/60 minutes. Plates were read at 420nm Biorad ELISA plate reader.

Table 7. In house anti- E and prM antibodies

Antibody	Origin	[c] (µg/ml)
749(2)A7_H2	in-house isolation	50.084
751-C5_H	in-house isolation	29.42
751-C10_H	in-house isolation	65.132
753(3)B11_H1	in-house isolation	703.532

Dot blot of VLP

A dot blot of the VLP produced was performed to assess whether the alanine substitutions affected ability of the VLPs to bind to prM and E.

2µl of supernatant of the VLPs from 293T cells was dotted onto a nitrocellulose membrane and allowed to air dry. The membranes were soaked in 5% skimmed milk in 1x PBS for 1 hour. The membranes were then washed 3 times in PBS. Primary antibody was added, either anti-prM (1H10) or anti-E (4G2), 1.5mls per membrane and incubated for 2 hours at room temperature. The membranes were washed 3 times in PBS-T for 5 mins each. Secondary antibody was then added to each membrane, anti-mouse polyclonal goat HRP (P0447) 1:1000 dilution in skimmed milk PBS, and membranes incubated for 1 hour at room temperature. The membranes were washed 3

times in PBS-T then 1.5 mls HRP substrate (Classico) added to each membrane for 3 minutes and blotted. The membranes were developed on photographic paper.

Results

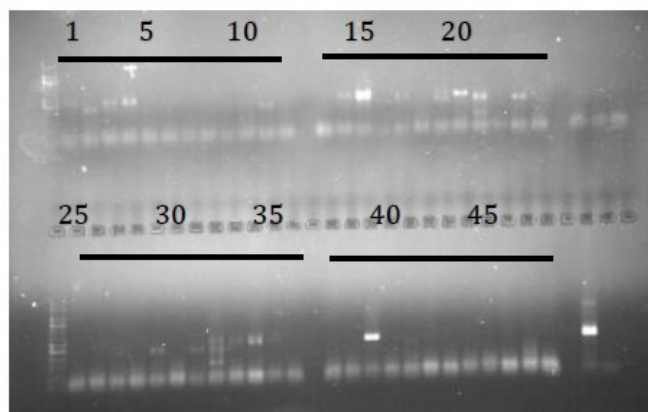
Isolation of activated B cells

To investigate the early immunoglobulin response to dengue virus, activated B cells, gated as CD19⁺ CD3/20^{low/-} CD27^{high} CD38^{high}, were isolated from a patient at day – 1 after defervescence. The lymphocytes were then sorted as single cells (n=48) at sample source in Thailand, frozen and transported to the UK. RT-PCR was performed to isolate the heavy, lambda and kappa chains from each cell. Nested PCR was then performed on the heavy and lambda chains to amplify the product. The kappa chains were sequenced before undergoing a cloning PCR.

Production of heavy and light immunoglobulin chains

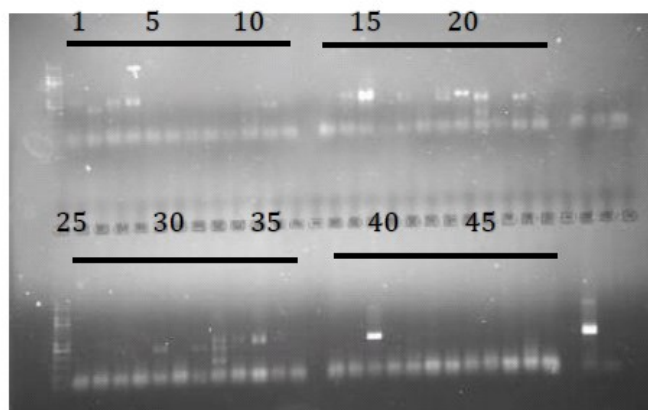
Nested PCR was then performed on the heavy and lambda chains to amplify the product. Positive reactions were identified by loading onto a 1% agarose gel. DNA was identified from 35 heavy chains (380bp), 22 lambda chains (405bp) and 20 kappa chains (350bp).

Fig 15. Immunoglobulin chain nested PCR. 1% agarose gels demonstrating immunoglobulin chains isolated and amplified from single sorted antibody secreting cells.



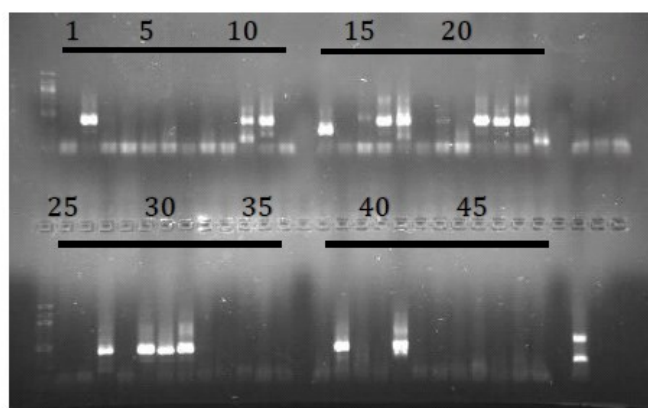
VH 380bp

Total 35



Vλ 405bp

Total 22

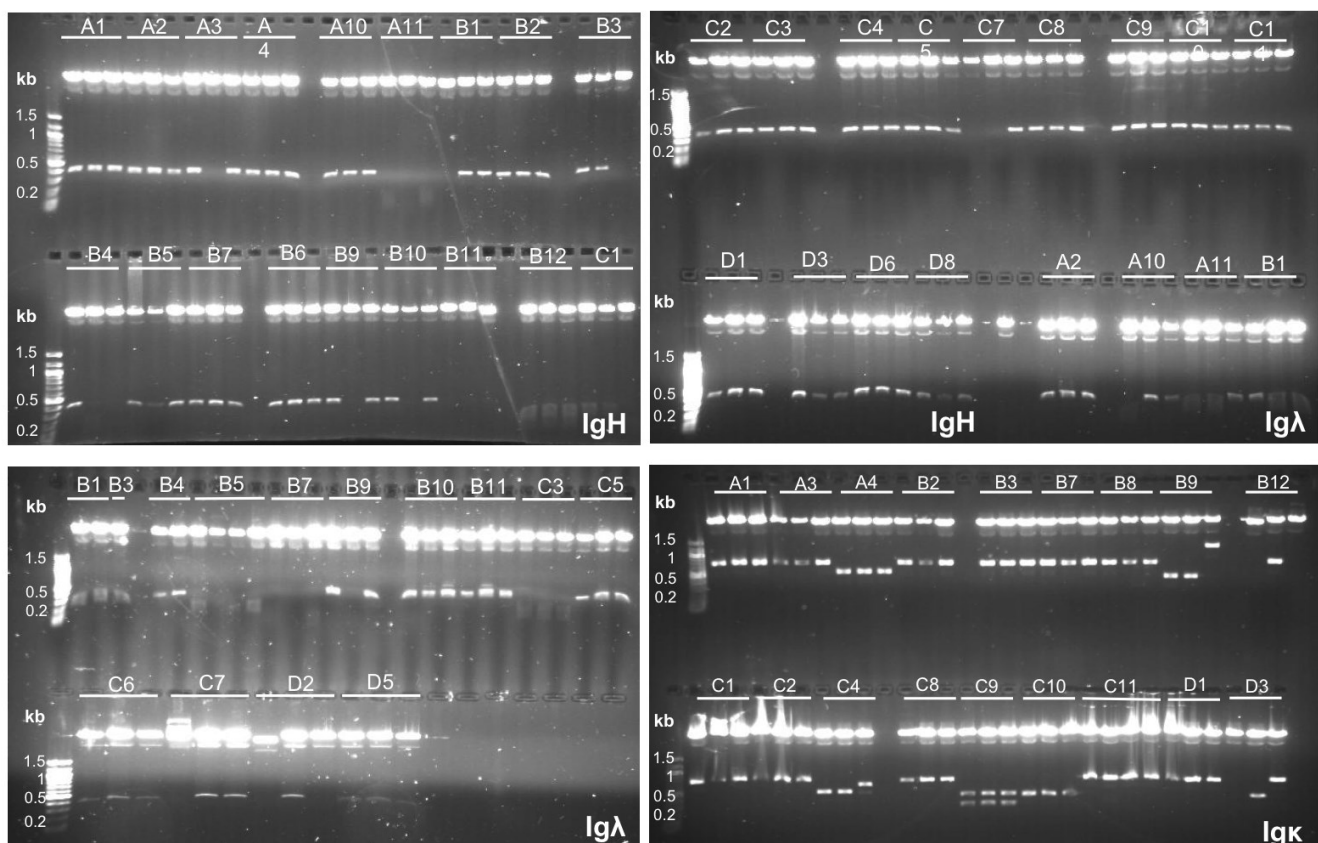


Vκ 350bp

Total 20

The kappa chains were sequenced before undergoing a cloning PCR. The purified PCR products of heavy and light chains were digested and expressed in human vectors, Ig γ 1 (heavy), Ig λ (lambda) and Ig κ (kappa), then used to transform competent DH5 α *E. coli*. At least 2 colonies of bacteria producing the required DNA were selected for each heavy and light chain (heavy 380bp, lambda 405bp, kappa 350bp). DNA produced by the *E. coli* was sequenced.

Fig 16. Vector ligation gel. A diagnostic digest was performed to confirm that the immunoglobulin chains had been correctly inserted into the appropriate vectors. Digests were performed with *Age*I and *Sal*I, *Age*I and *Xho*I, and *Eco*RI and *Hind*III for the IgH, Ig λ and Ig κ constructs, respectively. Heavy, lambda and kappa chains were identified at 380bp, 405bp, 350bp respectively.



Antibody expression by HEK293T cells and concentration measurement

In order to reproduce all potential antibodies (from the combination of heavy and light chains) HEK293T cells were co-transfected with all possible permutations of heavy and light chains produced from each single B cell, totalling 199 combinations. This comprised of 134 IgH-Ig λ and 65 IgH-Ig κ combinations from 48 single ASC.

Control transfections were performed with constructs containing DNA from anti-dengue (3-147 H2K1) (positive control) and anti-flu (2-8CH/ λ) (negative control) antibodies. The antibody supernatant was harvested after 4 days. A total IgG ELISA of the supernatant was then performed assessing the concentration of IgG produced by the transfection [Table 8]. In total 88/134 IgH-Ig λ and 44/65 IgH-Ig κ recombinant IgG antibodies were produced from the 199 transfected HEK293T cells. These recombinant IgG antibodies were produced from 22 single sorted cells, 13 with a lambda light chain and 9 with a kappa light chain.

Fig 17. Recombinant human IgG concentration ELISA. Supernatant from HEK293T cells was incubated on coated ELISA plates. Recombinant human IgG was detected by AP-conjugate anti-human IgG and measured at 405 – 420 nm. Concentrations were measured by a standard curve with purified human IgG: $[c] = (0.0259 * \text{Absorbance} - 0.0035) * 600$. The graph shows the conversion of absorbance to concentration.

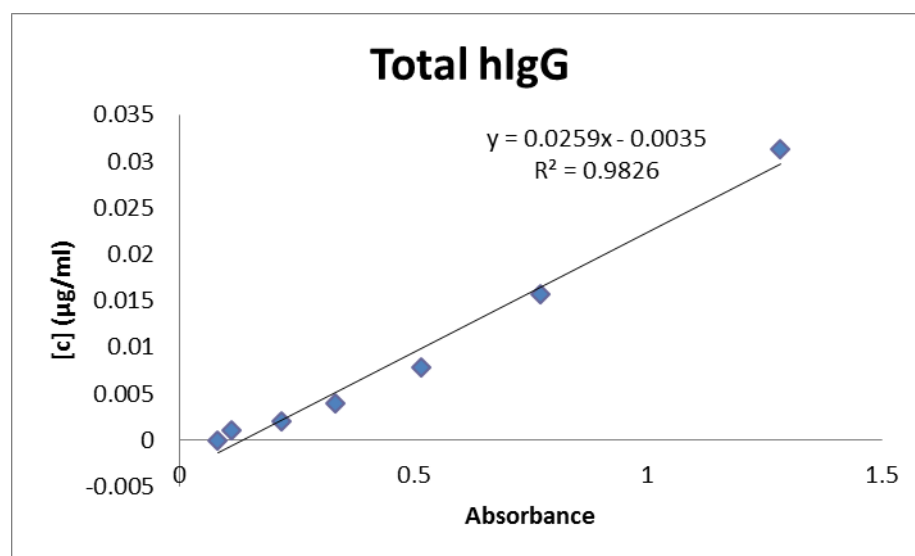


Table 8. Recombinant human IgG concentration ELISA. The table shows the samples chosen for further investigation and the concentrations of IgG calculated for each of these samples.

No.	Sample	Concentration ($\mu\text{g/ml}$)	No.	Sample	Concentration ($\mu\text{g/ml}$)
1	0623P3_A1_H2K2	6.23	28	Negative control	7.77
2	0623P3_A1_H2K3	8.17	29	0623P3_A2_H1L1	15.18
3	0623P3_A3_H1K2	11.61	30	0623P3_A2_H1L2	15.37
4	0623P3_A3_H1K3	13.56	31	0623P3_A10_H2L2	11.09
5	0623P3_B2_H2K1	9.09	32	0623P3_A10_H2L3	12.60
6	0623P3_B2_H2K3	9.73	33	0623P3_B1_H2L1	13.92
7	0623P3_B3_H1K2	12.13	34	0623P3_B1_H3L1	11.96
8	0623P3_B3_H1K3	13.64	35	0623P3_B4_H6L1	9.20
9	0623P3_B7_H1K2	13.18	36	0623P3_B4_H6L2	10.58
10	0623P3_B7_H1K3	10.50	37	0623P3_B5_H2L7	11.39
11	0623P3_B8_H4K1	11.26	38	0623P3_B5_H3L7	13.77
12	0623P3_B8_H4K3	9.06	39	0623P3_B9_H1L1	12.99
13	0623P3_C2_H2K1	10.77	40	0623P3_B9_H3L1	11.22
14	0623P3_C2_H3K3	10.33	41	0623P3_B10_H1L1	12.54
15	0623P3_C8_H1K3	9.60	42	0623P3_B10_H1L3	4.08
16	0623P3_C8_H2K2	8.62	43	0623P3_C3_H2L6	2.24
17	0623P3_C9_H2K3	6.62	44	0623P3_C3_H3L6	9.38
18	0623P3_C9_H3K1	4.85	45	0623P3_C5_H2L2	10.13
19	0623P3_C11_H1K1	5.14	46	0623P3_C5_H2L3	11.14
20	0623P3_C11_H1K2	4.60	47	0623P3_A4_H3K3	-1.66
21	0623P3_D1_H1K3	16.73	48	0623P3_B9_H3K3	-1.77
21	0623P3_D1_H3K2	18.60	49	0623P3_B12_H2K2	-0.95
23	0623P3_D3_H1K3	11.14	50	0623P3_C1_H1K2	-1.79
24	0623P3_D3_H2K3	15.12	51	0623P3_C4_H1K3	-0.65
25	0623P3_D8_H1K2	12.03	52	0623P3_C10_H1K2	-0.30
26	0623P3_D8_H2K2	9.69	53	0623P3_B3_H1L1	-1.56
27	Positive control	6.34			

A selection from the above 199 samples were then chosen for further investigation. We aimed to select 2 samples derived from each positive single B cell. Selections were made based on the concentration produced by transfection. Ideal samples had a concentration of greater than 10µg/ml. However, if concentrations produced did not reach this, a sample with the concentration of greater than 4µg/ml was used. A selection of one clone chosen randomly was made for negative samples. In total 44 positive samples were chosen for further assessment.

Identification of dengue specific antibodies

Dot blot of Recombinant antibodies

A dot blot was performed to assess whether the recombinant IgG produced was against dengue or Japanese Encephalitis (JE). The 53 samples above were incubated with UV inactivated Dengue 1-4, JE and mock (supernatant from non-infected C6/35 cells, negative control). Seven recombinant antibodies showed reactivity to dengue. Three antibodies (43%) reacted only to dengue 1. Whereas three (43%) showed cross reactivity to all four dengue serotypes, and a further antibody (14%) reacted to all four serotypes and additionally to JE. Samples 47 to 53, which were negative on IgG ELISA, were also negative by dot blot.

Fig 18. Anti-dengue activity and serotype specificity of 53 recombinant antibodies. Dot blot of UV treated dengue 1-4, JE and mock, were counter stained with recombinant antibody and detected with HRP-conjugated anti-human IgG.

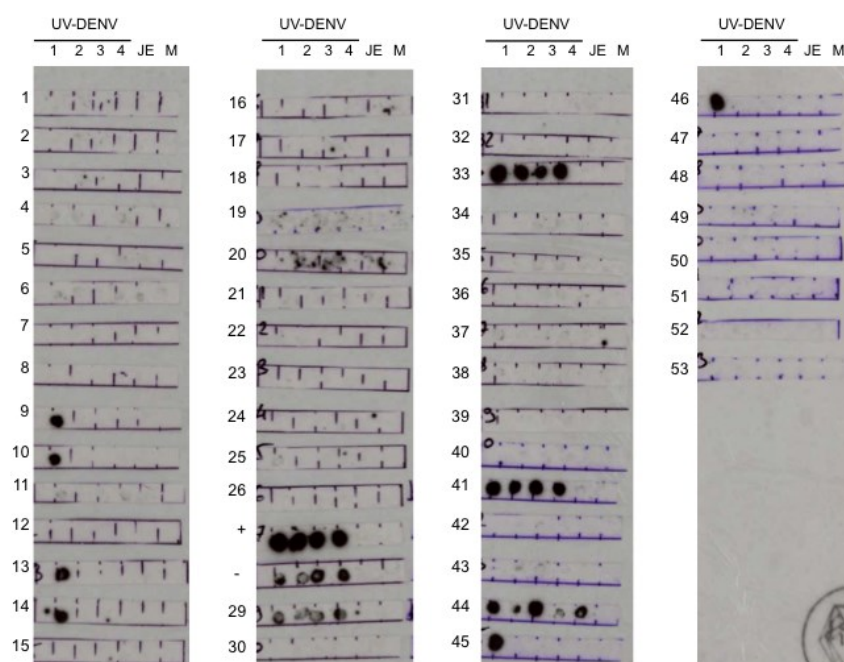


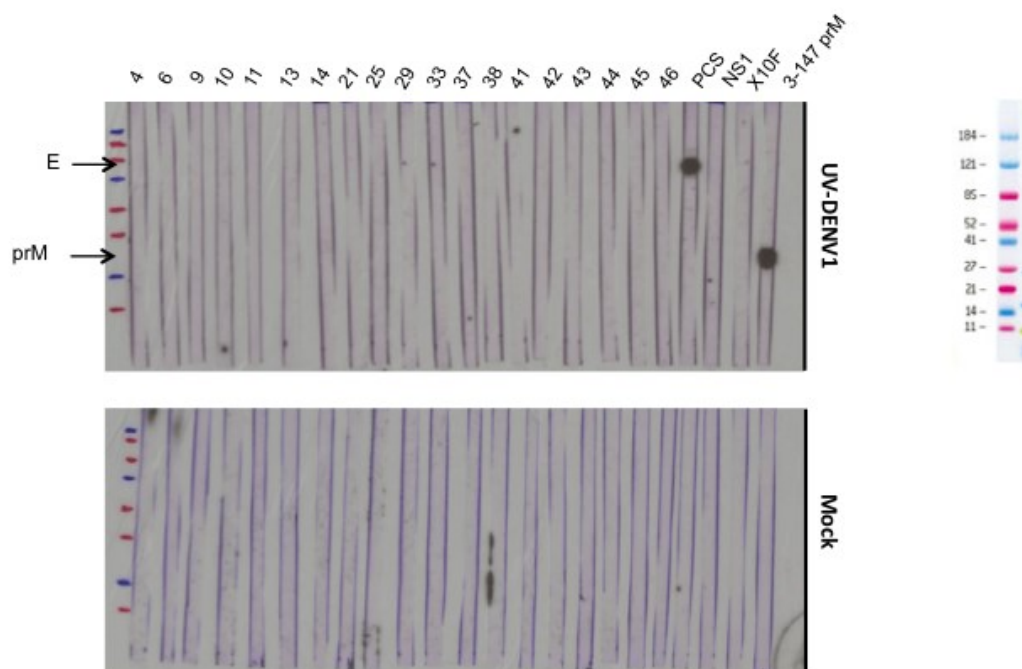
Table 9. Anti-dengue activity and serotype specificity of 53 recombinant antibodies. Table showing combined positive results of the dot blot.

No.	Sample	Positive reaction
9	0623P3_B7_H1K2	DENV 1
13	0623P3_C2_H2K1	DENV 1
29	0623P3_A2_H1L1	DENV 1,2,3,4
33	0623P3_B1_H2L1	DENV 1,2,3,4
41	0623P3_B10_H1L1	DENV 1,2,3,4
44	0623P3_C3_H3L6	DENV 1,2,3,4, JE
46	0623P3_C5_H2L3	DENV 1

Western blot of dengue specific recombinant antibodies

A western blot was performed in non-reducing conditions to assess the antibodies' epitope. The seven antibodies with reactivity to dengue were assessed against dengue 1. Dengue 1 was chosen as the patient had a confirmed dengue 1 illness and therefore it is likely that the most avid antibodies would be to dengue 1. Also, all seven recombinant antibodies reacted to dengue 1. No recombinant antibodies bound to either the E epitope or the prM epitope.

Fig 19. Western blot assessing specific epitopes of recombinant human IgG. Non-reducing Western blot of UV-treated DEN1 cell lysates showing reactivity to NS1, prM and E proteins. Controls of anti-E (pooled convalescent sample, PCS), anti-NS1, anti-prM (3-147 prM) and negative control (X10F, anti-flu) were used.

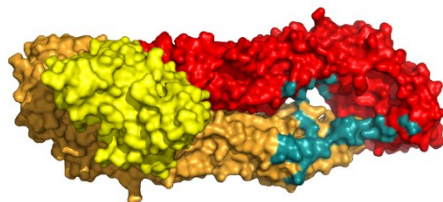


VLP alanine scan library production

An alanine scan library of dengue VLPs was produced. This library was developed to assess the precise residues within the dengue structural proteins that the antibodies bind to. Work in the laboratory had already produced a library to dengue 1 with 113 alanine substitutions. These 113 residues had been chosen according to published work on which dengue amino acids were present on the surface of the virus. However, crystallography performed on antibodies produced in the laboratory had identified a new epitope, the C10 epitope [118].

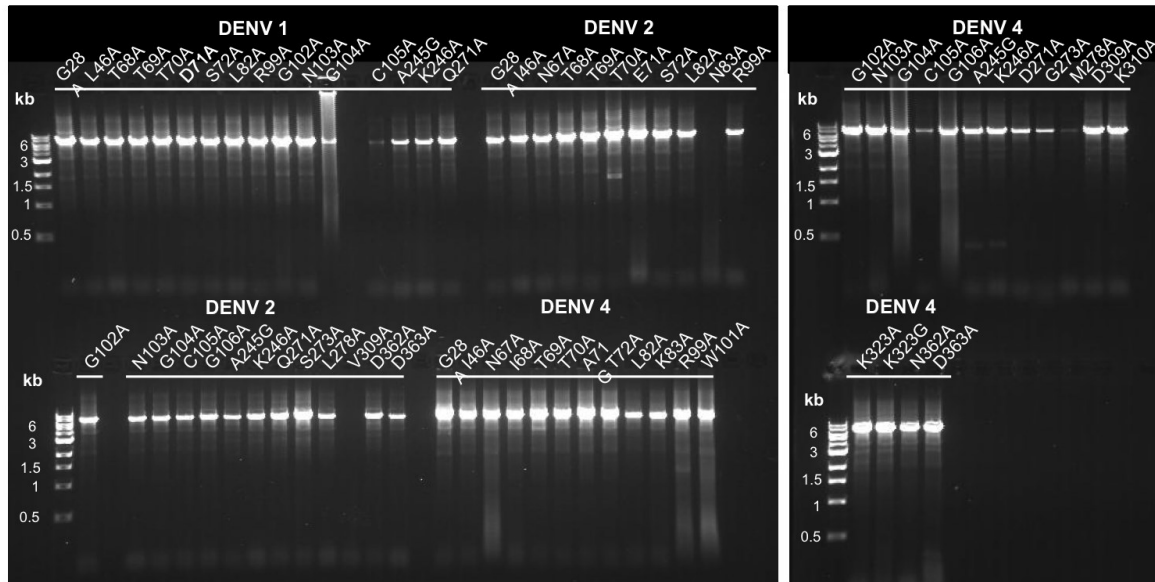
We produced a library to cover this epitope. There were 67 residues identified on Den 1, 2 and 4.

Fig 20. 3D structure of E protein dimer highlighting the C10 epitope. There are 2 E proteins, one in orange and the other in red and yellow. The blue area is the C10 epitope, which overlaps the 2 E proteins.



PCRs were performed for each set of primers. A southern blot was performed to confirm VLP production.

Fig. 21. Southern blot confirming presence of VLP products produced by site direct mutagenesis.



The VLPs were then used to transform DH5 α *E. coli*, and the VLPs produced were then sent for sequencing to confirm the alanine substitutions. A sample of which is demonstrated below showing just the alanine substitutions in the sequence. VLP mutants were produced to 67 residues across 3 dengue serotypes. We were able to produce mutants and confirm the correct sequence with alanine substitution to 65 of the 67 residues identified as part of the C10 epitope.

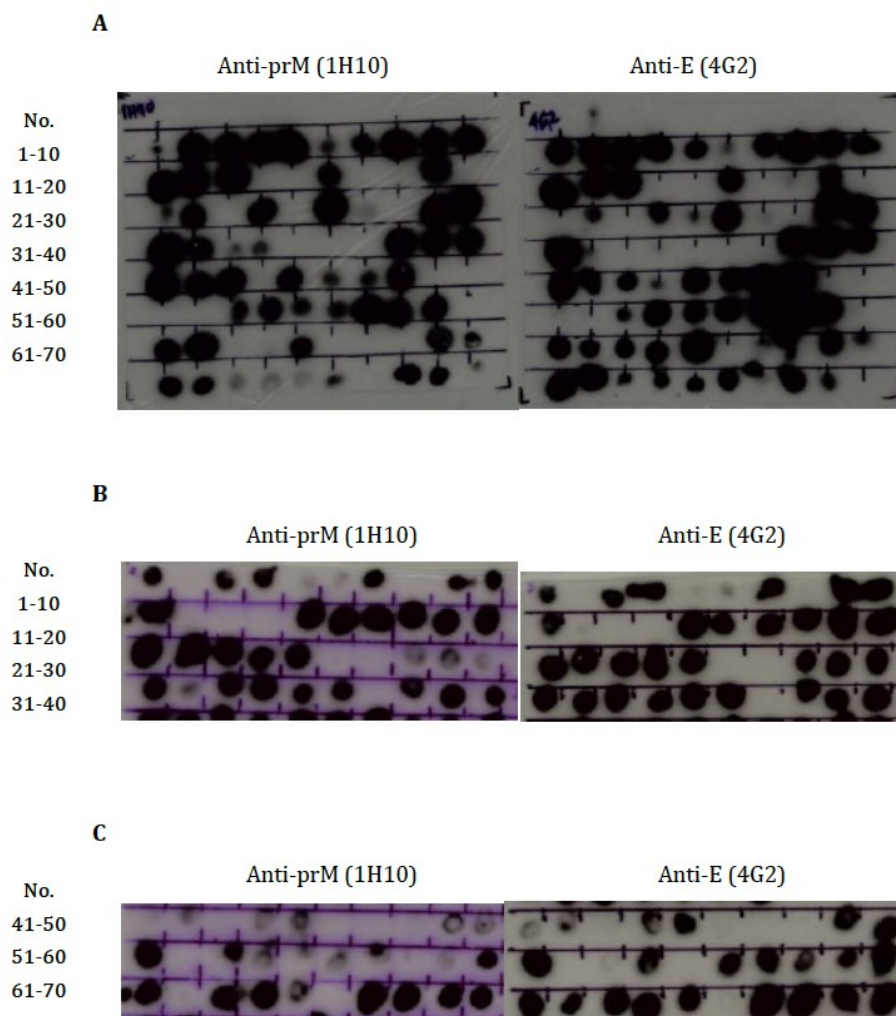
The VLP products were then used to transfect 293T cells. A dot blot was performed from the supernatant of the 293T cells to assess expression. The blot on the left shows reactivity to prM and the blot on the right to E. A second run was performed (B) with extra mutants identified after the initial run. A third transfection and dot blot (C) was performed with samples where expression was either weak or had failed on the initial run. Of the 65 mutants with the correct alanine substitution, 6 were not expressed in the 293T cell supernatant, as demonstrated by dot blot. It is hypothesised that the mutation insertion may affect excretion of the particle.

Table 10. Summary of VLP prME constructs and their expression by 293T cells as confirmed by dot blot. Expression of VLP construct is represented by +, absent expression by – and w meaning weak.

No.	Mutation name	Sequence	Expression by dot blot	No.	Mutation name	Sequence	Expression by dot blot
1	DV1_Emt_G28A_2	+	+	35	DV2_Emt_Q271A_3	+	+
2	DV1_Emt_L46A_3	+	+	36	DV2_Emt_S273A_4	+	+
3	DV1_Emt_T68A_1	+	+	37	DV2_Emt_L278A_7	+	+
4	DV1_Emt_T69A_3	+	+	38	DV2_Emt_V309A_2	+	+
5	DV1_Emt_T70A_1	+	+	39	DV2_Emt_D362A_3	+	+
6	DV1_Emt_D71A_2	+	+	40	DV2-JE K64A_R323A_4	+	+
7	DV1_Emt_S72A_2	+	+	41	DV4_Emt_G28A_3	+	+
8	DV1_Emt_L82A_6	+	+	42	DV4_Emt_I46A_1	+	+
9	DV1_Emt_R99A_6	+	+	43	DV4_Emt_N67A_4	+	-
10	DV1_Emt_G102A_3	+	+	44	DV4_Emt_I68A_3	+	+
11	DV1_Emt_N103A_3	+	+	45	DV4_Emt_T69A_2	+	-
12	DV1_Emt_G104A_9	+	+	46	DV4_Emt_T70A_3	+	+
13	DV1_Emt_C105A	-	-	47	DV4_Emt_A71G_1	+	+
14	DV1_Emt_A245G_3	+	+	48	DV4_Emt_T72A_2	+	+
15	DV1_Emt_K246A_3	+	+	49	DV4_Emt_L82A_1	+	+
16	DV1_Emt_Q271A_6	+	+	50	DV4_Emt_K83A_1	+	+
17	DV2_Emt_G28A_3	+	+	51	DV4_Emt_R99A_5	+	+
18	DV2_Emt_I46A_2	+	+	52	DV4_Emt_W101A_4	+	+
19	DV2_Emt_N67A_2	+	+	53	DV4_Emt_G102A_2	+	+
20	DV2_Emt_T68A_2	+	+	54	DV4_Emt_N103A_2	+	-

21	DV2_Emt_T69A_2	+	+	55	DV4_Emt_G104A_3	+	+
22	DV2_Emt_T70A_6	+	+	56	DV4_Emt_C105A_12	+	+
23	DV2_Emt_E71A_11	-	W	57	DV4_Emt_G106A_3	+	+
24	DV2_Emt_S72A_1	+	+	58	DV4_Emt_A245G_2	+	+
25	DV2_Emt_L82A	+	-	59	DV4_Emt_K246A_1	+	+
26	DV2_Emt_N83A_1	+	+	60	DV4_Emt_D271A_3	+	+
27	DV2_Emt_R99A_2	+	+	61	DV4_Emt_G273A_1	+	+
28	DV2_Emt_G102A_4	+	+	62	DV4_Emt_M278A_1	+	+
29	DV2_Emt_N103A_4	+	-	63	DV4_Emt_D309A_1	+	+
30	DV2_Emt_G104A_1	+	+	64	DV4_Emt_K310A_4	+	+
31	DV2_Emt_C105A_3	+	-	65	DV4_Emt_K323A_1	+	+
32	DV2_Emt_G106A_4	+	+	66	DV4_Emt_K323G_1	+	+
33	DV2_Emt_A245G_6	+	+	67	DV4_Emt_N362A_2	+	+
34	DV2_Emt_K246A_2	+	+				

Fig. 23 Expression of VLP prME constructs assessed by dot blot. Membranes were dotted with the VLP constructs and counter stained with anti-prM (1H10) and anti-E (4G2) antibodies. Each dot represents an individual VLP mutant (list in appendix 1). Fig A represents the initial sample run. Figure B represent further samples that were assessed in addition to run A. Figure C represents repeat sample runs for the samples that had either weak or absent expression in run A.



In summary, 65 prME VLP mutants were successfully produced with alanine substitutions for wild type amino acids. Of these 65, 59 samples were expressed by 293T cells. Thus a library was created against which recombinant antibodies can be tested to assess key binding residues within the C10 epitope

Discussion

Primary dengue infection has rarely been studied. In this preliminary study, I investigated the acute response to primary dengue haemorrhagic fever as a result of dengue 1 infection.

Monoclonal antibodies have been used to study the complex immune response to dengue virus. Traditionally, monoclonal antibodies have been raised in mice. However, recent advances in *in vitro* techniques have allowed the study of monoclonal antibodies in response to acute and convalescent infection.

The advances in *in vitro* techniques have meant that relatively large numbers of monoclonal antibodies have been able to be generated in humans. Thus allowing the study of human responses to dengue infection. One technique has been the EBV immortalisation of memory B cells and the other uses a sensitive single cell immunoglobulin PCR. The latter of which was used in this study. The disadvantages of EBV immortalisation are the inefficiency of transformation (1-3%) that is highly dependent on cell stage, and thus cannot be used to assess immunoglobulin usage within a cell population. Also, as only memory B cells can be assessed, the acute response to infection cannot be studied. The technique used in this study was chosen for a number of reasons. Firstly, it uses activated plasmablasts during an active infection. This means that many of the cells are antigen specific and reflective of the natural immune response. This method is also highly efficient and allows relatively easy production of large numbers of unbiased monoclonal antibodies.

Work using these new techniques has shown us that the human response to infection is quite different from that in the mouse model. In mice the antibody response is dominated by neutralising antibodies to the E protein, domain III (EDIII) [113] [114]

[115] [116]. In humans, EDIII antibodies are much less frequent. Through studies using immunodepletion, it has been shown that these antibodies have little effect on neutralising capacity. In fact, a large proportion of antibodies produced in humans are against prM and the fusion loop (a highly conserved short section of domain II) [120] [148] [149].

Acute phase antibody response to dengue

There have been three studies to date investigating the acute antibody response to dengue infection. Two of the studies are based on the same patient data. In the studies by Pipattanaboon and Setthapramote, four patients with acute infection (1 week after onset of fever) and five patients with convalescent infection were studied. From the four acute patients, 111 monoclonal antibodies were generated. Serological reactivity was assessed by immunofluorescence. This revealed that 90% of clones derived from acute phase patients were cross reactive to all four serotypes, whereas only 46.7% of clones from convalescent patients was cross reactive to all four serotypes. Epitope binding was assessed by immunofluorescence. The majority of antibodies were directed against E protein (89%), with only small numbers directed against prM (7%), NS1 (4%) and none to C protein. However, 60% of the antibodies were generated from one patient, so caution should be applied when assessing these figures. The patient that contributed most antibodies had an antibody response to all three proteins investigated, with 84% being against E, 12% being against prM and 4% being against NS1. Of the mAbs from the convalescent patients, two were reactive with E (17%), two with prM (17%), 8 with NS1 (66%) and none with C [150, 151].

In the second study, this by Sasaki, three patients with acute dengue (day 5-8 after onset of fever), one of whom had dengue haemorrhagic fever, were investigated. 17 monoclonal antibodies that were strongly neutralizing to all four serotypes were selected for investigation; therefore breadth of antibody response cannot be assessed from this study. All of the mAbs reacted to E protein. It was found by western blot, that 13 of the 17 mAbs had an epitope between residues 53 and 132, the epitope lying across

DI and DII of the E protein. The remaining 4 mAbs did not react with either truncated forms or the full length form, indicated that their binding is conformation dependent [126].

Convalescent sera

There have been three studies investigating the breadth of antibody response to dengue in convalescence. In the study by de Alwis, 1 patient with convalescent primary dengue was assessed for breadth of response. They found that 93% (13/14) of antibodies cross reacted with all four dengue serotypes. The majority of the clones mapped to prM (71%), with 7% mapping to E protein and a further 21% that were not reactive on western blot [148]. A second study isolated 70 mAbs from 5 donors, 3 with convalescent primary infection and 2 with secondary. Of the 70 mAbs, 47 (67%) were reactive to E protein. Of the patients with primary disease, mAb reactivity was as follows: DI/II 39%, DIII 20%, prM 12%, NS1 17%, NS3 10%, C 2%. Patients with secondary infection had the following mAb responses: DI/II 77%, DIII 17%, prM 3%, not classified 3%. In the third paper 40 mAbs were isolated with 48% being direct against E protein and 8% being directed against NS1 [143]. However, reactivity to other epitopes was not tested [152].

These studies demonstrate that the breadth of antibody response in both acute illness and convalescence varies markedly between individuals with some having a dominant response to E and other recognising a wide variety of epitopes.

Isolation of anti-dengue antibodies

In this study, I isolated recombinant antibodies from an acute case of dengue haemorrhagic fever. A dot blot was performed to assess whether the antibodies bound to dengue 1, or indeed any other serotype of dengue or Japanese encephalitis virus. I

found that despite the very early time point in infection (day -1 before defervescence), the patient was already producing antibodies against dengue. Of the 53 antibodies assessed, 7 (13%) showed reactivity to dengue. Three of the 7 clones (43%) reacted to dengue 1 only, 3 (43%) were cross reactive to all four dengue serotypes and a further clone (14%) cross reacted not only with all four dengue serotypes, but also Japanese encephalitis virus. It has been well known that in early convalescence following dengue infection, patients develop cross reactive antibodies to all four serotypes and that this acts as protection from a further dengue infection for up to 18 months. However, it is interesting to see that these antibodies are being produced so early in infection. This concurs with the data from Setthapramote showing a high degree of cross reactivity among antibodies produced in the acute phase. However from the results produced by this study, this is not the full story. It appears antibody responses are not all cross-reactive, but that there is a specific anti-dengue 1 immune response very early in infection.

Assessment of epitope binding of antibodies

A western blot was performed to assess whether the antibodies bound to either prM or E protein. Interestingly, we found that no antibodies bound to either structural protein. This suggests that either the antibodies produced were against another dengue protein, or else they bind across a complex epitope. Although the western blot was performed in non-reducing conditions, the proteins may have lost some of their natural conformation and therefore the results may be misleading. Further assessment of epitope binding will need to be performed, by repeating the western blot and assessing binding using an alanine scan. To this end a VLP library was generated.

prME VLP library and alanine scan

In order to identify the precise location of mAb binding within either the prM or the E proteins a VLP library was created. Each amino acid from the exposed surfaces of the E and prM proteins is substituted within an alanine amino acid. The mAbs are then tested by capture ELISA against these mutated proteins and a key residue for binding is found when binding to the protein is lost. Alanine is chosen for its small, chemically inert methyl group and its ability to mimic the secondary structure of many other amino acids. There are limitations to this technique. The prME VLPs are not homogenous in structure and most are smaller than the dengue virion, containing only 30E dimers, displaying a different arrangement to the 90E dimer virion. The difference in packing of structural proteins may therefore alter the ability of the alanine scan to detect quaternary binding. The use of alanine as a substitute may also be too conservative to assess epitope binding, because either the electrostatic charge is too weak or else the epitope binding may rely on binding to the main amino acid chain rather than the side chain. However, it had been used successfully to identify many epitopes for mAb binding [118].

A prME VLP library had already been generated within the laboratory too much of the prM and E proteins. However, a new epitope, the C10 epitope had recently been identified in the laboratory, and as such the library required extending. 67 residues were identified as part of the C10 epitope. Of these we managed to create 65 stable mutants, and express 59 of them through 293T cells. Transfection may have failed in 6 cases either because of a failure in transfection or because the mutations introduced to the VLPs affected their excretion from the cells. Therefore the transfection plates of the failed transfections were stored for later staining to determine whether the VLPs remained in an intracellular compartment. Unfortunately because of the time taken to generate the VLP library, we were unable to test our clones against the library. This work will be performed in the laboratory at a later date.

For the antibodies that appear particularly interesting, X-ray crystallography could be additionally used to assess epitope binding further. Further assessment of the antibodies generated would also include functional assessment of the mAbs. This would involve performing neutralisation and antibody-enhancement assays. Thus describing not only what early antibodies to primary infection bind to, but also whether they are helpful at controlling infection or are detrimental through virus enhancement.

Summary

In this study, monoclonal IgG antibodies were isolated from a patient with primary severe dengue whilst still in the febrile phase of the illness. Of the 53 monoclonal antibodies produced, seven were reactive against dengue. Of these, 43% were reactive to the infecting serotype only, whilst another 43% were reactive to all four dengue serotypes. This demonstrates that an IgG response is produced very early even in primary infection and that it is not only serotype specific, but some of the response is cross reactive. Western blot of the antibodies did not reveal the epitopes to which the antibodies react.

Further proposed work

Unfortunately, due to time pressure I was not able to complete all my investigations. As a result, I would like to propose future work that may be useful in studying the area further.

Firstly, I would like to investigate epitope binding further. In this, I would like to repeat the Western Blot and extend the epitopes tested against to include C, NS1 and perhaps other non-structural proteins. I would also like to test the antibodies produced against the VLP library I have generated. Hopefully, through these experiments, I would be able

to elucidate which epitopes the antibodies react against. The results would allow us to understand whether in severe primary disease, antibodies are contributing to pathogenesis, as prM antibodies are thought to enhance infection by rendering non-infectious particles infectious.

The second set of experiments I would like to undertake would be to assess the functionality of the antibodies. By performing neutralisation and enhancement assays, I could assess the effect of the antibodies on infection. Again help assess the contribution of antibodies to pathogenesis in severe primary disease.

Thirdly, I would like to return to the primary samples and assess IgM production and its contribution to anti-dengue antibodies and pathogenesis. I feel that this would be very instructive in understanding the relative contribution of antibodies to disease.

Fourthly, it would be very interesting to compare the results obtained from this patient with severe primary disease to that of a patient with primary dengue but without severe disease and ideally an asymptomatic primary patient as well. This in particular would give an insight into the role of antibodies in the development of clinical disease.

Studying patients with primary disease gives an interesting insight into the body's response to dengue and why some patients are incredibly ill, whilst most are asymptomatic. This is a fascinating area of research that should prove fruitful not only improving knowledge, but also in the design of safe and effective vaccination.

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Appendix

Appendix 1. Dengue E protein amino acid reference sequences

DENV-1

MRCVGIGNRDFVEGLSGATWVDVLEHGSCVTTMAKDKPTLDIELLKTEVTNPAVLRKLCIEAKISNT
TTDSRCPTQGEATLVEEQDANFVCRRTFVDRGWNGCGLFGKGS LITCAKFKCVTKLEGKIVQYENLK
YSVIVTVHTGDQHGVNETTEHGTIATITPQAPTSEIQLTDYGALTLDSPRTGLDFNEMVLLTMKEK
SWLVHKQWFLDLPLPWTSGASTPQETWNREDLLVTFKTAHAKKQEVVVLGSQEGAMHTALTGATEIQT
SGTTKIFAGHLKCR LKMDKLT LKGMSYVMCTGSFKLEKEVAETQHGTVLVQVKYEGTDAPCKIPFSTQ
DEKGV TQNGRLITANPIVTDKEKPVNIEAEPFGE SYIVVGAGEKALKLSWFKKGSSIGKMLEATARG
ARRMAILGDTAWDFGSIGGVFTSVGKLVHQIFGTAYGVLFSGVSWTMKIGIGILLTWLGLNSRSASLS
MTCIAVGMVTLYLGVMVQA

DENV-2

MRCIGMSNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYCIEAKLTN
TTTESRCPTQGEPSLNEEQDKRFVCKHSMVDRGWNGCGLFGKGGIIVTCAMFRCKKNMEGKVVPEN
LEYTIVITPHSGEEHAVGNDTGKHGKEIKITPQSSITEAELTGYGTVTMECSPTGLDFNEMVLLQM
ENKAWLVHRQWFLDLPLPWLPGADTQGSNWIQKETLVTFKNPHAKKQDVVVLGSQEGAMHTALTGAT
EIQMSSGNLLFTGHLKCR LKMDKLT LKGMSYSMCTGKFKVVKEIAETQHGTIVIRVQYEGDGSPCKI
PFEIMDLKRVHLGR LITVNP I VTEKDSPVNIEAEPFGDSYIIIGVEPGQLKNWFKKGSTLGKAF
STTLKGAQRLAALGDTAWDFGSIGGVFN SIGKAVHQVFGGAFRTLFGGMSWITQGLMGALLLWMGVN
ARDRSIALAFLATGGVLVFLATNVHALETSIAIIX

DENV-4

MNQRKKVVRPPFNMLKRERNRVSTPQGLVKRFSTGLFSGKGPLRMVLAFITFLRVLSIPPTAGILKRW
GQLKKNKA I KILTGRKEIGRMLN I LNGRKRSTMTLLCLIPTAMAFHLSTRDGEPLMIVAKHERGRPL
LFKTTEGINKCTLTAMD LGEMCEDTVTYECPLLVNTEPEDIDWCNLTSAWVMYGTCTQSGERRREKR
SVALTPHSGMGLETRAETWMSSEGAWKHAQRVESWILRNPGFALLAGFMAYMIGQTGIQRTVFFVLMM
LVAPSYGMRCVGIGNRDFVEGVSGGAWVDLVLEHGSCVTTMAQ GKPTLDFELIKTTAKEVALLRTYCI
EA

New insights into the immunopathology and control of dengue virus infection

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Abstract | Dengue virus poses a major threat to global public health: two-thirds of the world's population is now at risk from infection by this mosquito-borne virus. Dengue virus causes a range of diseases with a small proportion of infected patients developing severe plasma leakage that leads to dengue shock syndrome, organ impairment and bleeding. Infection with one of the four viral serotypes results in the development of homotypic immunity to that serotype. However, subsequent infection with a different serotype is associated with an increased risk of developing severe disease, which has led to the suggestion that severe disease is triggered by immunopathology. This Review outlines recent advances in the understanding of immunopathology, vaccine development and human monoclonal antibodies produced against dengue virus.

Aedes mosquito vector
The genus of mosquito by which dengue virus is transmitted; the primary vector being *Aedes aegypti* followed by *Aedes albopictus*.

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Dengue is a positive-sense RNA virus belonging to the Flaviviridae family. There are four distinct serotypes — dengue virus serotype 1 (DENV1) to DENV4 — and several genotypes within each serotype. Since the 1950s, there has been considerable expansion in the geographic spread of dengue and an exponential rise in disease incidence. It is estimated that the annual global incidence is 390 million cases, of which 96 million are clinically apparent¹. There are several factors that drive this pandemic, including globalization, the spread of the *Aedes* mosquito vector, inadequately planned urbanization and the absence of a licensed vaccine or anti-dengue therapeutics².

The clinical phenotype of dengue can vary depending on several factors, of which age, genetic predisposition and background immunity are major determinants. However, most clinical infections result in a self-limiting febrile illness termed dengue fever. The hallmark feature of severe disease is increased capillary permeability, causing plasma leakage, which can lead to haemodynamic compromise and dengue shock syndrome (DSS) (BOX 1). If untreated, severe disease can lead to a mortality rate of up to 20% of affected individuals but with expert management and primarily careful fluid replacement, this can be reduced to less than 1%².

The pathogenesis of severe disease is thought to be due, at least in part, to immune mechanisms in which antibody enhancement and T cell immunopathology probably have key roles. These theories have served to

explain the observation that the development of severe disease is associated with serial infection with dengue viruses of different serotypes. However, because of this risk of severe disease in sequential infections, developing a safe vaccine that provides protection against all four serotypes has been challenging. There have been several advances in understanding and tackling dengue virus infection over the past decade, including viral epitope mapping and defining conserved neutralizing antibodies, as well as the publication of the first Phase III vaccine efficacy trials. This Review focuses on the adaptive immune response to dengue virus infection, the challenges of developing a dengue virus vaccine and new insights from the study of human monoclonal antibodies (mAbs).

Dengue virus

Dengue virus is a relatively simple positive-sense single-stranded RNA virus that is 50 nm in diameter and has three structural proteins — capsid (C) protein, precursor membrane (prM) protein and envelope (E) protein — as well as seven non-structural (NS) proteins termed NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (FIG. 1). Studies using X-ray crystallography and cryo-electron microscopy (cryo-EM) have shed considerable light on structural aspects of the flaviviral life cycle^{3–9} (discussed later).

E protein and prM protein form the glycoprotein shell of the virus, and E protein is responsible for host cell binding and entry¹⁰. Dengue E protein has two N-linked glycosylation sites and is divided into three domains

Box 1 | Clinical manifestations and treatment of dengue virus infection

- The majority of patients infected with one of the four dengue virus serotypes are either asymptomatic or present a self-resolving febrile illness, termed dengue fever, that lasts 4–8 days.
- Key clinical manifestations include high fever, headache, retro-orbital pain and muscle pain, as well as other well-recognized signs including rashes, abdominal pain, vomiting and mucosal bleeding.
- The defining feature of severe disease is increased capillary permeability, which causes plasma leakage that can occasionally lead to shock and death¹⁴⁶.
- Other severe manifestations include haemorrhage and organ impairment, such as hepatitis, myocarditis and encephalitis.
- Clinical symptoms vary by age of the infected individual, with children being more at risk of shock and adults more likely to develop organ impairment and bleeding¹⁴⁷.
- The treatment of dengue is supportive care, in the form of intravenous fluids for patients with haemodynamic compromise from plasma leak and for those unable to tolerate oral rehydration.
- There have been several recent clinical trials investigating novel antiviral and immune modulators; however, none of these treatments has demonstrated any clinical benefit in dengue to date¹⁴⁸.

termed EDI, EDII and EDIII⁵. During viral assembly in the endoplasmic reticulum, 180 copies of E protein associate with 180 copies of prM protein to form 60 trimeric (heterohexameric) spikes, which gives immature virions their characteristic spiky appearance¹⁰ (FIG. 2a). In the *trans*-Golgi network, prM protein is cleaved at a membrane proximal site by host-encoded furin protease, resulting in a membrane-anchored M stump and a pr molecule that remains associated with the virion until it is secreted. Upon secretion from the host cell, pr protein disassociates to leave the 'mature virion', which has a smooth structure (FIG. 2b).

The cleavage of prM protein is not complete in all dengue virions, leaving a proportion of partially mature dengue virions that contain a varying amount of cleaved and uncleaved prM protein^{11–14}. The cleavage of prM protein is more efficient in certain cell types, particularly primary human cells such as dendritic cells, compared with the cleavage event in insect cells or tumour cell lines such as Vero^{15,16}. Fully immature dengue viruses contain regular trimeric E–prM protein spikes and are non-infectious^{17,18}. By contrast, the partially mature forms, some of which are infectious, have a less regular structure, with areas that are spiky and contain prM protein–E protein trimers and areas that are smooth and are proposed to contain E protein dimers¹⁹.

Finally, virions attach to cells, possibly through EDIII¹⁰. Several host proteins — such as heparan sulfate, dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN; also known as CD209), heat shock protein 70 kDa (HSP70), HSP90, mannose receptor, CD14, T cell immunoglobulin and mucin domains (TIMs; also known as HAVCRs), TAM receptor protein tyrosine kinases and laminin — have been suggested to have a role in dengue virus binding, but there is no single receptor yet defined that is necessary for entry²⁰. Following endocytosis, acidification in the early endosome triggers a dramatic reorganization of the viral envelope, from the dimer form to a new trimeric conformation.

This exposes the fusion loop at the tips of the trimeric spikes, which then fuses with the endocytic membrane and allows the viral nucleic acid to be released into the host cell cytoplasm¹⁰ (FIG. 1).

Immune involvement of dengue pathogenesis

There are several key observations that suggest a role for the immune system in dengue pathogenesis: first, severe dengue usually, but not exclusively, occurs with secondary infection, implying that priming of the adaptive immune system to one dengue virus serotype leads to disease enhancement during a secondary infection with a different serotype^{21,22}. Second, the peak in symptoms occurs when viral loads are rapidly decreasing, implying that immunopathology may be driven as a consequence of viral control^{23,24}. Third, higher viral loads and/or soluble NS1 levels in patients with dengue haemorrhagic fever versus patients with dengue fever predispose to increased disease severity, which manifests when the virus is controlled and viral loads fall^{25–28}. Finally, the peak in symptoms, vascular leak and viral control coincide with a cytokine storm, which is characterized by high circulating levels of many pro-inflammatory mediators such as tumour necrosis factor (TNF), soluble TNF receptor 1 (sTNFR1), sTNFR2, interferon- γ (IFN γ), CXC-chemokine ligand 8 (CXCL8), CXCL9, CXCL10, CXCL11, CC-chemokine ligand 5 (CCL5) and vascular endothelial growth factor A (VEGFA), as well as the anti-inflammatory cytokine interleukin-10 (IL-10)^{24,26–30}. This cytokine storm has been proposed to trigger increased vascular permeability and resolves relatively quickly upon patient convalescence.

The exact mechanisms underlying the capillary leak are probably more complex than a cytokine storm, which is also associated with other diseases with very different clinical phenotypes, such as graft-versus-host disease³¹, septic shock³² and influenza virus infection³³. Dengue virus may cause increased vascular permeability through binding to the endothelial glycocalyx layer, which lines the luminal surface of microvessels, providing vital barrier functions to capillaries. Both the virus and NS1 protein have been shown to bind to heparan sulfate, a major component of the glycocalyx layer^{34,35}, which may in turn alter the permeability of capillaries (FIG. 3).

NS1-mediated complement activation may also play a part in the altered capillary permeability through the generation of the anaphylatoxins C3a and C5a and the terminal complement complex C5b–C9 (REF. 23). High C5b–C9 levels in plasma and pleural fluid have been demonstrated in patients with dengue shock, and patients who were high producers of C5b–C9 had more signs of plasma leakage²³. Furthermore, NS1 may have an immunomodulating role by binding to and reducing the functional capacity of C4, thereby altering the classical and lectin complement pathways³⁶.

Innate immune cells also have a role in dengue pathogenesis; for example, several studies suggest that mast cells have a role in both protection against and pathogenesis of dengue. Upon interaction with dengue virus,

Cytokine storm
An excessive production of pro-inflammatory cytokines that occurs during acute dengue infection and that has been proposed to drive the vascular leak.

mast cells release chemokines that recruit T cells, natural killer (NK) cells and NKT cells to control the infection³⁷. By contrast, the interaction also leads to the secretion of mediators such as chymases — proteases that may contribute to vascular leakage. The level of chymases is correlated with disease severity³⁷.

Overall, the well-established association of severe dengue with secondary infection, the evidence of extreme immune activation in severe infection and the coincidence of viral control with the peak of symptoms has led to the detailed study of both T cell- and B cell-mediated adaptive immune responses, which are reviewed here.

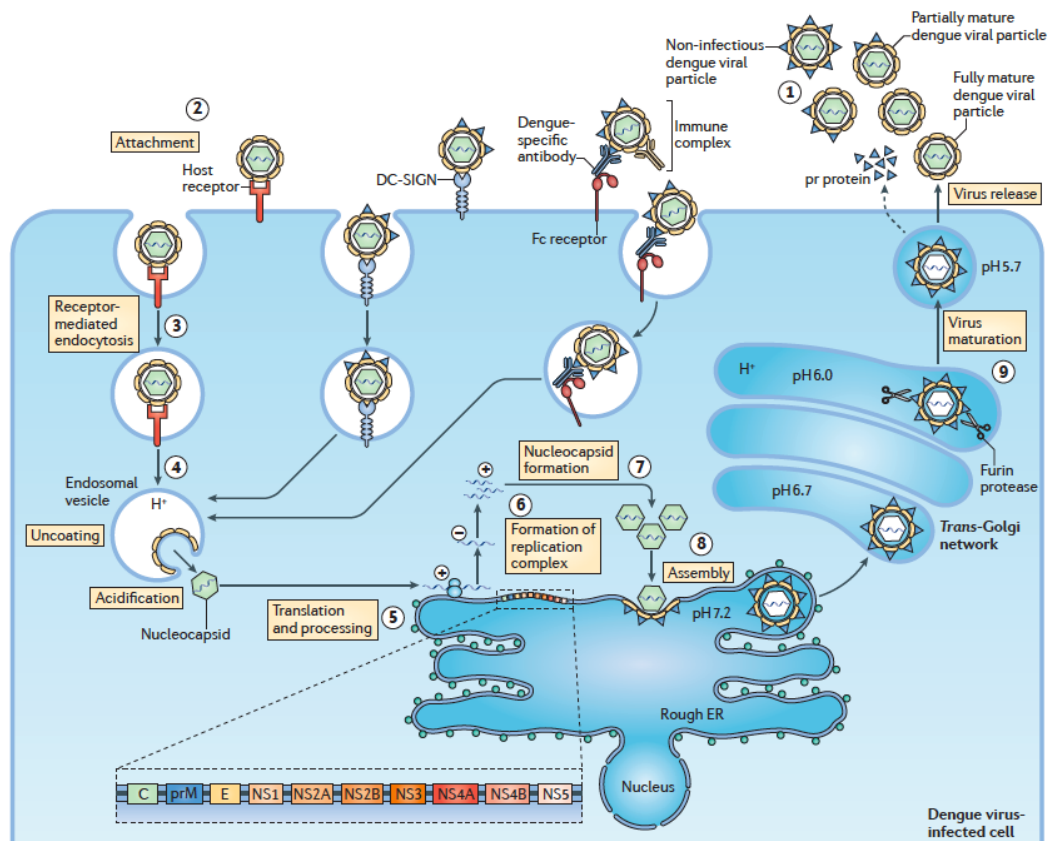


Figure 1 | The life cycle of dengue virus. Dengue virus exists as a number of different viral forms depending on the degree of precursor membrane (prM) protein cleavage. Fully immature dengue virus particles contain a full complement of prM proteins and are non-infectious, whereas all prM proteins are cleaved in fully mature virus particles. A number of intermediate, partially mature forms exist in which some prM proteins have been cleaved and some remain intact. Fully mature and some of the partially mature virus particles are infectious (step 1). The dengue viral replication process begins when a virion attaches directly to a diverse group of host cell receptors or when the Fc portion of a dengue virus-containing immune complex attaches to a Fc receptor on the target cells (step 2) and subsequently enters the cell by receptor-mediated endocytosis (step 3). Acidification of the endosomal vesicles triggers conformational changes in the virion, resulting in an irreversible trimerization of the viral envelope (E) protein (not shown). This exposes the fusion peptide and mediates fusion between the viral and the endosomal membranes, allowing the release of the nucleocapsid into

the cytoplasm. The viral RNA is released into the cytoplasm and presented to the rough endoplasmic reticulum (ER) (step 4). At the ER, viral RNA is translated into a single polyprotein that is processed by viral and host proteases (step 5). After the viral replication complex is synthesized, viral RNA translation switches off, and RNA synthesis begins by the transcription of an antisense viral RNA followed by the amplification of viral RNA (step 6). The newly synthesized RNA is subsequently packaged by capsid (C) protein, forming a nucleocapsid (step 7). Virus assembly occurs on the surface of the ER when the nucleocapsid buds into the ER lumen, resulting in non-infectious, immature viral particles (step 8). Immature viral particles are transported through the Golgi into the trans-Golgi network, where acidification induces conformational changes of the virion and exposes the furin cleavage sites. The host protease furin cleaves between pr protein and M protein, with the pr protein remaining associated until the virion is released in the neutral pH of the extracellular milieu (step 9). DC-SIGN, dendritic cell-specific ICAM3-grabbing non-integrin; NS proteins, non-structural proteins.

REVIEWS

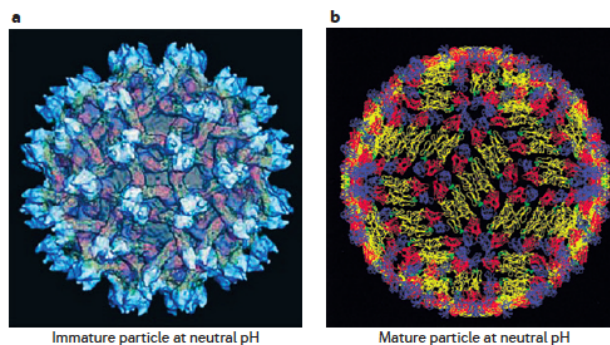


Figure 2 | Structure of the dengue viral particle. a | The structure of an immature dengue viral particle shows the arrangement of envelope (E) proteins in pink and precursor membrane (prM) proteins in light blue. b | The mature dengue virus shows the arrangement of the E proteins into a smooth particle. Figure in part a from REF. 149, Nature Publishing Group. Figure in part b reprinted with permission from REF. 3, Elsevier.

Tetramer staining

A technique used to track antigen-specific T cells by flow cytometry. Biotinylated monomeric MHC molecules are folded *in vitro* together with a specific peptide in the binding groove. These peptide–MHC complexes are tetramerized using a fluorescently labelled streptavidin molecule. The tetramers bind T cells that express T cell receptors specific for the cognate peptide–MHC complex.

Original antigenic sin

An immune response to serial exposure to two pathogens of similar yet distinct sequences. Upon a second infection, rather than making an entirely new immune response to antigens of the secondary infecting pathogen, cross-reactive and potentially suboptimal memory cells that were generated during the first infection are expanded. Original antigenic sin was first described for antibody responses but has also been demonstrated in T cell responses.

The role of T cells in dengue pathogenesis. The association of severe dengue symptoms with a rapid decline in viral loads and a peak of pro-inflammatory cytokine secretion have led to the proposal of a role for a T cell-mediated immune response in driving immunopathology in severe dengue. There has been much study of T cell responses in dengue, but the contribution of these responses to pathogenesis and protection remains controversial³⁸. Reports have demonstrated potent T cell responses in dengue-infected individuals, and many dengue T cell epitopes have been mapped in humans and mouse models^{39–46}. Responses to NS3 seem to be immunodominant in humans, although CD8⁺ and CD4⁺ T cells that respond to epitopes across the whole virus proteome have been observed^{39,41,44,46–50}.

The magnitude of the T cell response positively correlates with disease severity^{39,42,43,51,52} with many cells during the acute disease displaying markers of both proliferation and programmed cell death⁴². Some scientists have questioned the relevance of T cells to dengue pathogenesis and control because of the relatively low numbers of circulating T cells seen during acute infection that increase rapidly following viral clearance^{42,53,54}. However, a recent study showed that skin blister fluid taken from dengue-infected patients contained more dengue-specific T cells than peripheral blood, suggesting that dengue-specific T cells may migrate from the peripheral blood to the skin during acute infection and return upon viral clearance⁵⁵.

During secondary dengue virus infection, responding T cell populations rapidly expand and can account for up to 20% of circulating T cells⁵³, and cross-reactive T cells in dengue have been well studied^{39,42,43,46,50,54,56,57}. Using double tetramer staining with tetramers containing peptides from either the primary or secondary infecting viruses, we and others have studied the cross-reactivity of CD8⁺ T cell responses^{42,43,54}. These studies provide evidence for original antigenic sin in

secondary T cell responses to dengue virus. During a secondary infection, there is a large increase in the number of T cells that bind to tetramers loaded with peptides derived from the primary dengue virus infection⁴². Some of these cells cross-react with tetramers loaded with peptide derived from the secondary infecting virus, whereas others show lower avidity for the tetramer from the secondary virus and no functional responses to the secondary virus-derived peptides. Original antigenic sin has previously been reported for CD8⁺ T cell responses to influenza virus⁵⁸ and lymphocytic choriomeningitis virus (LCMV)⁵⁹, and also appears to operate in dengue virus infection⁴². Some dengue-specific T cell populations that expand during secondary infection have been shown to be fully cross-reactive to antigens of the serotypes of the primary and secondary infections, whereas other populations show preference for the primary-infecting serotype^{42,43,50}. The expansion of these low-avidity cells specific for the primary infection may delay viral clearance and thereby lead to higher viral loads.

In another study, the presence of original antigenic sin was demonstrated in the dengue T cell response, but the magnitude of the response did not seem to be compromised⁴⁴. This study differed from the one described earlier in that it measured functional responses to peptide stimulation and did not measure non-functional cells with tetramer staining or formally show cross-reactivity, which was inferred on the basis of the similarity of peptide sequences. Thus, in the absence of a good animal model of disease, it remains controversial whether the expansion of low-avidity cross-reactive T cells in secondary dengue infection contributes to disease pathogenesis.

Study of the function of dengue-specific T cells has revealed an interesting difference between mild and severe infections. During milder dengue fever illness, CD8⁺ T cells degranulate but produce lower levels of pro-inflammatory cytokines³⁹. By contrast, during severe primary and secondary infections, a high proportion of CD8⁺ T cells produce the cytokines TNF and IFN γ but do not undergo marked degranulation, potentially negatively affecting virus control while enhancing disease pathogenesis and immunopathology³⁹. CD4⁺ T cell populations also expand during dengue virus infection and, similarly to CD8⁺ T cells, the magnitude of the CD4⁺ T cell response correlates with disease severity^{39,46}. Interestingly, several studies have reported the generation of dengue-specific CD4⁺ T cells that express degranulation markers or can lyse peptide-pulsed target cells, which could have a role in the disease^{39,46,57,60}.

As mentioned above, the role of T cells in dengue disease pathogenesis and protection is debated. On the one hand, potent T cell responses skewed to cytokine production and not degranulation are associated with, and potentially drive, severe disease^{42,43}. On the other hand, in milder disease, CD4⁺ or CD8⁺ T cells may have a beneficial role in controlling virus replication. An analysis of T cell responses in individuals from a large Sri Lankan dengue cohort has suggested a role for T cells in protecting from dengue: higher-amplitude

T cell responses were observed for epitopes presented by protective HLA alleles⁴⁴. However, most HLA association studies in dengue have been small and not repeated⁶¹. Furthermore, depletion and adoptive transfer of CD8⁺ and CD4⁺ T cells in mouse models have suggested a role for these cells in reducing viral loads and protecting from severe disease^{45,62,63}. Other investigators who question the importance of T cells in dengue pathogenesis point out that severe dengue can occur during a primary dengue infection in which cross-reactive T cells and original antigenic sin would not be operative⁶⁴.

Finally, it has been speculated that the suboptimal efficacy of the recent Sanofi Pasteur vaccine in clinical trial (see later) may in part be driven by the lack of a protective T cell response, as all of the NS proteins in this tetravalent vaccine derive from the yellow fever virus. We suggest that the generation of an early T cell response to dengue virus may be protective, whereas the late generation of T cell populations that have a proportion of low-avidity T cells and are skewed to inflammatory cytokine production in the absence of degranulation may predispose to immunopathology in the presence of high viral or antigen loads and contribute to the cytokine storm and vascular leak³⁹ (FIG. 3).

Antibody-dependent enhancement. The theory of antibody-dependent enhancement (ADE) attempts to explain why severe dengue is usually associated with secondary infections in children and adults^{21,22,65}. The ADE hypothesis posits that pre-existing heterologous antibodies generated in response to a primary infection may not be of sufficient avidity or concentration to neutralize a secondary infection by a different dengue serotype, in which the sequence of E protein may vary by 30–35% at the amino acid level. Instead, the virus may be opsonized and targeted for uptake into Fc receptor (FcR)-bearing cells, such as monocytes and macrophages, which are major sites of dengue virus replication *in vivo* (FIG. 3b). It has also been shown that dengue viruses interact with leukocyte immunoglobulin-like receptor B1, which leads to inhibition of FcR-mediated signalling induced by dengue virus-specific antibody complexes and the downstream induction of antiviral type I IFN-stimulated genes⁶⁶.

ADE can be readily demonstrated *in vitro* using FcR-expressing cells and has been shown to drive higher viral loads in both mouse and primate models of dengue virus infection⁶⁷. ADE has also been suggested to lead to increased disease severity in infants experiencing primary dengue virus infection during the first year of life when the levels of passively transferred maternal dengue-specific antibodies fall below neutralizing levels^{21,68–70}. In mouse models, ADE can increase disease severity, leading to vascular leakage and lethality^{67,71}. Polymorphisms in the gene encoding the low-affinity FcR for IgG (FCGR2A) have been suggested to have a role in ADE; there is an association of the H/H and H/R alleles at position 131 with severe infection, whereas the 131 R/R allele is associated with protection from severe disease^{72–74}.

Antibodies directed against prM protein can facilitate FcR-mediated uptake of immature viral particles which, although non-infectious in the absence of antibody, can undergo furin-mediated prM protein cleavage following endocytosis in the host cell, rendering them infectious^{15,18,75}. A similar phenomenon has also been demonstrated following ADE driven by antibodies specific for E protein⁷⁶. At sub-neutralizing concentrations, almost all antibodies specific for E and prM proteins can enhance viral uptake, with maximum levels of enhancement occurring at roughly half the concentration required for neutralization^{77,78}. In infections with West Nile virus, a structurally related flavivirus, it has been estimated that around 15–29 E protein-specific antibodies bound to a virion will promote ADE⁷⁸. The exact role of ADE in human infection is much debated and has been a great concern in vaccine studies. The very recent data (described later) from the Sanofi Pasteur vaccine trials may be the first evidence to substantiate this concern.

In summary, antibodies and T cells have been implicated in the generation of both protective and pathogenic responses during dengue virus infection. Optimizing protective responses while minimizing pathogenic responses are the goals for successful vaccines.

Dengue vaccines

The exponential rise in dengue virus infections over the past few decades has made the search for a dengue vaccine an imperative, but achieving this goal has proved enormously challenging. Any successful vaccine would need to induce a protective and durable immune response to all four dengue serotypes, preferably with one or two doses, in individuals who have been unexposed to dengue virus, as well as those who have had a previous dengue virus infection. At the same time, a vaccine would need to avoid eliciting or enhancing pathogenic immune responses.

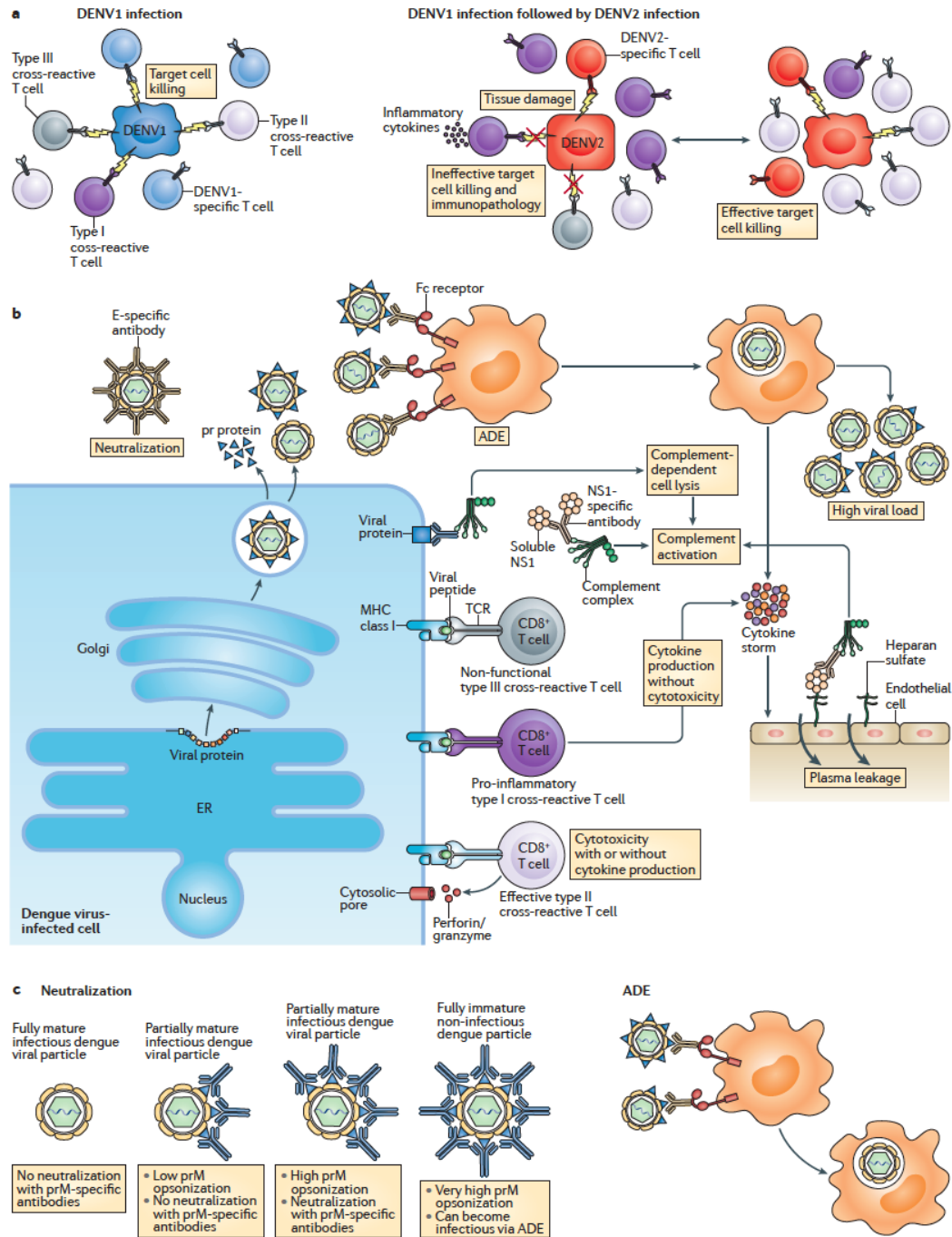
As primary dengue virus infection does not give long-term protection to re-infection with the other three viral serotypes^{79,80}, it has been generally held that a vaccine will need to induce protective responses against all four serotypes, mandating a tetravalent formulation. Efforts to develop vaccines have been pursued for almost 50 years, beginning in Thailand with work to produce live attenuated viruses by serial passage of viral strains representative of the four serotypes⁸¹. A particular challenge has been to develop attenuated forms of the viruses that are not too virulent to induce overt dengue disease but are not too over-attenuated to be unable to incite a protective immune response. Another challenge has been to produce a tetravalent formulation in which all four viruses are delivered together, replicate equally and induce a balanced immune response against all four serotypes rather than resulting in good responses to some serotypes but poor responses to one or more serotypes^{82–84}.

There have been substantial advances in recent years in vaccine development with three live attenuated vaccines currently undergoing clinical trials: CYD-tetravalent dengue vaccine (CYD-TDV), National Institutes of Health (NIH) live attenuated tetravalent vaccine (LATV) Δ30 and DENVax^{85–88} (FIG. 4). There are also

Antibody-dependent enhancement

Antibodies that may not be of sufficient avidity or concentration to neutralize a virus instead opsonize it and promote viral uptake into Fc receptor (FcR)-bearing cells, leading to enhanced viral replication.

REVIEWS



Heterologous prime–boost strategies

Vaccination strategies in which different immunogens are given sequentially to boost and focus an immune response to a given antigen. This is achieved by utilizing a variety of platforms — viral, DNA or protein — with the same antigen being delivered sequentially.

various other vaccines in preclinical studies. The most advanced in clinical trials is the Sanofi Pasteur vaccine CYD-TDV. This is a chimaera vaccine using the related yellow fever 17D vaccine strain as a backbone, with dengue virus prM protein and E protein genes replacing those from yellow fever virus. The vaccine contains a mixture of four recombinant viruses representing each serotype (termed CYD1 to CYD4)^{89,90}. Initial clinical trials demonstrated good serological responses to the vaccine, with seropositivity ranging from 66.5% to 100%^{91–93}. However, the overall efficacy (that is, protection from infection) of a Phase IIb trial in Thailand was below expectations, with the lowest efficacy being against DENV2 (TABLE 1). Phase III trials of this vaccine in Asia and Latin America have been more promising; however, the results still showed suboptimal efficacy ranging from 35% to 78% and, again, the efficacies against DENV2 were lowest^{94,95}. Further analysis revealed that the vaccine gave better protection to vaccinees who were already immune to one or more serotypes before vaccination, and the protection offered by the vaccine against severe and haemorrhagic disease was between 80% and 91%^{94,95}.

◀ **Figure 3 | Adaptive immune responses to dengue virus infection.** a | The phenomenon of original antigenic sin occurs when memory T cell activation in a second infection (for example, with dengue virus serotype 2 (DENV2) in middle and right panels) leads to the domination of that response by cross-reactive T cells generated during a primary infection (for example, DENV1 in the left panel) with a different dengue virus serotype. Three types of cross-reactive T cells are illustrated: type I produce only inflammatory cytokines during secondary infection, type II can degranulate and potentially kill DENV2-infected target cells effectively, and type III cross-reactive T cells are of low avidity and can recognize DENV2-infected cells but do not induce a response. If there is a high proportion of type I cross-reactive T cells that are skewed to inflammatory cytokine production without cytotoxicity, it may predispose to immunopathology on secondary infection (middle panel), whereas if there is a predominance of type II cells that can degranulate (right panel), the virus may be more rapidly cleared. b | The mixture of newly produced dengue virions can be neutralized by the optimum concentration of dengue virus-specific antibodies. In addition, dengue virus proteins — such as envelope (E) protein, precursor membrane (prM) protein and non-structural protein 1 (NS1) — presented on the surface of dengue virus-infected cells, or soluble NS1, can be recognized by dengue virus-specific antibodies. These antibodies can promote complement-dependent cell lysis following complement activation by the classical pathway. Soluble NS1 can also bind to heparan sulfate, a major component of the endothelial glycocalyx layer that regulates capillary permeability. The subsequent recognition by NS1-specific antibodies triggers complement activation and anaphylatoxin formation, which may contribute to the disruption of this layer and vascular leak. Presentation of viral peptides on MHC class I or MHC class II molecules activates DENV-specific CD8⁺ T cells or CD4⁺ T cells, respectively. The recognition of MHC–peptide complexes by DENV-specific T cells or effective type II cross-reactive T cells (only cross-reactive CD8⁺ T cells are shown for simplicity) results in target cell lysis with or without cytokine production, whereas peptide recognition by ineffective DENV cross-reactive T cells (type I and type III) causes only cytokine production or no response. Sub-neutralizing concentrations of E- and/or prM-specific antibodies can bind to the dengue virus and mediate dengue virus infection of Fc receptor-expressing target cells, such as monocytes and macrophages, resulting in antibody-dependent enhancement (ADE) and increased viral load. The cytokines secreted by dengue virus-infected cells and those produced by T cells can be amplified by the high viral load and cause a cytokine storm and plasma leakage in patients infected with dengue virus. c | prM-specific antibodies show poor neutralization but are potent at inducing ADE. Fully mature particles contain no prM protein and will not be neutralized by prM-specific antibodies, whereas low-prM protein-containing particles do not contain enough prM protein for neutralization but can be opsonized for ADE. Some partially mature particles with higher levels of prM protein will be neutralized, whereas fully immature particles are non-infectious but can be made to infect cells via ADE. ER, endoplasmic reticulum; TCR, T cell receptor.

The first report from the long-term follow up of the CYD-TDV vaccine trials has recently been published⁹⁶ and shows that older children in the vaccinated group (aged 9–16 years) continue to benefit. However, in the under-9-year age group, there appears to be an increase in hospitalization at 3 years post-vaccination when compared with control unvaccinated subjects⁹⁶. This increase may represent disease enhancement, possibly by ADE, in individuals who were uninfected at enrolment and who have been primed but not protected by the dengue vaccine. There is now much urgency in the field to understand why the initially promising *in vitro* correlates of vaccine immunity^{85,89–93} did not always translate to good *in vivo* efficacy^{94–96} and to develop more representative *in vitro* correlates of protection to inform future trials.

In the Phase IIb trial of CYD-TDV in Thailand, although the efficacy of the vaccine against DENV2 was low, the seropositivity to DENV2 at 1 year after vaccination was high⁹⁰ (TABLE 1). Several reasons are postulated for this mismatch: first, the vaccine strain might be a poor immunological match for viral strains circulating during the study period, although preclinical trials showed that the vaccine candidate antigens induced neutralizing antibody activity against a broad range of viral strains⁸⁵. Second, T cells may be important for protection, and because the CYD-TDV vaccine contains NS elements from yellow fever virus, it may not produce an effective dengue virus-specific T cell response. Third, the CYD-TDV vaccine does not contain dengue virus NS1, which was suggested to induce protective immunity in mouse models^{97,98}. Finally, the current plaque reduction neutralization test (PRNT), which uses viruses generated from cell lines, may not be a good surrogate for protection *in vivo*, and better, more predictive assays are urgently being sought.

The tetravalent formulation does not mimic the natural situation in which the vast majority of infections are with a single serotype followed by reinfection months to years later^{79,80}. Although studying sequential infections in the population is difficult, surveillance studies suggest symptomatic cases caused by third or fourth infections are rare^{99–101}. This implies that following secondary infection there is some degree of cross protection to the remaining serotypes. Inducing a balanced immune response to all four serotypes using a tetravalent formulation is clearly a major challenge, and heterologous prime–boost strategies have been suggested to overcome this. The observation in the recent Sanofi Pasteur trials that efficacy was better in individuals who had previously been exposed to dengue virus, compared with the uninfected cohort, may also be instructive in this regard, implying that the vaccine can boost pre-existing dengue virus immunity but is poor at producing a protective immune response *de novo* in dengue virus-naïve vaccinees⁹⁴.

Sequential priming and heterologous boosting with the recombinant viruses CYD1 to CYD4 has been investigated in primates¹⁰². CYD2 and CYD3 were inherently less immunogenic, with lower numbers of responders, than CYD1 and CYD4. Priming with CYD1 and CYD2 followed by boosting at day 56 with CYD3 and CYD4 gave better responses when compared with priming and boosting with the tetravalent vaccine, especially with

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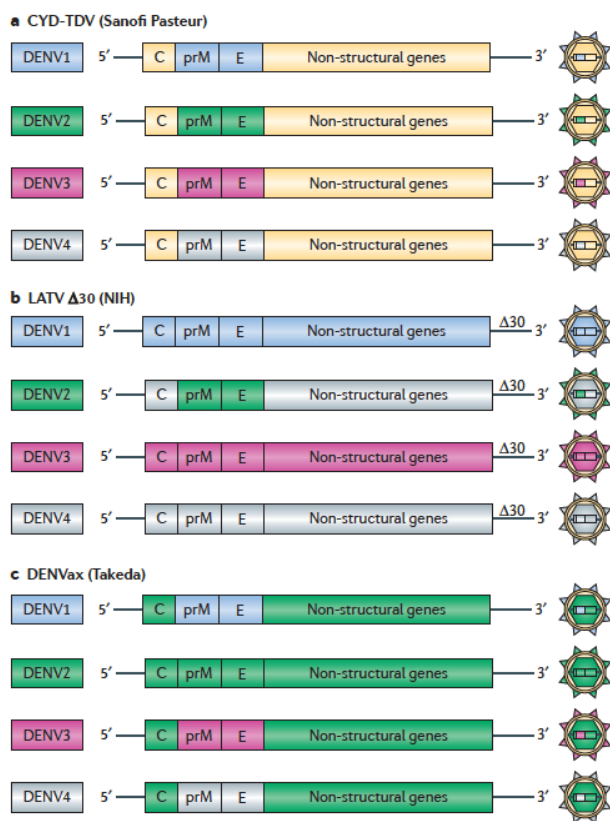


Figure 4 | Dengue vaccines. a | The Sanofi Pasteur vaccine CYD-TDV contains four chimeric live flaviviruses, each derived from the genome of the yellow fever virus 17D vaccine strain (shown in yellow) with the precursor membrane (prM) and envelope (E) gene segments replaced by the corresponding gene segments of each of the four dengue virus serotypes (DENV1 to DENV4). b | The US National Institutes of Health (NIH) live attenuated tetravalent vaccine (LATV) contains a mixture of four recombinant dengue virus genomes; the DENV2 component is a chimeric dengue virus derived from a DENV4 genome with prM and E gene segments replaced by those of DENV2. The vaccine strains were attenuated by deleting 30 nucleotides ($\Delta 30$) from the 3' untranslated region of the dengue viral genome. c | The DENVax vaccine from Takeda contains a mixture of four recombinant DENV2 genomes, each derived from the genome of an attenuated DENV2 virus with prM and E gene segments replaced by the corresponding gene segments of DENV1, DENV3 and DENV4.

regard to responses to DENV2 (REF. 102). Heterologous priming and boosting has also been investigated in humans, in which CYD1 and CYD3 were used to prime and CYD2 and CYD4 used for boosting at 15 weeks¹⁰³. In contrast to the primate study, the sequential bivalent immunization strategy was not superior to the repeated tetravalent immunization approach. It remains possible that the timing between priming and boosting and the serotype combinations used in these two studies may have influenced the outcome.

Of the other live attenuated vaccine candidates, a Phase I trial of the NIH LATV $\Delta 30$ vaccine, which has a 30-nucleotide deletion in the 3' untranslated regions of the cloned cDNA (FIG. 4b), showed that seropositivity across all serotypes was over 90% after one dose of the vaccine; however, around 60% of vaccinees developed a rash and 73% experienced viraemia^{86,104}. The tetravalent live attenuated vaccine DENVax (developed by Takeda) has undergone a Phase I trial⁸⁷, in which more than half of the volunteers developed measurable antibodies to all four virus serotypes after a single dose^{87,105}. Finally, despite the demonstration that the TDEN vaccine candidate developed by the Walter Reed Army Institute of Research (WRAIR) and GlaxoSmithKline induced antibody responses to the four serotypes in a Phase II trial, development has been stopped¹⁰⁶.

Despite the difficulties, there remains a pressing need to pursue vaccine candidates as well as to test whether live attenuated vaccines can be improved to provide a more balanced protection, and whether these responses are durable will be determined by longer-term follow up of clinical trials.

New insights from monoclonal antibodies

A large number of mouse antibodies have been generated against dengue virus, and the epitopes for several of these have been mapped by systematic or random mutagenesis to the E protein^{107–116}. More recently, human mAbs have been generated from dengue virus-infected patients; the main epitopes recognized by mAbs are described in this section (TABLE 2).

Antibodies targeting the fusion loop epitope of E protein.

The fusion loop epitope (FLE) is a major epitope targeted by antibodies produced in both humans and mice. mAbs specific for the FLE are exemplified by mAb E53, which was originally isolated from West Nile virus-infected mice but is fully cross-reactive with dengue viruses¹¹⁷. Mutation of E protein W101 blocks the binding of antibodies directed to the FLE and reduces the reactivity of polyclonal human serum^{118–120}. mAbs specific for FLE show good neutralization of dengue viruses grown in insect cells, which contain high levels of prM protein¹⁶. FLE-specific mAbs bind better in the presence of prM protein which, by virtue of changing the virion architecture, better exposes the FLE¹³. The binding of FLE-specific mAbs to more mature virions produced in primary human cells, which contain lower levels of prM protein, and their subsequent neutralization is not particularly efficient, with neutralization levels typically failing to exceed 60–80% even at high antibody concentrations¹⁶. The induction of antibodies specific for FLE, although commonly generated in humans and often cross-reactive between serotypes, may not therefore be the ideal response to target by vaccines. Another antibody, 1C19, which recognizes the BC loop (amino acids 73, 78 and 79) close to the FLE, potentially neutralized all four serotypes, suggesting that the epitope may be a good target for vaccines. However, it is not clear whether the epitope for 1C19 is prM-dependent in common with other FLE-specific mAbs, as the epitope for 1C19 lies close to the FLE¹²¹.

Table 1 | Dengue vaccine candidates

Vaccine candidate*	Phase	Trial Geographical region	Age of vaccinee	Trial design	Results†	Side effects	Refs
CYD-TDV (Sanofi Pasteur)	Phase III (N=20,869)	Latin America	9–16 years	- Vaccination at 0, 6 and 12 months - Follow up 25 months after final vaccination - Incidence density of dengue: 2.9% per year	- Overall efficacy: 60.8% - Efficacy: 50.3% against DENV1; 42.3% against DENV2; 74.0% against DENV3; 77.0% against DENV4 - Seropositivity at baseline: 83.7% - Seronegativity at baseline: 43.2% - Protection from severe disease: 91.7%; protection from DHF: 90%	- Four SAEs: asthma, urticarial, acute peripheral polyneuropathy and viral meningitis - Seizure - No long-term sequelae	94
CYD-TDV (Sanofi Pasteur)	Phase III (N=10,275)	Asia Pacific	2–14 years	- Vaccination at 0, 6 and 12 months - Follow up 25 months after final vaccination - Incidence density of dengue: 4.7% per year	- Overall efficacy: 56.5% - Efficacy in 2–5 years old: 33.7%; 6–11 years old: 59.5%; 12–14 years old: 74.4% - Efficacy: 50.0% against DENV1; 35.0% against DENV2; 78.4% against DENV3; 75.3% against DENV4 - Seropositivity at baseline: 74.3% - Seronegativity at baseline: 35.5% - Protection from severe disease: 80.8%; protection from DHF: 88.5%	- One SAE: acute disseminated encephalomyelitis - No long-term sequelae	95
CYD-TDV (Sanofi Pasteur)	Phase IIb (N=4,002)	Thailand	4–11 years	- Vaccination at 0, 6 and 12 months - Follow up 25 months after final vaccination	- Overall efficacy 30.2% - Efficacy against DENV1 55.6%, DENV2 9.2%, DENV3 75.3%, DENV4 100% - Seropositivity 1 year after third dose: DENV1 77%, DENV2 85%, DENV3 89%, DENV4 94%	No attributable SAE	90
TDEN (WRAIR and GSK)	Phase II (N=120)	Thailand	20–24 years	- Vaccination at 0 and 6 months - Compared placebo to two formulations: F17 and F19 - Follow up 31 days after final vaccination	- Seropositivity pre-vaccination: 76.5% (F17) and 78.9% (F19) to all four serotypes - Seropositivity post-vaccination: 97.1% F17 and 100% F19 to all four serotypes	- Viraemia in 5 of 80 vaccinees - Rash on >50% of body area in 5% of vaccinees	88
LATV Δ30 (NIH) [§]	Phase I (N=113)	USA	18–50 years	- Single dose of four different admixtures tested (TV001 to TV004) - Vaccinees were flavivirus-naïve	TV003 was the best formula with 45% of vaccinees with neutralizing antibodies against all four serotypes	- Viraemia in 73% of vaccinees - Rash in 64.2% of vaccinees	104
DENVax (Takeda) [§]	Phase I (N=96)	Colombia	18–45 years	- Comparison of placebo versus low and high dose of DENVax and intradermal versus subcutaneous administration - Dengue- and yellow fever-naïve volunteers	71% seroconverted to all four serotypes in the intradermal group - Neutralizing antibody levels to DENV2 were highest	- No SAE - Overall viraemia ranged from 43% (low dose) to 85% (high dose)	87

DENV, dengue virus serotype; DHF, dengue haemorrhagic fever; E protein, envelope protein; GSK, GlaxoSmithKline; LATV, live attenuated tetravalent vaccine; NIH, National Institutes of Health; NMRC, National Medical Research Council; prM, precursor membrane; SAE, serious adverse event; WRAIR, Walter Reed Army Institute of Research. *Soluble E protein (Merck), purified inactivated virus (WRAIR) and DNA-expressing prM protein and E protein (NMRC) vaccine candidates are in Phase I clinical trials. †Efficacy is expressed in % with a 95% confidential interval. §Under Phase II trial.

E protein domain III. Antibodies targeting EDIII have been frequently isolated from mice⁷¹ and are among the most potent antibodies described to dengue virus, with 50% *in vitro* neutralization (NT50) levels at concentrations in the low picomolar range^{71,107–110,112–115}. These antibodies are frequently specific for a single dengue

virus serotype, although the EDIII-binding mAb 4E11 has been engineered to bind to and potentially neutralize all four dengue virus serotypes¹²². The epitopes for some EDIII-binding mAbs have been mapped in detail by mutagenesis, crystallography and cryo-EM. The mAb E16 binds West Nile virus, a flavivirus related

REVIEWS

Table 2 | Characteristics of human monoclonal antibodies

Antigen	Antibody name	Cross-reactivity	NT50 $\mu\text{g ml}^{-1}$	In vivo protection	Structure accession number	Refs
Epitopes on a monomer						
prM protein	Several mAbs	DENV1–DENV4*	Weak (lack of full neutralization)	Not determined	Not applicable	15, 134–136
FLE (amino acids 98–113)	Several mAbs	DENV1–DENV4*	0.016 to >10	Not determined	Not applicable	16,121, 134,150, 151
BC loop E protein domain II (amino acids 73, 78 and 79)	1C19	DENV1–DENV4	0.01–0.05	Prophylactic activity against DENV1 and DENV2 in AG129 mice†	Not applicable	121
EDIII	Several mAbs	Serotype-specific and crossreactive	Strong (mostly serotype-specific) to weak (cross-reactive) <0.07 to >10	Not determined	Not applicable	134,135, 137
Quaternary epitopes						
E protein herring-bone epitope (EDI, EDI–EDII hinge and EDIII)	HM14c10	DENV1	0.005–1.503	Prophylactic and therapeutic activity against DENV1 in AG129 mice†	EMD-5268	139
E protein herring-bone epitope (EDI–EDII hinge, EDII fusion loop and EDIII)	5J7	DENV3	0.1	Not determined	EMD-5935	152,136, 143
E protein (monomer EDI, EDI–EDII hinge and EDIII; intact virion only)	1F4	DENV1	0.11	Prophylactic activity against DENV1 in AG129 mice†	EMD-2442 and PDB 4C2	140
EDE (EDI, EDII and EDIII)	752-2 C8	DENV1–DENV4	0.59–0.17	Not determined	PDB 4UTA	16,141
EDE (EDI, EDII and EDIII)	753(3) C10	DENV1–DENV4	0.012–0.28	Not determined	PDB 4UT9	16,141
EDE (EDI, EDII and EDIII)	747(4) B7	DENV1–DENV4	0.015–1.27	Not determined	PDB 4UT6	16,141
EDE (EDI, EDII and EDIII)	747(4) A11	DENV1–DENV4	0.011–1.17	Not determined	PDB 4UT7	16,141
EDE (EDI, EDII and EDIII)	2D22	DENV2	0.08	Prophylactic and therapeutic activity against DENV2 in AG129 mice†	EMD-2967, EMD-2996, EMD-2997, EMD-2999, EMD-2968, EMD-2998, and EMD-2969	130,152

DENV, dengue virus serotype; ED, envelope protein domain; EDE, envelope protein dimer epitope; E protein, envelope protein; FLE, fusion loop epitope; mAb, monoclonal antibody. *A majority of antibodies in this group recognise all 4 dengue virus serotypes. †Mice that lack type I and type II interferon receptors.

to dengue virus, where the epitope lies on the lateral ridge of EDIII⁷¹. Although not binding to dengue virus, E16 defines a class of antibodies binding to the lateral ridge of EDIII and shows potent neutralization of West Nile virus *in vitro* and *in vivo* in both prophylactic and therapeutic settings¹²³. Another EDIII-specific antibody, 1A1D2, binds to the A strand (amino acids 305–312) of dengue virus EDIII¹²⁴. In contrast to the lateral ridge of EDIII, the A strand is more conserved among the dengue viruses, allowing 1A1D2 to cross-react between DENV1, DENV2 and DENV3 (REF. 111).

Interestingly, the epitope recognized by 1A1D2 is not fully exposed in the smooth mature form of the dengue virion, and cryo-EM has shown that a temperature-dependent conformational change allows EDIII to

hinge up from its flat orientation in the smooth mature virion and facilitates mAb 1A1D2 binding¹²⁴. Because of the potency of the EDIII-specific mAb and the general serotype specificity of the response, EDIII has been proposed as a potential immunogen in a variety of vaccine formulations. However, the contribution of EDIII to the human antibody response appears to be more limited; it has been shown that the depletion of EDIII-specific antibodies from human serum does not reduce its neutralization potential *in vitro* and *in vivo*^{77,125}.

prM protein and virus maturity. As described above, immature dengue virus particles contain prM protein, which acts as a chaperone for E protein and prevents premature fusion to host cell membranes before virus release.

prM protein is cleaved by furin protease in the Golgi and falls away when mature virus particles are released from cells^{47,10}. However, prM cleavage is frequently incomplete, with a range of partially mature virus forms produced with intermediate levels of prM protein¹¹. This produces a challenge for the host immune response, as mature and immature virus particles present markedly different structural determinants at the virion surface. Studies of primary human cells infected with dengue virus suggest that, following an insect bite, the human host is first infected by viruses that have a high prM content; however, viruses produced in human cells will have potentially lower prM content, although the prM content of dengue virus in *ex vivo* samples has not been measured^{15,16}. Several dengue virus-specific antibodies, such as those targeting the FLE or prM protein, lack the ability to neutralize more mature, low-prM protein viruses^{15,16}.

Alternative conformations of the virion surface. The binding of antibodies to viruses is a complex process, in which the packing of E protein and prM protein into the virion lattice affects the accessibility of the target epitopes to antibodies. Several antibodies that bind well to recombinant monomeric E protein fail to show good binding or neutralization of intact virions because the viral epitopes are poorly exposed^{14,17,71,124,126}. The binding of such antibodies may be enhanced by the prM protein content of the virions, as described above for the FLE-specific antibodies, but may also be enhanced *in vitro* by prolonged incubation periods or increased temperature¹²⁷. This has led to the proposition that the traditional view of the virion as an invariant or rigid, smooth or spiky entity (which is necessarily imposed by the solution of cryo-EM structures) is not accurate. Instead, it is proposed that the virion is a dynamic entity that is capable of adopting many different conformations with different thermodynamic stabilities, which is often referred to as virion breathing^{124,128,129}. In this regard, an alternative 'bumpy' form of DENV2 particles has been recently described, in which there is disruption of the regular packing of the E protein dimers at the virion surface at 37 °C^{128,129}. This has implications for antibody binding, as shown by cryo-EM structures of mAb 2D22 binding to DENV2 particles, in which the valency of binding to the bumpy form of the virus was lower than that to the more regular form¹³⁰. The display of an 'ensemble' of different E protein and virion conformations can be continuously sampled and captured by antibodies, explaining the increase in binding of antibodies to dengue virus particles in a temperature- and time-dependent manner^{12,127}.

Human mAbs. The study of antibody responses in humans has been revolutionized by the development of various techniques that have allowed the generation of large numbers of human mAbs. Three main techniques have been used: expansion and Epstein–Barr virus immortalization of memory B cells; single-cell sorting, cDNA cloning and antibody expression from plasmablasts isolated from acutely infected individuals; and optimization by electrofusion of traditional hybridoma technology to make antibodies from memory B cells^{131–133}.

Antibodies to prM protein. prM protein-specific antibodies appear to be a major component of the memory B cell response to dengue virus infection; however, these antibodies show poor neutralization (maximum 30–50%) even at high concentrations^{15,134–137}. prM protein-specific antibodies do not bind to fully mature virions as they lack prM protein, and many partially mature particles do not contain a high enough density of prM protein to allow neutralization; furthermore, they may be sufficient to promote ADE^{15,18}. We have speculated that the inefficient cleavage of prM protein may be an immune evasion strategy, leading to the generation of poorly neutralizing antibodies directed to prM protein, as well as to the FLE, which also form a major component of the human antibody response^{15,16,118}.

The high frequency, low potency and high ADE potential of antibodies directed to prM protein have implications for vaccine design. All attenuated vaccines at an advanced stage of development contain prM protein, whereas the ideal vaccine would focus responses to the E protein and minimize the prM protein component of the response to reduce the potential for ADE. One possible route to this would be the generation of attenuated chimeric viruses with prM protein derived from third party flaviviruses such as Japanese encephalitis or West Nile virus, in which the anti-prM protein response cross-reacts poorly with dengue virus^{15,138}.

Antibodies targeting quaternary epitopes on the virion. Some of the most potent human antibodies bind to conformational and quaternary determinants that are only reproduced on intact dengue virions^{16,130,139–141}. One well-characterized epitope, the so-called 'herringbone epitope', has been described by cryo-EM for the DENV1-specific mAb HM14c10 (REF. 139). This antibody binds to an epitope that bridges between two adjacent head-to-tail E protein dimers, which form a herringbone-like conformation on the mature virion (FIG. 5a). Several other conformationally sensitive antibodies have been mapped by cryo-EM; the DENV1-specific mAb 1F4 binds to a single E protein monomer, but only in the context of the virion, whereas the DENV3-specific mAb 5J7 binds across three adjacent E protein monomers with a major component of the interaction across the hinge between EDI and EDII of the central E protein monomer^{140,143}. These conformational antibodies show potent neutralization but are nevertheless serotype specific.

We have recently described the cloning of a large series of E protein-specific mAbs from patients infected with dengue virus¹⁶. One-third of the antibodies bind to a complex epitope present only on intact virions. Interestingly, although most of the panel of human antibodies showed good neutralization of high-prM protein viruses produced in insect cells, only binding of mAbs to the complex epitope on intact virions could fully neutralize low-prM protein virions produced in primary human cells.

These broadly neutralizing antibodies bind to the basic repeating E protein dimers that make up the virion surface lattice, and the epitope is termed the E protein dimer epitope (EDE)^{16,141} (FIG. 5b,c). The antibodies bind

REVIEWS

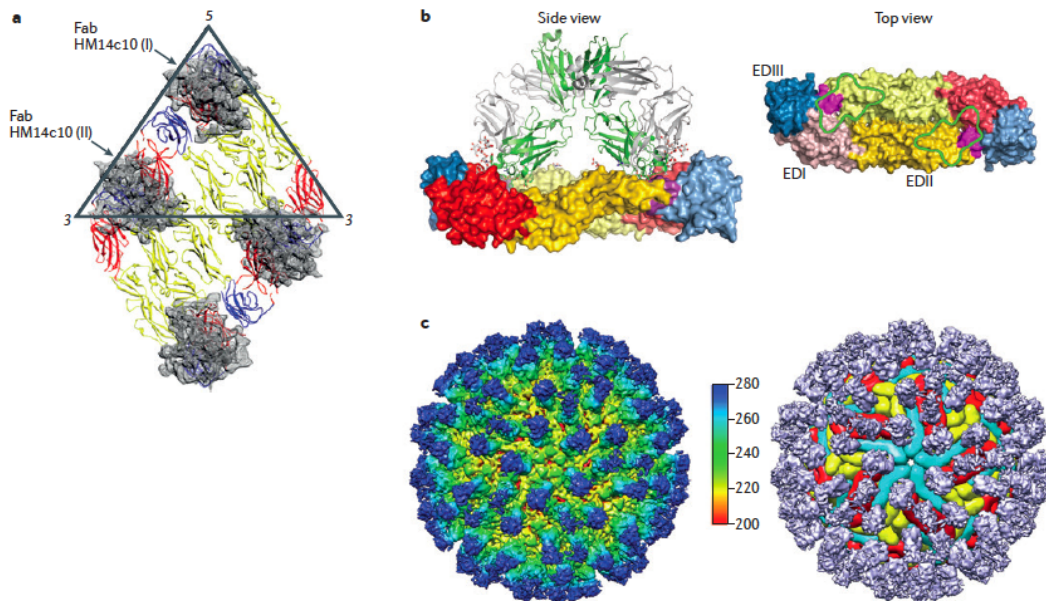


Figure 5 | Quaternary epitopes. The cryo-electron microscopy (cryo-EM) structure shows where HM14c10 binds to the epitope between two envelope (E) protein monomers of adjacent dimers (part a). The structure (part b) and the cryo-EM reconstruction (part c) of the DENV2 particle in complex with an E protein dimer epitope (EDE)-specific monoclonal antibody (mAb) — 747(4) A11 and 747(4) B7 for part b and part c, respectively — demonstrate that the epitope of the antibody is across two E proteins within one dimer. The colour scale in part c indicates the radial depth from inside (red) to the outer shell (yellow, green and blue). Domains I, II and III of the E protein (EDI–EDIII) are indicated in red, yellow and blue, respectively. In part b top view, the green outline shows the footprint of the EDE-specific mAb. Figure in part a from Teoh, E. P. *et al.* The structural basis for serotype-specific neutralization of dengue virus by a human antibody. *Sci. Transl. Med.* 4, 139ra83 (2012). Reprinted with permission from AAAS. Figure in part b from REF. 141, Nature Publishing Group. Figure in part c from REF. 16, Nature Publishing Group.

in a valley formed between the two monomers of E protein, making up the 90 head-to-tail dimers of the virion, and overlap with the footprint on E protein where prM protein sits on the immature dengue virus particle⁴. The antibodies make contact with several conserved amino acid side chains and main chain atoms in the E protein peptide backbone, which explains the cross-reactivity and broad neutralization of the four virus serotypes by these antibodies. Another recently reported mAb, 2D22, binds to E dimers, although the epitope is slightly shifted towards EDIII compared with the epitope described above, which results in 2D22 being specific for just DENV2 and not broadly cross-reactive¹³⁰.

The discovery of the EDE opens up a number of interesting future possibilities for dengue treatment. Current vaccination strategies use tetravalent formulations with the aim of raising single serotype-specific responses against all four serotypes. The demonstration that potent and broadly neutralizing antibodies are produced during dengue virus infection indicates that the development of such antibodies should be a goal for the next-generation vaccines. Furthermore, it may be possible to design a universal, rather than tetravalent, formulation to achieve this

response or to use heterologous prime–boost strategies. Importantly, as the response is limited to the E protein dimer, it opens the way for subunit vaccines that consist of E protein dimers alone. To achieve this, the E protein dimer will need to be stabilized, as E protein only forms dimers naturally at relatively high protein concentrations. A similar situation has been observed with respiratory syncytial virus, in which potent neutralizing antibodies bind to the trimeric pre-fusion forms of the fusion protein (F protein)¹⁴⁴. Efforts to stabilize a soluble F protein trimer have been successful either by cavity-filling hydrophobic substitution or by covalent linkage of the F protein monomer, which allows for the generation of a novel subunit immunogen that is capable of inducing a neutralizing response against the F protein trimer in mouse and primate models of respiratory syncytial virus infection¹⁴⁵.

As described here, the neutralization of dengue viruses is a complex process with several different virion forms produced during infection. Successful vaccines need to target potentially neutralizing epitopes, such as those found on EDIII or quaternary epitopes, and minimize the targeting of poorly neutralizing epitopes, such as those on prM protein or the FLE.

Conclusion and future direction

Despite nearly 50 years of work, we still do not have a fully efficacious dengue virus vaccine, and it remains to be determined whether the Sanofi Pasteur CYD-TDV live attenuated vaccine will be licensed and deployed in endemic countries. However, the burden of dengue continues to increase and, despite promising advances in vector control strategies and an increasingly active search for antiviral drugs that target dengue virus, an effective vaccine is seen by many as the only realistic strategy to control the spread of this disease and reduce the burden it has on the health-care systems in endemic countries.

There are a number of second-generation live attenuated vaccines that are approaching larger-scale clinical trials. The challenge of inducing protection against all

four virus serotypes using a tetravalent formulation is formidable, and heterologous prime-boost strategies, which mimic natural infection but have given conflicting results, may need to be evaluated further. The recent description of potent human neutralizing antibodies in dengue gives insight into the sort of responses that should be targeted by vaccines. However, major challenges in how to preferentially boost immune responses to these complex quaternary epitopes remain. Finally, the recent Sanofi Pasteur clinical trials have demonstrated the need for robust *in vitro* correlates of protection, which would guide the development of future vaccine trials, and there is now interest in developing dengue virus-challenge studies in humans to guide future vaccine development.

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Competing interests statement

The authors declare no competing interests.



New opportunities for control of dengue virus

Catherine H. Roberts, Juthathip Mongkolsapaya, and Gavin Screaton

Purpose of review

Dengue is a rapidly spreading vector-borne disease estimated to infect 400 million people worldwide. To date, there are no licensed treatments or vaccines. The last few years have seen significant developments in dengue control strategies. In this review, we will address four key areas: vaccines, vector control, antivirals and immunotherapeutics.

Recent findings

The first generation of dengue vaccines is able to induce good serological responses in test individuals. However, the recent Sanofi-Pasteur trial in Thailand found that a good serological response did not correlate with clinical protection. This trial did not demonstrate an increase in cases of severe disease following immunization, suggesting that concerns over vaccine-related immune enhancement may have been overcome. The bacterium *Wolbachia* appears to control dengue proliferation in *Aedes* mosquitoes, and field studies are underway. A large number of antivirals are in early-stage development and may prove useful in epidemics. Monoclonal antibodies have been postulated to have a clinical role. Whether their clinical application is feasible has yet to be seen.

Summary

Marked improvements in our knowledge of dengue have been made over the recent years. Sadly, clinical application remains some years away.

Keywords

control, dengue, review, therapeutics

INTRODUCTION

Dengue is an important emerging disease causing approximately 390 million dengue infections worldwide annually, with 96 million that are clinically manifest [1]. There are four dengue serotypes circulating that vary by approximately 30% of their genetic sequences. Consequently, immunity to one serotype does not confer immunity to other serotypes. Severe dengue occurs in 1–5% of cases and predominantly, but not exclusively, occurs in secondary infection [2].

After an incubation of 4–10 days, severe disease has three phases, febrile, critical and recovery (Fig. 1). The critical phase is usually marked by cessation of fever and viraemia. In this period, lasting usually less than 48 h, severe plasma leak may occur, which can result in shock, organ impairment and metabolic acidosis [2].

The pathogenesis of severe disease is hypothesized to be immune driven, caused by an over-exuberant immune response resulting in cytokine release and vascular leak. The immune-mediated pathogenesis hypothesis is derived from a number of observations: first, that severe disease commences as viraemia clears; and second, that severe disease

occurs more frequently in patients with secondary dengue infection and in infants with circulating maternal dengue antibodies. In the 1970s, antibody-dependent enhancement (ADE) was suggested by Halstead and O'Rourke [3] as a mechanism for the development of severe disease. In this model, following primary infection, an antibody response is mounted to envelope proteins on the infecting virus. Antibodies directed against one serotype may not confer completely neutralizing protection to another serotype. Thus, it is proposed that in secondary infection, bound antibodies, rather than neutralizing virus, target the uptake of opsonized virus, driving higher viral replication. A number of other mechanisms may contribute to severe disease. During acute illness, dengue-specific T cells are highly activated and the

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KEY POINTS

- A number of live attenuated vaccines have been produced giving good serological responses in vaccines. However, clinical trials of CYD-TDV have shown disappointing clinical protection against dengue. Of other vaccines in development, a balanced serological response appears difficult to achieve and some are marred by symptomatic viraemia.
- Vector control through the use of the insect bacterium *Wolbachia* is a new approach. It reduces dengue proliferation in the mosquito and seems to prevent its entry into the salivary glands. A field trial is currently in progress.
- A large array of antiviral therapies are in early-stage development. A number of drugs licensed for other uses are also under investigation such as ivermectin and chloroquine.
- Steroids have long been suggested for the treatment of severe dengue disease. However, a clinical trial found no reduction in the number of cases. Of other immunotherapeutics, mAbs developed for investigation of the immune response have been considered for clinical application. However, it is unlikely that they could be administered early enough in the pathogenesis of the disease to have an impact on disease progression.

magnitude of the T cell response seems to correlate with the severity of the illness. In severe illness, T cells that are cross-reactive and produce proinflammatory cytokines are dominant [4^{***}]. Viral virulence and host genetic susceptibility are also likely to play a role. In Latin America, the more benign American DENV2 strain has been replaced with the Asian strain and there has been an associated increase in severe cases [5]. A number of human leukocyte antigen (HLA) variants and blood antigens are associated with susceptibility to severe disease [4^{***}].

Current management of dengue involves early diagnosis and supportive therapy. The WHO has produced guidelines detailing current best practice. Shock is managed with judicious crystalloid according to haematocrit, and haemorrhage is treated with fresh red cells. Expert clinical care can reduce the mortality from severe disease to below 1% [2]. In clinical use, there are no effective vaccines and no antivirals.

In this review, we will focus on four exciting areas in dengue control. The first two topics will be on dengue prevention: vaccination and mosquito control. The second two areas will be dengue therapeutics: antiviral compounds and immune therapies.

VACCINES

Vaccination against dengue virus is challenging. Vaccines need to elicit a protective, balanced and durable immune response to all four serotypes whilst avoiding generating detrimental or enhancing immune responses. There are a number of vaccine strategies currently under investigation. The most advanced are the live attenuated vaccines (LATVs). There are four LATVs currently undergoing clinical trials: CYD-TDV, DIPV, Δ30 and DENVax (Fig. 2). Although serological responses to some of the vaccines have been promising, it is proving difficult to produce a balanced immune response. In addition, the recent clinical trial in Thailand has shown that serological responses do not necessarily correlate with clinical response.

CYD-TDV (Sanofi-Pasteur, Lyon, France) is the most developed vaccine, with data from three phase 2 clinical trials published: in Peru (children) [6], Singapore (children and adults) [7] and Thailand (children) [8^{***}]. The CYD-TDV vaccine contains four recombinant viruses (CYD-1–4), each using the yellow fever 17D vaccine strain as a backbone with genes encoding dengue (serotypes 1–4) prM and prE inserted. Although the serological response in children was good, 84–95% (across all studies), the response in adults was disappointing, 56.7% of 18–45 year olds (Singapore study) [7]. Clinical response over a 2-year follow-up was particularly disappointing with a 30% overall efficacy (subtype efficacy: dengue 1, 55.6%; dengue 2, 9.2%; dengue 3, 75.3%; and dengue 4, 100%) [8^{***}]. Despite the disappointing efficacy, reported side effects were few and no increase in cases of severe dengue was seen during the trial. However, long-term safety cannot be assessed without prolonged follow up [9]. The practicability of a 12-month vaccination schedule should also be considered, and whether if vaccinees fail to return for their final dose, there will be reduced disease protection or perhaps an increased risk of severe disease.

Two further LATVs have undergone recent clinical trials; both are hampered by a mixed serological response and their side effect profile. The Walter Reed Army Institute of Research (WRAIR) and GlaxoSmithKline vaccine (DPIV) underwent a small-scale phase II clinical trial [10]. This vaccine is derived from four live vaccines attenuated by serial passage through primary dog kidney cells followed by three passages through foetal rhesus lung cells and formulated with a carbohydrate stabilizer. The monovalent vaccines were then combined into a tetravalent preparation. Serological response rates to two formulations were 60 and 66.7%, respectively. Side effects were prominent, with vaccinees with preexisting antidengue antibodies experiencing

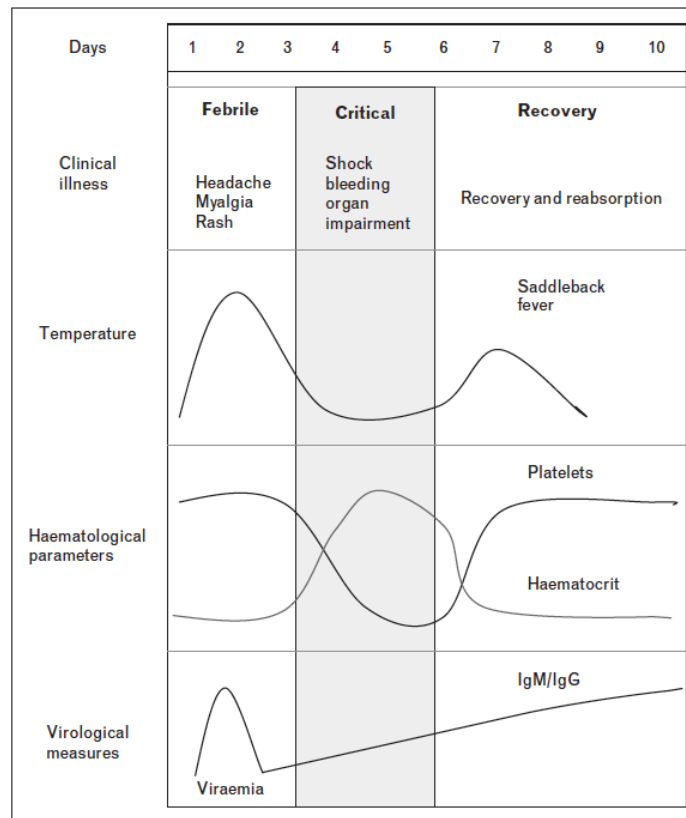


FIGURE 1. Measures of dengue illness. Clinical illness, temperature, haematological parameters, virological parameters. Adapted with permission from WHO guidelines [2].

symptomatic viraemia, including arthralgia, headache, myalgia, fatigue and rash. The next LATV is produced by the US National Institutes of Health, (National Institute of Allergy and Infectious Diseases). This vaccine is based on an infectious clone of a DENV-4 strain (814669 Dominica/81). A 30-nucleotide deletion ($\Delta 30$) in the 3' UTR of cDNA clones was produced. Four admixtures of different combinations of monovalent vaccines are used to create a tetravalent vaccine. A mixed serological response was produced (DENV-1 and DENV-4 seropositivity in 100%, DENV-3 in 85% and DENV-2 in 50%). However, 64.2% developed a maculopapular rash and low-level viraemia was frequent (73%). The poor serological response in both vaccines and the high rate of side effects suggest that neither are currently feasible vaccine candidates [11].

DENVax (Inviragen, Fort Collins, CO) is an LATV constructed from a cDNA clone derived from

dengue 2-PDK-53 and expressing the *prM* and *prE* genes of wild-type (wt) DENV-1 16007, DENV-3 16562 or DENV-4 1036. Some of the formulations produced showed good immunogenicity in mice. A clinical trial is currently underway and is due to complete in late 2013 [12].

These LATVs, although appearing initially promising, are proving difficult for establishing durable immune responses to all four serotypes. Therefore, a number of other vaccine strategies are under investigation: dengue antigens inserted into Venezuelan equine encephalitis virus replicon particles (VRP) [13]; inserting domain III (DIII) from dengue 1 and P64K from the C terminus of menin-gococcus into nucleocapsid-like particles from dengue 3 [14]; using an HIV-derived VLP (virus-like particle) with DIII and CD16-RIG inserted [15]; plasmids containing *prM/E/NS1* with or without granulocyte colony stimulating factor (GCSF) [16].

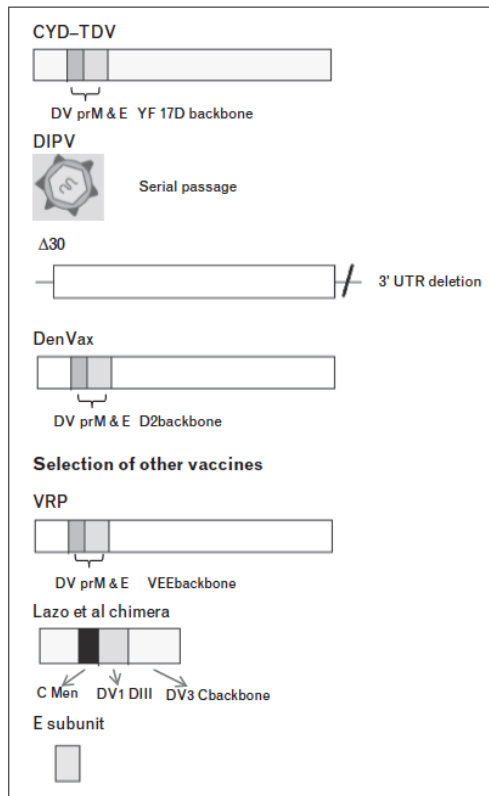


FIGURE 2. Vaccine constructs. CYD-TDV, DIPV, Δ30, DenVax, selection of other constructs, VRP, Lazo *et al.* chimera, E subunit. Diagram drawn by CH Roberts. Adapted from text of cited articles.

These all show some promise, although serological responses in animal models have often been disappointing. Truncated dengue envelope proteins (DEN-80E) are able to induce neutralizing antibody responses in animal models [17]. A single dose of subunit vaccine, using domain III of the E protein delivered by a recombinant lipoprotein with adjuvant properties, was able to elicit neutralizing antibodies in a study from Taiwan [18]. A strategy employed by Crill *et al.* [19] uses DNA vaccines with CD4⁺ T cell immune enhancing epitopes from West Nile virus introduced. This study showed some success in protecting mice [19]. The obstacles to translating all of the preclinical results described here are substantial.

To date, a durable and well tolerated vaccine inducing a balanced and protective immune

response remains elusive. Whether second-generation vaccines can overcome the problems of the first generation has yet to be determined.

VECTOR CONTROL

In the 1970s, the use of DDT spraying resulted in a marked reduction in the mosquito population and a consequent fall in dengue transmission [20]. However, safety concerns and the subsequent ban of DDT resulted in a rise in both the mosquito population and dengue. A new vector control strategy is the use of *Wolbachia*. *Wolbachia* is a maternally inherited intracellular bacterium that naturally infects *Aedes* mosquitoes. Strains of this bacterium are able to reduce dengue transmission (wAlbB and wMelPop with *Aedes aegypti*, wMel with *Aedes albopictus*) [21,22], through mechanisms not yet determined. Some evidence suggests that *Wolbachia* prevents the spread of dengue virus into the salivary glands, thus preventing the mosquitoes' ability to infect humans [21]. There is also evidence that high concentrations of *Wolbachia* in body tissues prevent dengue infection [23]. This may be due to chronic immune upregulation, production of reactive oxygen species and induction of antiviral mechanisms or competition for the same host resources [24,25].

Modelling the use of *Wolbachia* for dengue control suggests that dengue may be well controlled in areas where R_0 (the basic reproductive number) is not large [26]. Field studies using both wMel and wMelPop strains are currently underway.

ANTIVIRALS

The duration of viraemia in dengue disease is short, lasting approximately 3–4 days. As a result, the time to administer antivirals is brief. It has been shown that patients with a high peak viraemia are more likely to develop severe disease [27], and if antivirals can reduce this peak viraemia then the chances of severe disease may be diminished. It has been proposed that antivirals would be most useful during an epidemic, when mass deployment could allow early administration for febrile illnesses.

A number of compounds are currently under investigation for their antidengue properties. Many of these compounds remain in the early phases of development. Antidengue compounds can be broadly grouped by their site of action: entry inhibitors (fusion inhibitors, glycosidase inhibitors, carbohydrate binding agents, heparin mimetics); virus/endosomal fusion; NS3/NS5 inhibitors; virus maturation inhibitors (Fig. 3) [28–44,45*,46–53].

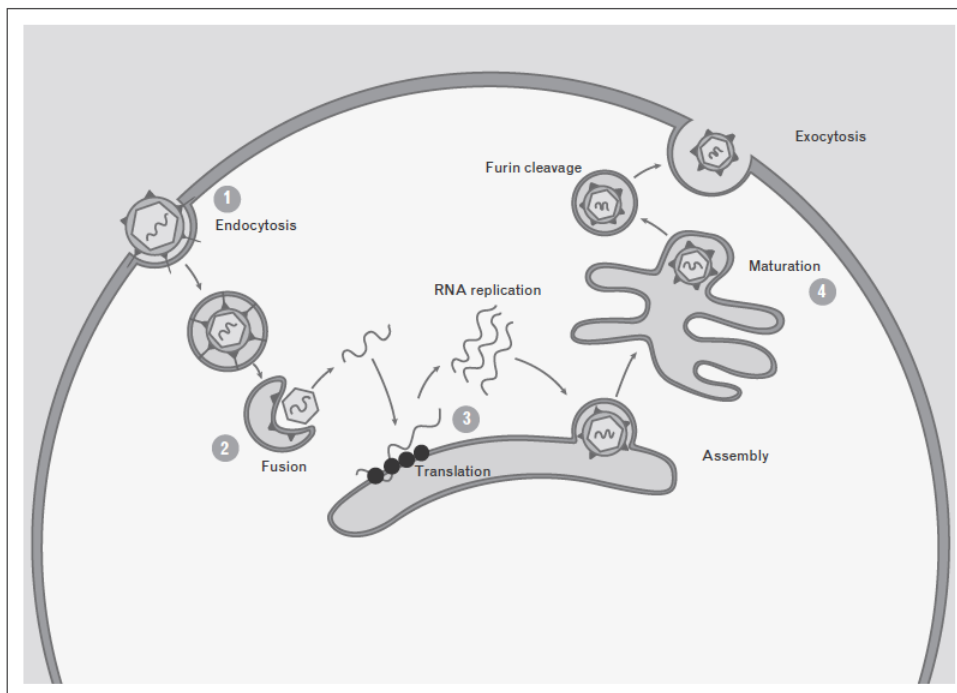


FIGURE 3 Cell cycle of dengue with antiviral therapy targets. (1) Entry inhibitors, bind to E protein hydrophobic pocket: small molecules/peptides [28], DN59 [29], IOAN1 [30], tetracycline derivatives [31], doxorubicin derivative (SA-17) [32], NITD 448 [33]; act on clathrin mediated pit: siRNA to CD14/CLTC and DNM2 [34]; glycosidase inhibitors: castanospermine, DNJ [35], NN-DNJ [36]; carbohydrate-binding agents: plant lectins HHA, GNA, UDA [37]; heparan mimetics: suramin [38], fucoidan [39], PI-88 [38], PPS [38], dextran sulphate [40], sulphated galatomannan [41], α -D-glucan [42], GAG [43], heparin [43]. (2) Endosomal fusion: chloroquine [44]. (3) RNA/protein production: NS3 inhibitors: ivermectin [45], retropeptides [46–48]; NS5 inhibitors: GTP binding site inhibitors [49–51]. (4) Viral maturation inhibitors: chloroquine [44], celgosivir [52,53]. Diagram drawn by CH Roberts. Adapted from text of cited articles.

ENTRY INHIBITORS

A number of entry inhibitors are under investigation (Fig. 3) [28–44,45*,46–53]. Entry inhibitors may prevent initial viral entry; however, they may have little effect on ADE. Most studies have not assessed this. A teicoplanin analogue (LCTA-949) has broad antiviral effects and anti-E opsonized dengue particles were also found to have reduced cell binding in the presence of LCTA-949 suggesting that it may prevent ADE [45*]. It would be interesting to assess the effects of this compound in the presence of enhancing anti-prM antibodies.

Endosomal fusion

The antimalarial drug chloroquine increases the pH of endosomes. It has been proposed that through this mechanism, chloroquine might interfere with

virus/endosomal fusion and furin-dependent virus maturation. Although effective *in vitro* [54], a randomized controlled trial in Vietnamese adults showed no reduction in viraemia. The median time of disease onset to administration of chloroquine was 49 h; earlier administration may result in a more effective clinical response [44].

NS3 inhibitors

The development of new and highly efficacious drugs against the flavivirus, hepatitis C, has changed the landscape of this disease. A number of these drugs act against the protease encoded by NS3/4, i.e., telaprevir [55] and boceprevir [56]. It is proposed that anti-NS3 agents may be efficacious in dengue. NS3 contains the virus protease and the helicase. Structural searches for agents against the NS3/2B

proteins have been performed. Early studies show promise, with retro-tripeptides showing both activity and selectivity against this molecular target [46,47]. A trial of the hepatitis C drug, balapiravir, in dengue patients was not found to be effective at controlling viraemia [57]. A docking search of compounds to NS3 identified the antihelminthic drug, ivermectin, and in-vitro studies showed that ivermectin inhibited the replication of dengue virus [45].

NS5 inhibitors

NS5 is an RNA capping enzyme with methyltransferase and guanylyltransferase activity. The GTP-binding site appears to be important for this enzyme's function. Screening of small molecular compounds has led to the identification of a molecule, (E)-[3-[5-(4-tert-butylbenzylidene)-4-oxo-2-thioxo-1,3-thiazolidin-3-yl] propanoic acid] (BG-323), which competitively binds to the GTP-binding site and shows in-vitro antiviral activity against a range of flaviviruses.

Viral maturation inhibitors

Celgosivir is a water-soluble oral pro-drug of the alkaloid castanospermine (Cast), derived from the Moreton Bay chestnut tree. Celgosivir inhibits α -glucosidase I and II, resident enzymes of the endoplasmic reticulum. By inhibiting these enzymes, viral proteins misfold and cause a series of downstream effects resulting in cell death. Celgosivir was initially trialled against hepatitis C and HIV [58]. In dengue, Celgosivir has been shown to have activity against all four serotypes by acting on NS1 and reducing infectious particles produced in ADE. It reduces viraemia in mice [52,53] and a phase 1b study in dengue is underway in Singapore.

STEROIDS

Steroids are a controversial therapy for all-cause shock [59]. A randomized controlled trial was conducted in Vietnam assessing the use of short course (3 days) prednisolone (0.5 or 2mg/kg) in early dengue infection (<72h). This trial found no effect of prednisolone on clinical, haematological or virological endpoints. However, with only 225 patients recruited, the likelihood of finding a clinical difference in progression to severe disease was small [60].

MONOCLONAL ANTIBODIES

Antibodies play a key role in the immune response to dengue infection. Some hypothesize that they are

responsible for the antibody-dependent enhancement and therefore severe disease. A number of research groups use monoclonal antibodies (mAbs) as a strategy to explore immune responses. If an mAb is strongly neutralizing and weakly cross-reactive, it has been proposed that the mAbs could potentially treat severe disease. These mAbs bind epitopes on the E protein [fusion loop [61,62], domain III (DIII) [63], DI [64], DI-DII hinge region] [65]. Antibodies engineered to prevent Fc γ R binding, through insertion of an LALA mutant in the CH2 region of the mAb, were found to neutralize infection without causing enhancement and were used successfully as postexposure therapy in a mouse model [66]. Although mAbs have found broad uses throughout the medical therapeutics world, whether they will find a place in the treatment armory against dengue has yet to be seen. They will likely be expensive to produce and are thought to be required very early in dengue infection to have a clinical effect.

CONCLUSION

The last few years have seen a dramatic increase in the size of the dengue epidemic. The Sanofi-Pasteur trial provided disappointing clinical protection, but did not appear to elicit infection enhancing immune responses. Work is now needed to design a second generation of vaccines. Furthermore, a better understanding of correlates of clinical protection in humans is needed to evaluate vaccines. No doubt the current wealth of research into dengue immunology will prove informative. The use of *Wolbachia* as a dengue control strategy appears promising. Field trials are currently underway and modelling suggests that judicious use of *Wolbachia* in areas with moderate dengue activity may reduce transmission significantly. Mass deployment of antivirals during an outbreak may prove efficacious, and currently there are a number of promising candidates in development. As a result, the next 10 years may see a change in the landscape of dengue, but until an effective vaccine is deployed it is unlikely that the spread of this rapidly emerging disease will be stymied.

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Conflicts of interest

There are no conflicts of interest.

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