Dengue Virus Infection
of Human Microvascular Endothelial Cells

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ABSTRACT

Dengue virus (DENV) infection is an important public health problem, being globally the most highly prevalent mosquito-borne viral illness. The World Health Organization (WHO) has estimated 2.5 billion people are at risk of DENV infection and each year 50-100 million infections are reported, with over 500,000 hospitalisations and 25,000 deaths. DENV consist of four serotypes, each of which are capable of producing a wide spectrum of syndromes varying from mild dengue fever (DF) to more severe dengue haemorrhagic fever (DHF). Vascular leakage resulting from increased micro vessel endothelial permeability is one of the life-threatening complications in DHF. The relationship between DENV infection and the endothelium has previously been investigated both in vitro and in vivo experiments. Nevertheless, the mechanisms by which dengue infection leads to increased vascular permeability remain unclear. Understanding the underlying mechanisms of vascular leakage is required to identify better control strategies for DHF/DSS.

To study DENV-induced vascular leakage, an appropriate microvascular endothelial cell type derived from organs naturally targeted by DENV infection is required to produce a more physiologically relevant model. In this thesis, we investigated the susceptibility and responses of the human brain endothelial capillary cell line (hCMEC/D3) and the liver sinusoidal endothelial cell line (LSEC) to DENV infection. LSECs and hCMEC/D3s were permissive to DENV infection to varying degrees. Additionally DENV infection activated LSECs and hCMEC/D3s, as indicated by increased expression of ICAM-1 (intercellular adhesion molecule) and VCAM-1 (vascular cell adhesion molecule),
suggesting that in the liver and brain, endothelial cells could play a role in initiating or enhancing the inflammatory response.

Productive infection of LSECs by DENV induced apoptosis in more than half of the LSEC population and leads to disruption of the endothelial barrier. Furthermore DENV infection induced LSECs to produce higher levels of cytokines, chemokines and vascular growth factors such as, IL-6, IL-1β, TNF-α, CXCL8, CCL4, CCL3, CCL2, CCL11, CXCL8, CXCL10, G-CSF and GM-CSF. Despite the higher levels of soluble mediators observed in DENV-infected LSEC supernatants did not alter permeability of LSEC monolayers. On the other hand, DENV-infected monocyte-derived macrophage (MDM) supernatants caused a transient permeability increase in LSEC monolayers.

Permissiveness of hCMEC/D3 cells to DENV infection has not been previously reported. Non-productive infection of hCMEC/D3s led to enhanced production of soluble immunomediators such as IL-6, IL-1β, TNF-α, CXCL8, CCL4, and G-CSF. Neither DENV-infected hCMEC/D3 nor MDM supernatants altered permeability of hCMEC/D3 monolayers. The inability of MDM supernatants to alter permeability, may be due to the tight junction organisation maintained by hCMEC/D3 monolayers. Tight junctions are abundant in brain endothelia compared to the endothelia lining other organs, which contributes to extremely low permeability of the endothelial barrier.

Gene expression analysis of LSECs and hCMEC/D3s revealed many differentially expressed genes, gene clusters and host response pathways, that were upregulated during DENV infection. Specific genes regulated in response to DENV, such as miR-155, USP18 and OAS genes have been implicated in DENV infection of microvascular endothelial cells for the first time. Over expression of miR-155 may be a factor
contributing to the limited DENV replication of hCMEC/D3s. The USP18 gene, identified in LSECs interferon response network, is a negative regulator of IFN mediated responses and inhibition of USP18 can enhance the protective role of IFN. Therefore, USP18 may be a potential target for antiviral therapies.

Overall, this is the first study to show that human microvascular endothelial cells from the liver alter their permeability in response to local DENV infection. DENV-induced apoptosis is one factor that was responsible for permeability alterations. Additionally, enhanced production of soluble mediators by DENV-infected endothelial cells caused activation of endothelial cells, as shown by increased expression of CAMs, although it did not alter permeability in endothelial monolayers. On the other hand, MDM supernatants contain vasoactive factors that caused transient permeability changes in LSEC monolayers. Therefore, this study suggests that both apoptosis of endothelial cells and vasoactive factors produced by cells of monocytic lineage are responsible for transient vascular leakage during DENV infection. Further, analysis of host cell gene expression found new antiviral targets such as OAS family members, USP18 and miR-155 genes, which may be promising candidates for future DENV therapeutic studies.
STATEMENT OF ORIGINALITY

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Maheshi Obeysekera

July 2015
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It was a long journey! There are many who deserve to be mentioned here as part of my unforgettable expedition. First of all, I would like to express my sincere thanks to my principle supervisor, Professor Suresh Mahalingam. He has been a tower of strength. His perseverance and enthusiasm for his discipline have encouraged and profoundly influenced me since my arrival in Griffith University.

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<td>$\alpha$</td>
<td>Alpha</td>
</tr>
<tr>
<td>$\beta$</td>
<td>Beta</td>
</tr>
<tr>
<td>$\mu$g</td>
<td>Microgram</td>
</tr>
<tr>
<td>ADE</td>
<td>Antibody dependent enhancement</td>
</tr>
<tr>
<td>APCs</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
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<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
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<td>BDV</td>
<td>Border disease virus</td>
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<tr>
<td>BHK</td>
<td>Baby hamster kidney</td>
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<tr>
<td>BVDV</td>
<td>Bovine viral diarrhea virus</td>
</tr>
<tr>
<td>C protein</td>
<td>Capsid protein</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<tr>
<td>CAMs</td>
<td>Cell adhesion molecules</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>CSFV</td>
<td>Classical swine fever virus</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DCSIGN</td>
<td>Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin</td>
</tr>
<tr>
<td>DENV</td>
<td>Dengue virus</td>
</tr>
<tr>
<td>DHF</td>
<td>Dengue haemorrhagic fever</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DSS</td>
<td>Dengue shock syndrome</td>
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<tr>
<td>E protein</td>
<td>Envelope protein</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>EBM</td>
<td><em>Endothelial Basal Medium</em></td>
</tr>
<tr>
<td>ECIS</td>
<td>Electrical Cell-substrate Impedance Sensing</td>
</tr>
<tr>
<td>ECV304</td>
<td>Endothelium cell of vessel 304</td>
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<tr>
<td>EDTA</td>
<td>di-sodium ethylene di-amine tetra-acetate</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ESDN</td>
<td>Endothelial and smooth muscle cell-derived neuropilin</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
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<tr>
<td>FcγR</td>
<td>Fc-gamma-receptor</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GMCSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GSEA</td>
<td>Gene set enrichment analysis</td>
</tr>
<tr>
<td>GSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GSL</td>
<td>Glycosphingolipid</td>
</tr>
<tr>
<td>HBMEC</td>
<td>Human brain microvascular endothelial cell</td>
</tr>
<tr>
<td>HCAECs</td>
<td>Human coronary artery endothelial</td>
</tr>
<tr>
<td>HCMEC/D3</td>
<td>Human brain endothelial capillary cell line</td>
</tr>
<tr>
<td>HMEC-1</td>
<td>Human dermal endothelial cell line</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>ICTV</td>
<td>International committee of taxonomy of viruses</td>
</tr>
<tr>
<td>IFA</td>
<td>Immunofluorescence assay</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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</table>
IFNAR  IFN-α/β receptor
Ig       immunoglobulin
ISGF3    interferon stimulating gene factor 3
JAK      Janus kinases
JAMs     junctional adhesion molecules
JEV      Japanese encephalitis
LCMV     Lymphocytic choriomeningitis virus
LSECs    Liver sinusoidal endothelial cells
LSIGN    liver/lymph node-specific ICAM-3 grabbing non-integrin
MCP-1    Monocyte chemotactic protein 1
MDA-5    melanoma differentiation associated gene-5
MDM      Monocyte-derived macrophages
MIP      Macrophage inflammatory protein
MMPs     Matrix metalloproteinases
MVEV     Murray valley encephalitis
NK cells Natural killer cells
NO       Nitric oxide
NS       Non-structural
ORF      Open reading frame
PAMPs    pathogen-associated molecular patterns
PBMCs    Peripheral blood mononuclear cells
PBS      Phosphate buffered saline
PDGF     platelet-derived growth factor
PECAM-1  platelet–endothelial cell adhesion molecule
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Pen/Strep</td>
<td>Penicillin streptomycin</td>
</tr>
<tr>
<td>Pfu</td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td>PrM</td>
<td>Pre-membrane protein</td>
</tr>
<tr>
<td>RIG-I</td>
<td>retinoic acid inducible gene-I</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RIN</td>
<td>RNA integrity number</td>
</tr>
<tr>
<td>Rtp</td>
<td>Room temperature</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TBEV</td>
<td>Tick-borne encephalitis</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TEER</td>
<td>Transendothelial electrical resistance</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF-receptor associated factors</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal dUTP Nick end-labelling</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VE Cadherin</td>
<td>Vascular endothelial cadherin</td>
</tr>
<tr>
<td>VECAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>WNV</td>
<td>West Nile virus</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>YFV</td>
<td>Yellow fever virus</td>
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<td>ZO</td>
<td>Zona occluden</td>
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Chapter I

Literature review
1.1 Family *Flaviviridae*

1.1.1 History

The *Flaviviridae* family comprises 60 globally distributed members that are responsible for a significant number of diseases in both humans and animals. In the 1800s, yellow fever virus (YFV) caused continuous epidemics in the Caribbean region, with a high death rate. In the 1900s, Walter Reed’s investigations proved decisively that YFV is transmitted to humans by mosquitoes. In 1927, a virus was isolated from infected rhesus monkeys and identified as YFV causing yellow fever. Subsequently the viral group was named “*Flavi*”, as yellow fever is associated with jaundice, and “*flavus*” means “yellow” in Latin (Schweitzer et al. 2009).

Prior to 1984, the Flaviviruses was considered to be ‘group A arbovirus’ and was later reclassified as ‘group B arbovirus’ within the family *Togaviridae*. With the increasing knowledge of distinct patterns of *Flavivirus* replication, structure and biochemistry, later it was placed in a separate family. The family was named *Flaviridae* after the prototype yellow fever virus (Schweitzer et al. 2009).

1.1.2 Classification of *Flaviridae*

The family *Flaviridae* comprises 60 species within four genera: *Flavivirus* (53 species), *Hepacivirus* (one species), *Pestivirus* (four species) and (recently included) *Pegivirus* (two species), according to the current update by the International Committee of Taxonomy of Viruses (ICTV 2015). The representative viruses in each genera are listed in table 1.1.
**Flavivirus** is the largest genus in the family **Flaviviridae** and was classified under the family **Togaviridae** prior to 1984. The 39 species of the genus are transmitted by arthropod vectors, including mosquitoes and ticks, while another fourteen species are not known to be transmitted by arthropods (Schweitzer et al. 2009).

The genus **Hepacivirus** consists of the hepatitis C virus species, which was discovered in 1989. During the 1970s, a mild form of chronic hepatitis was reported frequently in recipients of blood transfusions and was not associated with hepatitis A or hepatitis B virus, which were discovered during the 1960s and 1970s. Further studies on the causative agent of ‘non A, non B’ hepatitis discovered a new RNA virus which was named hepatitis C virus. It was classified as the type species in the new genus **Hepacivirus** under the family **Flaviviridae** (Stapleton et al. 2011). The genus includes seven different genotypes, with differences in antigenic variability and geographical distribution. Further virological, clinical and immunologic evidence is required to consider these as different species (Simmonds 2013).

The genus **Pestivirus** consist of four species namely: bovine viral diarrhea virus-1 (BVDV-1), BVDV-2, classic swine fever virus (CSFV), and border disease virus (BDV). **Pestiviruses** are important pathogens of cattle, sheep, and pigs and cause significant economic losses worldwide. The genus was named after the affected host species, and differences in natural host range, disease, and pathology have assisted in the classification of **Pestiviruses** (Becher et al. 2003).

In 2011, Stapleton et al. (2011) demonstrated the need for a new genus within the family **Flaviviridae** to assigned GBV-A-like viruses, GBV-C and GBV-D based on their phylogenetic relationships, genome structure and pathogenic features (Stapleton et al.
During 2012, the new genus, *Pegivirus*, was created within the family *Flaviviridae* and designated *Pegivirus A* as the type species of the new genus. The GB-viruses was renamed as *Pegivirus* to reflect their host origin.

1.2 Dengue virus

1.2.1 Introduction

Dengue is the most prevalent mosquito-borne viral disease in the world today. The causative agent, dengue virus (DENV), a member of the *Flavivirus* genus of the *Flaviviridae* family, comprises four serotypes, each of which is capable of causing dengue fever (DF) with or without warning signs and severe dengue (dengue haemorrhagic fever (DHF), dengue shock syndrome (DSS) and severe organ impairment). The pathogenesis of dengue is multifactorial, and the disease severity depends on both viral and host factors. The geographical spread of both the mosquito vector and virus has created a socio-economic burden in dengue endemic countries, especially as effective antivirals, vaccines or vector control management for DENV remain elusive.

1.2.2 Dengue virus serotypes

There are four DENV serotypes; DENV-1, DENV-2, DENV-3 and DENV-4. All four serotypes can infect humans in varying degrees, depending on viral virulence (Kuhn et al. 2002; Lewis et al. 1993). DENV-2 is more often associated with more frequent and severe epidemics than other serotypes; hence it has been studied in more detail. Phylogenetic studies have suggested that DENV-2 was the first to evolve from a common progenitor (Rico-Hesse 2003). A study of the phylogenetic relationships of DENV 2 collected 40 strains of DENV 2 over a 45 year period from different tropical regions, and
showed that the DENV 2 serotype can be classified into five genetic clusters or genotypes by comparison of E/NS1 gene junction sequences (240 nucleotides) (Loroño-Pino et al. 2004; Rico-Hesse 2003) (Table 1.2).

1.2.3 Structure of dengue virus

The spherical shaped DENV virion, has a diameter of approximately 50 nanometre (nm) and consists of an RNA genome encapsulated in a nucleocapsid of 30 nm surrounded by a lipid bilayer-envelope (Figure 1.1). The nucleocapsid is composed of multiple copies of capsid (C) protein and the envelope is made of 180 copies from each of the two structural proteins: envelope glycoprotein (E) and membrane protein (M) (Fibriansah et al. 2013; Whitehead et al. 2007).

1.2.4 Dengue virus genome

The DENV genome is a positive-sense, single-stranded RNA molecule with a 10.7 kilobase length. The RNA genome serves as the mRNA for translation. The single open reading frame (ORF) of the DENV genome is flanked by a capped 5’ untranslated region (UTR) and a nonpolyadenylated 3’ UTR. The non-coding first ~100 nucleotides at the 5’-end and ~384-466 nucleotides at the 3’-end are highly structured and contain regulatory regions involved in viral replication and translation. The ORF encodes a single polyprotein that is processed by cellular and viral proteases to produce three structural (C, prM and E) and seven non-structural (NS; NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) proteins. (Figure 1.2) (Bartenschlager and Miller 2008; Xie et al. 2015).
1.2.5 Life cycle of DENV

DENV is transmitted to humans through the bite of an infected female mosquito of the *Aedes* species (Figure 1.3). *Aedes aegypti* serves as the primary vector of DENV but *Aedes albopictus* can also transmit the virus (Whitehead et al. 2007). Infected humans are the main carriers and multipliers of the virus and serve as a source of virus for uninfected mosquitoes. When a mosquito bites an infected person for a blood meal, DENV enter into the mosquito and infects its midgut epithelium and spreads through other systems, including salivary glands. A mosquito can be infectious eight to ten days after feeding and continue to be infectious throughout its entire life. The bite of an infectious mosquito can transmit the virus to susceptible individuals in the course of subsequent blood meals (WHO 2009). The virus circulates in the blood of infected humans for two to seven days and is coincident with fever. Subsequently DENV will enter into a wide range of cells through cellular receptors for replication.

1.2.6 Attachment and entry

The initial step of DENV infection occurs when the virion binds to the surface of the target cell. This virion-receptor interaction induces virion entry to the host cells by receptor-mediated endocytosis (Alen and Schols 2012; Arevalo et al. 2009; Pokidysheva et al. 2006). Distinct and multiple host-cell receptors are involved in DENV infection of permissive cells. Table 1.4 summarises candidate DENV receptors on mammalian cells that have been identified in previous studies. Hidari and Suzuki categorise these cellular receptors into four groups as follows (Hidari and Suzuki 2011).
i. Carbohydrate molecule receptors

The carbohydrate molecules, including sulphated glycosaminoglycans (GAGs) and glycosphingolipid (Liew et al.) are co-receptor molecules for DENV. Heparan sulphate, a GAG and neolactotetraosylceramide (nLc4Cer), which is a GSL without sulfation, has been reported as vital for virus absorption and for virus attachment to the host cells respectively (Aoki 2006; Chen et al. 1997; Lee et al. 2006a).

ii. Carbohydrate binding proteins — lectins

Lectins, expressed on dendritic cells (DCs) and macrophages, contribute to DENV entry into cells. Viral entry into DCs and macrophages is shown to be supported by both DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DCSIGN) receptors and liver/lymph node-specific ICAM-3 grabbing non-integrin (L-SIGN) receptors. The mannose receptor specifically supports virus-macrophage interaction (Miller et al. 2008; Navarro-Sanchez et al. 2003; Pokidysheva et al. 2006; Tassaneetrithep et al. 2003).

iii. Folding protein receptors — heat shock proteins (HSPs)

HSPs are a family of highly conserved molecular chaperones that function as co-receptors for virus-host cell interaction. HSP90 and HSP70 at the cell surface membrane facilitate DENV entry into the cells, including HepG2 cells, SH-SY5Y cells and macrophages (Reyes-del Valle et al. 2005).

iv. Other proteins

Several independent studies have showed other proteins such as high-affinity laminin receptors and CD14-associated proteins may also support DENV entry into several cell types, including monocytes and macrophages (Hidari and Suzuki 2011; Tio et al. 2005).
Some of the candidate receptors bind all four DENV serotypes, whereas some putative receptors may only recognise a specific DENV serotype. The DENV use multiple receptors to gain entry into different types of host cells, and the receptor can even vary between different DENV serotypes (Hidari and Suzuki 2011).

This virus receptor interaction induces virus entry to the host cells by receptor-mediated endocytosis. Subsequently, fusion of DENV E with the endosomal membrane occurs in the late endosome and the viral RNA genome is released into the cytosol (Alen and Schols 2012; Pokidysheva et al. 2006).

1.2.7 Translation and cleavage of polyprotein

The DENV genome released into the host cell cytoplasm dissociates from the C protein and functions as mRNA for translation in the rough endoplasmic reticulum (ER). The genome contains of a single ORF that encodes a polyprotein which is processed by viral and cellular proteases both co- and post-translationally. The cleavage of polyprotein at N-termini is processed by the host signal peptidase located in the lumen of the ER and cleaved prM, E, NS1 and NS4B. The C-terminus C protein and most of the non-structural proteins are cleaved by viral protease in the cytoplasm of infected cells. NS3 and, NS2B as a cofactor, form the viral protease and cleave the junctions between C/prM, NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4A/NS4B and NS4B/NS5. The NS1/NS2A junction is cleaved by an unknown resident protease in the ER. During virion maturation, prM is cleaved to M by the furin protease located at the Golgi apparatus. The three structural proteins are components of the virion and the seven non-structural proteins modulate viral replication (Alcaraz-Estrada et al. 2010; Xie et al. 2015).
1.2.8 The functions of dengue virus proteins

The three structural proteins are important in infectious virion assembly. The E protein is essential for viral attachment and entry into host cells. It contains most of the antigenic determinants for the *Flavivirus* group — DENV complex, DENV sub-complex and the DENV serotype. Non-glycosylated M is formed from a glycosylated pre-membrane protein (prM) precursor during virus maturation. Un-cleaved prM stabilises the E protein to prevent conformational changes by forming a prM-E heterodimeric complex during virus maturation. The role of M in the mature virion is not known. The nucleocapsid, composed of capsid protein (C) is the first protein to be synthesised during translation. The C protein, once translated, associates with newly synthesised RNA in an unknown mechanism to form the RNA-C complex (Alcaraz-Estrada et al. 2010; Leopoldo et al. 2011).

Most of the NS proteins are associated with viral RNA replication. NS1 exists at multiple cellular locations including being associated to the cell-membrane, in vesicular compartments within the cell or on the cell surface and as a secreted extracellular species. Intracellular NS1 plays a cofactor role in virus replication while secreted and cell-surface associated NS1 are associated in disease pathogenesis (Muller and Young 2013). NS5 is the RNA polymerase which contains an N-terminal methyl transferase domain (MTase) and a C-terminal RdRp domain that are important in formation of the RNA cap. NS3 is the viral serine protease which is important in polyprotein cleavage and it requires NS2B as the cofactor for functioning. A little is known about the other NS proteins including NS2A, NS4A and NS4B (Bartenschlager and Miller 2008). The known functions of DENV proteins are summarised in Table 1.3.
1.2.9 Assembly of dengue virus particles

The assembly of synthesised viral proteins with new viral RNA occurs in the lumen of the ER, where RNA-C complex buds into the ER lumen and is covered with the lipid bilayer. At this stage the virion can be seen as an immature particle in the ER. The new virions are released from the ER by budding into the Golgi apparatus for glycosylation. The structural transitions from immature to mature take place at a late stage of virion assembly in the trans-Golgi network, and mature virions are subsequently released (Martínez-Gutierrez et al. 2011; Mondotte et al. 2007) (Figure 1.4).

1.3 Epidemiology of dengue virus infection

1.3.1 Dengue epidemiology

More recent global resurgence of epidemic DF and emergence of DHF are consequences of the geographical dissemination of both the mosquito vectors and the virus (Mackenzie et al. 2004). The World Health Organization (WHO) has estimated 2.5 billion people are at risk of dengue infection in over 100 endemic countries and in new areas where DENV can be transmitted (WHO 2009). Prior to the 1970s, DHF was reported only in five countries, and since then it is has been endemic over 100 countries (Whitehead et al. 2007; Guzman and Harris 2015). Many of these countries have not reported dengue for 20 or more years and several have no known history of the disease (Guha-Sapir and Schimmer 2005). Annually, 96 million infections are reported, which is more than three times higher than WHO’s 2012 estimate of the rate of DENV infection (Guzman & Harris, 2015), with over 500,000 hospitalisations and 25,000 deaths (Chuansumrit & Chaiyaratana, 2014). DF and DHF are most common in urban and suburban areas in the
Americas, South-East Asia, the Eastern Mediterranean and the Western Pacific, and in rural areas in Africa (WHO 2009) (Figure 1.5).

1.3.2 Dengue in Australia

In Australia, DF has been reported in the states of Queensland and New South Wales, where the mosquito vector *Aedes aegypti* is established. Queensland has a history of dengue epidemics dating back to 1879, and since 1885 thirteen dengue epidemics have been reported. The dengue vector *Aedes aegypti* is most abundant in the urban areas in the north-east of the state, and locally acquired infections have been reported. In recent years DENV outbreaks have been confined to northern Queensland, including Townsville, Charters Towers, Torres Strait, Mossman, Innisfail, Tully and Cairns. The largest outbreak in the last years was in 2009–2010 in Cairns and Townsville, with over 1000 confirmed cases. The percentage of imported dengue cases in 2010 and 2011 increased by 298% and 155% respectively compared to the five year mean. In 2012, 144 cases per month were reported during the first seven months of the year (Knope et al. 2013). DENV infection notifications by place of acquisition between 1991 and 2012 are shown in Figure 1.6.

Even though local outbreaks of DENV infection by imported cases are restricted to urban areas of Queensland where the vector mosquito is present, there is a risk of DENV infection becoming endemic in the area (Knope et al. 2013). A significant increase in the distribution and incidence of dengue infection in Australia is anticipated with climate change. An expansion of the dengue risk zone is predicted, which would mean a 2–3 °C rise would spread DENV to Brisbane, and a 3–4 °C rise would extend dengue infection as far as Sydney (Russell et al. 2009).
1.4 Clinical manifestations and pathogenesis of dengue virus infection

1.4.1 Dengue fever with or without warning signs

DENV affects infants, young children and adults, and causes a broad spectrum of illnesses, ranging from a mild febrile illness to the most severe dengue (Figure 1.7). DF can present with or without warning signs. Probable DF presents with a sudden onset of high fever accompanied by nausea, vomiting, skin rash, aches and pains and leukopenia. This can be accompanied with warning signs such as abdominal pain or tenderness, persistent vomiting, mucosal bleeding, lethargy, restlessness, liver enlargement (>2cm) and fluid accumulation. (Gubler 1998; Murphy and Whitehead 2011; Simmons et al. 2012; WHO 2009).

1.4.2 Severe Dengue

The severe form of dengue infection can present as severe plasma leakage, severe haemorrhage or severe organ impairment. Severe plasma leakage leads to dengue shock syndrome (DSS) and fluid accumulation with respiratory distress. During severe organ impairment liver, central nervous system, heart and other organs can get involved (figure 1.7). Severe haemorrhage, which is potentially a deadly complication, is characterised by all the symptoms of DF along with haemorrhagic manifestations, such as coagulopathy, thrombocytopenia and increased vascular fragility and permeability (Figure 1.8). Onset is usually three to seven days after DF symptoms, followed by severe abdominal pain, vomiting, blood in vomit, bleeding gums, rapid breathing, fatigue and restlessness (Simmons et al. 2012; WHO 2009).
1.4.2.1 Vascular leakage

The endothelial cell lining is the structural barrier between the vascular space and tissues in all blood vessels in the circulatory system. It regulates a range of functions, including vascular smooth muscle tone, host-defence reactions, angiogenesis, and tissue fluid homeostasis. Failure of the endothelium to function as a semi-permeable barrier results in the passage of macromolecules and fluid from blood into the interstitial space, which leads to tissue inflammation (Basu and Chaturvedi 2008; Mehta and Malik 2006). In DHF/DSS, alteration of the vascular fluid barrier of the endothelium leads to vascular leakage, which is a hallmark of severe DENV infection (Dalrymple and Mackow 2012). The underlying molecular mechanism of micro-vascular plasma leakage in DENV infection is unknown.

1.4.2.1.1 Structure of the vascular endothelium

The well-constructed anatomical structure of blood vessels is composed of 3 layers: tunica intima, tunica media and tunica adventitia. Innermost tunica intima is composed of a monolayer of endothelial cells and it lines the entire circulatory system of humans. The endothelial cells are connected to each other by adherens, tight and gap junctions (Wallez and Huber 2008).

The adherens junctions are important in initiation and stabilisation of cell–cell adhesion, intracellular signalling and cytoskeleton regulation. Adherens junctions mainly consist of interactions between the classical cadherin family and catenin family members (Figure 1.9). Catenin family members — p120-catenin, β-catenin and α-catenin — connect the
cadherin complex to the actin cytoskeleton. Vascular endothelial (VE)-cadherin is the major protein in the formation of adherens and its extracellular domain binds with the adjacent cells’ VE-cadherin extracellular domains and glues neighbouring cells together. In addition to VE-cadherin, cell-cell contacts are maintained by E-cadherin, junctional adhesion molecules (JAMs), and platelet–endothelial cell adhesion molecules (PECAM-1), where their specific role in endothelial barrier function is unknown (Hartsock and Nelson 2008; Wallez and Huber 2008; Yuan and Rigor 2010).

The tight junctions are composed of interactions of claudin and occludin proteins (Figure 1.10). Tight junctions are considerably variable along different vascular beds, due to the variation of composition and complexity of the occluding strands. Comparatively capillaries have less complex tight junctions although opposing to other capillaries blood brain barrier (BBB) are rich with tight junctions. Claudin and occludin have four transmembrane domains and two extracellular loop domains. The extracellular domains of these proteins bind with the extracellular domains of like molecules in adjacent endothelial cells to seal the intracellular gap (Yuan and Rigor 2010). Members of the claudin family are the major constituents of tight junctions, 24 claudins have been studied in mammals and 7 of these are found in liver, namely claudin-1, -2, -3, -4, -5, -7 and -10 (Lee and Luk 2010). Brain endothelial cells have claudin-5 and claudin-12 and possibly some other claudins. Claudins are important to selectively limit paracellular ion movement to produce a high electrical resistance in the BBB (Stamatovic et al. 2008). Occludins are associated with tight junction barrier function, as is highly expressed on BBB although it is not a mandatory claudin in the formation of tight junctions. Occludin degradation has been associated with increased vascular permeability. Claudins and occludens are connected with intracellular proteins, including zona occludens proteins
(ZO-1, ZO-2, ZO-3), AF-6, PAR-3, cingulin and 7H6. ZO proteins are important in binding tight junctions to the cytoskeleton and in signal transmission (Hartsock and Nelson 2008; Yuan and Rigor 2010).

Gap junctions are mainly found in larger vessels and do not significantly involve endothelial barrier function in micro-vessels. In addition to junctional proteins, glycocalyx is considered an important structural determinant of endothelial barrier function (Hartsock and Nelson 2008; Yuan and Rigor 2010). Glycocalyx is a negatively charged fibre matrix, which lines the lumen of vascular endothelium. This mesh-like glycocalyx coats the vascular surface with proteoglycans and glycoaminoglycans. Glycocalyx contributes to endothelial barrier function by limiting the passage of differentially charged macromolecules, exerting a charge-specific exclusion to the specific regions of the endothelial cell surface. Also it regulates nitric oxide (NO) production and activation of intracellular signals important in mediating barrier function (Arevalo et al. 2009).

1.4.2.1.2 Vascular endothelium damage during severe dengue infection

Life threatening DSS in severe DENV infection is a consequence of plasma leakage in the capillary endothelium. Clinical, *in-vitro* and *in-vivo* evidence has suggested that various mechanisms, such as: enhancing antibodies (Basu and Chaturvedi 2008); cross-reacting antibodies (Lin et al. 2002; Lin et al. 2003); soluble immune mediators such as cytokines and chemokines (Chen et al. 2007; Dewi et al. 2004; Lin et al. 2005); complement and its products (Basu and Chaturvedi 2008); and virulent DENV strains and virus virulence (Chen et al. 2007), may induce vascular permeability leading to plasma leakage (Basu and Chaturvedi 2008).
In-vitro studies have shown that endothelial damage by DENV can be caused by several factors, including infectious DENV, cytotoxic reagents, and pathophysiological mechanisms mediated by immune responses (Basu and Chaturvedi 2008). Infection of human endothelial cells from different vascular beds (Liver Sinusoidal Endothelial Cells (LSECs), dermal endothelial cell line — HMEC-1, and Human Umbilical Vein Endothelial Cells (HUVEC)) are susceptible to DENV in varying degrees. Infection causes apoptosis in some endothelial cell types, leading to vascular leakage due to the loss of vascular barrier integrity. Permeability analysis on endothelial monolayers shows an increase of permeability with the infection of DENV (Avirutnan et al. 1998; Basu and Chaturvedi 2008; Dewi et al. 2004; Huang et al. 2000; Peyrefitte et al. 2006; Talavera et al. 2004). DENV-induced cytokine production by endothelial cells is another mechanism contributing to permeability changes in endothelial cell monolayers in in-vitro models of vascular permeability (Andrews et al. 1978; Avirutnan et al. 1998; Dewi et al. 2004; Huang et al. 2000; Talavera et al. 2004).

Evidence from clinical presentations of DHF/DSS patients has demonstrated that there is extensive endothelial cell dysfunction during DENV infection all over the body, including lung alveoli, lung pleura, pericardium, abdomen, liver, brain, gut, urinary tract and reproductive tract (Basu and Chaturvedi 2008). High numbers of dengue viral RNA copies in endothelial cells of the liver and lungs in biopsy and autopsy samples from DHF/DSS patients suggests viral replication in endothelial cells (Jessie et al. 2004). The study done by Limonta et al. (2007) showed that DENV induced apoptosis of pulmonary, intestinal and cerebral endothelial cells in DHF/DSS patients in Cuba (Limonta et al. 2007). In addition, it has been shown that an up-regulation of several cytokines and the shift of helper T-cell type (Th) 1 response to the Th2 response during severe dengue
infection is responsible for plasma leakage (Chaturvedi 2009; Chaturvedi et al. 2000). In DENV-infected AG129 mice, endothelial damage caused defects in spleen, liver and intestine due to plasma leakage (Shresta et al. 2006) and in C57BL/6 mice, the tumor necrosis factor (TNF) induced endothelial cell death (Chen et al. 2007). This in-vivo evidence also suggests an indirect or direct effect by DENV on vascular endothelial damage.

1.4.3 Cellular tropism of dengue virus infection

Cells of hematopoietic lineage, including monocytes, macrophages and DCs, are primary targets of DENV natural infection (Kyle et al. 2007; Pham et al. 2012). Human autopsy studies have described DENV RNA in splenic macrophages and peripheral blood monocytes (Jessie et al. 2004) and DENV NS3 protein in splenic macrophages, lymph node macrophages, and alveolar macrophages (Balsitis et al. 2009). Blood-derived DCs are reported to be tenfold more permissive for DENV than monocytes or macrophages (Wu et al. 2000). It has been shown that DENV infection is initiated by infection of resident cutaneous Langerhans DCs at the site of a mosquito bite (Pham et al. 2012).

Several other cells, including hepatocytes, B and T lymphocytes, endothelial cells and fibroblasts, have been implicated as potential targets for DENV infection in in-vitro studies (Jessie et al. 2004). Some early studies reported DENV infection or antigens in lymphocytes (Boonpucknavig et al. 1976; King et al. 1999) while a few recent in-vitro studies have challenged lymphocytes as a target of DENV and have confirmed primary human splenic macrophages as primary DENV targets, and not B or T lymphocytes (Balsitis et al. 2009; Blackley et al. 2007). A recent study demonstrated that overall
dissemination of DENV 2 was prevented in the absence of hematopoietic primary cells, and suggested that non-hematopoietic primary cells are not productively infected and that macrophages or DCs are essential for DENV dissemination (Pham et al. 2012).

1.4.3.1 Cells of the immune system

DCs are antigen presenting cells (APCs) and ‘sentinels’ of the immune system. According to ‘Langerhans cell paradigm’, immature DCs residing in peripheral tissues capture viruses through phagocytosis, macropinocytosis and receptor-mediated endocytosis. These immature DCs contain receptors for pathogen-associated molecular patterns (PAMPs), Toll-like receptors (TLRs), RIG-I like receptors, C-type lectin receptors, nucleotide-binding oligomerization domain (NOD) proteins, cytokine receptors and chemokine receptors. Signalling through these receptors triggers DC migration to secondary lymphoid organs as well as the DC maturation process. Mature DCs present antigens to T lymphocytes and activate the adaptive immune system (Palucka 2000; Villadangos and Schnorrer 2007).

DCs have been shown as early cellular targets of DENV infection (Boonnak et al. 2008). DENV injected into the skin through an infectious mosquito bite will undergo replication in the skin’s Langerhans cells (Espada-Murao and Morita 2011). Additionally monocyte-derived DCs also act as targets of DENV infection, and immature monocyte-derived DCs were reported to be 10-fold more permissive to DENV than macrophages or monocytes (Palucka 2000; Wu et al. 2000). In-vivo observations of DENV-infected DCs, in interferon (IFN) receptor-deficient mice, also suggested the involvement of DCs in DENV infection (Kyle et al. 2007).
The DC-specific DC-SIGN molecule — a cell-surface, mannose-specific and C-type lectin — has shown to be essential for the productive infection of DCs by mosquito-cell-derived DENV (Navarro-Sanchez et al. 2003). A recent study on interaction of DENV produced in DCs with C-type lectin molecules (DC-SIGN and L-SIGN) showed that DENV produced in DCs are capable of infecting cells expressing L-SIGN; however, the virus does not infect DC-SIGN-expressing cells, suggesting a switching of lectins during DENV infection (Dejnirattisai et al. 2011). The incapability of DC-produced DENV to infect DC-SIGN molecules expressing DCs suggests that DCs may be a site of initial infection by mosquito-derived DENV, but they do not participate in sequential infection by DC-produced virus. (Dejnirattisai et al. 2011).

The association between DC-SIGN and antibody-dependent enhancement (ADE) infection of DCs with respect to DENV has been recently studied, since DCs are targets of DENV and require DC-SIGN molecules to enter DCs (Boonnak et al. 2008). Boonnak et al.’s study showed that ADE is inversely correlated with DC-SIGN expression in DCs. The researchers found that both immature and mature DCs express similar Fcγ receptor levels, which are essential in ADE infection. Immature DCs which express higher levels of DC-SIGN are unable to support ADE infection of DENV, despite expression of Fcγ receptors. In contrast, mature DCs which express low levels of DC-SIGN support ADE infection. This study therefore demonstrated two different entry routes for DENV into mature DCs: complex entry through DENV-antibodies via Fcγ receptors, or direct DENV entry via DC-SIGN receptors (Boonnak et al. 2008).

Infected DCs then migrate from the infection site to the draining lymph node subsequently triggering the host immune response to the invading pathogen. This then results in a
primary viremia, where DENV becomes disseminated through the lymphatic and vascular systems throughout the body. After the primary viremia, circulating monocytes in the blood and macrophages in the liver, spleen and bone marrow and myeloid DCs become targets of DENV. During a heterologous secondary infection, DENV-specific immunoglobulin G (IgG) binds with newly produced DENV, and form virus-IgG complex. This complex gains entry into mononuclear cells, through biding of Fc portion of the antibody to the Fc receptors on mononuclear cells (Martina et al. 2009). The number of cells infected influences the level of cytokine, chemokine and other mediator production.

1.4.3.2 Endothelial cells

*In-vitro* and *in-vivo* studies have suggested that human endothelial cells are potential targets of DENV replication (Balsitis et al. 2009). These studies have found DENV antigens in the endothelium of different tissues (Balsitis et al. 2009; Jessie et al. 2004). DENV NS3 antigen was detected in splenic endothelium, sinusoidal endothelium and in larger vessels in a human autopsy study (Balsitis et al. 2009). In another study, DENV antigens in Kupffer and sinusoidal endothelial cells of the liver and vascular endothelium in the lung were detected by immunohistochemistry and *in-situ* hybridisation analysis of tissue specimens from DENV patients (Jessie et al. 2004).

Many studies have used endothelial cells to investigate underlying mechanisms in fatal dengue infection which is a result of permeability changes in the vascular endothelium (Basu and Chaturvedi 2008). The variable levels of infectivity of endothelial cells, reported as being from 1% to 90%, may possibly be due to the use of different viral
strains, endothelial cells from different vascular beds and different methods of analysing (Arevalo et al. 2009). Early vascular permeability studies were widely carried out in an ECV304 cell line which was initially proposed as a model for investigating DENV-induced DHF/DSS (Bonner and O'Sullivan 1998; Bosch et al. 2002; Peng et al. 2009; Wei et al. 2003). ECV304 cells were later identified as a non-endothelial derived cell line, and thus proposed molecular mechanisms of vascular leakage using this cell line were no longer reliable (Kiessling et al. 1999).

Detection of DENV antigens or productive dengue viral infection in endothelial cells in autopsy studies has been limited due to the rapid occurrence of DHF/DSS after defervescence and clearance of viremia. Due to the paucity of a suitable animal model of dengue infection, in-vivo evidence of pathology of the DENV-infected endothelium has also been limited. Under these circumstances, in-vitro endothelial cell models have become valuable in investigating vascular leakage in DENV infection. In this context, investigation of endothelial cells that can be productively infected by DENV, and the effect of DENV on inducing the release of immune modulators by infected endothelial cells which may contribute to vascular leakage, remain to be identified. This study aims to investigate endothelial cells from different vascular beds, including the human brain endothelial capillary cell line (hCMEC/D3) and the liver sinusoidal endothelial cell line (LSEC), in order to characterise DENV infection.

The human brain endothelial capillary cell line, hCMEC/D3, has been developed as a model for the human blood-brain barrier (BBB). It is the first stable, fully characterised and well differentiated human brain endothelial cell line. It is widely used in studies of BBB (Cucullo et al. 2008), of the responses of brain endothelium to inflammatory and
infectious stimulations, and the associations between brain endothelium and lymphocytes (Aguet et al. 1995; Cucullo et al. 2008). These cells express chemokine receptors, up-regulated cell surface adhesion molecules and junctional markers (Weksler et al. 2005). The properties of hCMEC/D3 cells allow it to be used in assessing permeability changes of cell monolayers (Aguet et al. 1995; Cucullo et al. 2008). The DENV infection of hCMEC/D3 cells has not been previously reported and this study will be the first to characterise DENV infection in hCMEC/D3 cells.

The liver is formed by hepatocytes and sinusoids surrounding a central vein. The endothelial lining of liver sinusoids is formed by liver sinusoidal endothelial cells (LSEC) (Jacobs et al. 2010). Open fenestration without a diaphragm or basement membrane is a typical feature of LSECs, in which it differs from all other capillaries (Braet and Wisse 2002; Elvevold et al. 2008). Zellweger et al. (2010) recently reported a large percentage of DENV-infected LSECs in AG129 mice that correlated directly with disease severity (Zellweger et al. 2010). Human LSECs have been previously shown to be susceptible to DENV infection, as in the productive replication by Peyrefitte et al. (2006). This is the only study in which human LSECs were investigated for DENV infection, and the study was limited to detection of functional changes in LSECs. This study will therefore aim to characterise DENV infection in LSECs.
1.5 Host immune modulators

1.5.1 Innate immunity

The innate immune system is the first line of defence to prevent viral invasion or replication before the specific adaptive immune response is initiated (Koyama et al. 2008). The innate immune system provides a non-specific immediate defence against a wide variety of pathogens. Innate immune responses to infection include surface defence, cytokine production, complement activation and phagocytic responses (Tosi 2005). Once the host cell recognises a virus through a variety of innate immune receptors, the innate immune system is activated soon after infection and provides protection against the invader through a number of cells and soluble factors. Innate immune responses are simultaneously modulated by several factors, including products secreted by innate immune cells, hormones and by the central nervous system (Giroir 2006).

Pattern recognition receptors such as toll-like receptors (TLRs) and cytoplasmic helicases are among the first lines of defence in the innate immune response to pathogen-associated molecular patterns (Figure 1.11). Among TLRs, TLR3, TLR7 and TLR8 are important in sensing invasion of double-stranded and single-stranded RNA. Recognition of DENV RNA by TLR3 after endosomal acidification or of TLR7 in DC-induced production of CXCL8 and type 1 IFN inhibits DENV replication. Recognition of DENV RNA by TLR3 induced phosphorylation of TIR-domain-containing adapter-inducing interferon-β (TRIF) which in turn interacts with TNF-receptor associated factors (TRAF) 3 and TRAF6. TRIF binds with TRAF3-TANK-binding kinase 1 (TBK1) complex that leads to IRF3 phosphorylation, dimerisation and translocation to nucleus which, in turn induces type 1 IFN expression. TRIF-induced TRAF6 activate AP-1 and initiate Ikk1/Ikk2
Dephosphorylation of IkB to activate NF-κB to induce proinflammatory cytokine expression. (Green et al. 2014) (Lee et al. 2012).

Cytoplasmic RNA sensors, retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation associated gene-5 (MDA-5) with TLR3 synergistically play an important role in restricting DENV infection. Increased expression of RIG-I and MDA-5 upon DENV infection can initiate IFN production. RIG-I and MDA-5 signal through mitochondrial proteins to activate TRAF3 and TRAF6. As described above, TRAF3 activation translocates IRF3 and IRF7 to produce IFNα/β, while TRAF6 translocates NF-κB to produce type 1 IFNs (Green et al. 2014; Nasirudeen et al. 2011).

A major component of the innate immune response is the induction of IFNs, which are strong inhibitors of DENV infection. Most cells produce type I and type II IFNs to modulate production of proinflammatory and antiviral molecules (Clyde et al. 2006; Espada-Murao and Morita 2011; Rodenhuis-Zybert et al. 2010). The viral infection induces cells to secrete IFN α/β, which signals to adjacent cells about the infection and induces an autocrine induction of antiviral responses. Secreted IFNs bind to IFN-α/β receptor (IFNAR) and activate Janus kinases (JAK), TYK2 and JAK1 adaptor molecules to auto-phosphorylation. Activated JAK molecules attract various signal transducer and activator of transcription (STAT) molecules, including STAT1, STAT2, STAT3, and STAT5, and results in phosphorylation and dimerisation. Dimerised STAT1 and STAT2 bind with IRF9 and form an interferon-stimulating gene factor 3 (ISGF3) complex that translocates to the nucleus. The complex binds to the promoter region of IFN-stimulated genes and induces transcription of more than 100 different genes of antiviral proteins and pro-inflammatory cytokines (Green et al. 2014; Navarro-Sánchez et al. 2005).
IFN activity appears to be critical for innate immune responses to resist DENV infection. Mice deficient in IFN receptors are extremely susceptible to DENV infection, while IFN α/β-mediated action can limit DENV replication and spread to the central nervous system. The role of type I IFNRs is critical for early immune responses to DENV infection, while IFN γ receptor-mediated responses act at later stages of infection for clearance of DENV (Shresta et al. 2004).

Several clinical studies have also observed a correlation between type I IFNs and disease severity (Espada-Murao and Morita 2011). The type I IFNs were shown to inhibit viral dissemination in mild dengue cases, while a suppression of viral inhibition was observed during severe dengue cases (Long et al. 2009; Ubol et al. 2008). These changes in immune responses of IFNs can be due to the differential activation of IFN-producing immune cells during disease progression; plasmacytoid DCs decrease in DHF patients compared to DF patients (Long et al. 2009; Pichyangkul et al. 2003).

Compared to DF patients, higher plasma IFN γ levels have been reported in DHF patients (Libraty et al. 2002; Priyadarshini et al. 2010). Additionally, DHF cases that progress to vascular leakage have also shown significant increases in IFN γ levels (Libraty et al. 2002; Priyadarshini et al. 2010). A similar result was demonstrated in an in-vitro assessment of human endothelial cells, where a direct effect of IFN γ level and endothelial cell permeability was reported (Dewi et al. 2004). Nevertheless, IFN γ was originally produced for the protection of the host, as it may result in disease pathogenesis in severe dengue clinical cases.

Several cells of the innate immune system, including monocytes, DCs, natural killer (NK) cells, basophils and neutrophils, play a protective role during DENV infection (Giroir
DCs are targets of DENV infection and are important in both innate and adaptive immunity. They offer protection against DENV, mainly via production of type I IFNs (Degli-Esposti and Smyth 2005).

NK cells play a key role in host defence against DENV infection (Azeredo et al. 2006a; Vidal et al. 2011). Even before the activation of an adaptive immune response, NK cells limit viremia through their activation and inhibitory receptors expressed on their surface. Activation receptors mediate cytokine release, including IFN \(\gamma\), tumor necrosis factor (TNF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and several chemokines (Azeredo et al. 2006a; Vidal et al. 2011). Additionally, NKs secrete type II IFN which activates macrophages and DCs, and it stimulates an adaptive immune response against DENV (Degli-Esposti and Smyth 2005; Navarro-Sánchez et al. 2005).

Elevated levels of NK cells during early DENV infection are associated with mildness of DENV cases and viral clearance in collaboration with CD8+ T cells (Azeredo et al. 2006a).

Monocytes and macrophages are heterogeneous bone marrow-derived cells that are important members of the innate immune system. Providing the first line of defence against invading organisms, macrophages phagocytose pathogens and present pathogen associated molecular patterns (PAMPs) on their cell membrane to activate the adaptive immune system. Despite their protective mechanism, during DENV infection, macrophages serve as host cells for DENV replication, increasing the severity of the disease and complicating immune functions (Chaturvedi et al. 2006). Monocyte subsets also produce inflammatory cytokines and chemokines in response to DENV infection, including IL-1\(\beta\), IL-6, CXCL8, TNF \(\alpha\), IFN \(\gamma\), CCL2, 3 and 4 (Kelley et al. 2011; Wong
et al. 2012). Elevated levels of these immune mediators secreted by DENV-infected monocytes are associated with severe disease conditions such as haemorrhagic manifestations, thrombocytopenia, coagulation disorders, vascular leakage, and shock syndrome (Azeredo et al. 2010; Kelley et al. 2011). Additionally, DENV-infected monocytes can produce factors that control DENV replication and intense immuno-activation (Azeredo et al. 2010).

The success of innate immunity in providing protection against DENV infection therefore lies in the balance between the induction of efficient anti-viral effector mechanisms and the avoidance of detrimental tissue damage.

1.5.2 Adaptive immune response

Adaptive immunity involves recognising the virus and producing neutralising antibodies to the virus which prevent the virus binding to cell surface receptors. However, all antibodies may not neutralise the viruses and they may enhance viral replication in host cells by aiding the virus’s entry into target cells (Eaton et al. 2010). This phenomenon is termed as ADE or antibody-mediated enhancement of viral infection.

When DENV infects and replicates in a host the viral proteins that immediately come in contact with the host are E and prM proteins. NS1 becomes a target after replication of DENV. These three proteins are involved in both protection and pathogenesis. Other non-structural proteins also become targets of the immune system rather slowly. E protein contains three distinct functional structural domains and domain III is an immunoglobulin-like domain that is exposed on the surface of the virion. These epitopes in domain III are DENV serotype-specific, sub complex-specific and complex-specific
epitopes. Domain III is the major target of neutralising antibodies, which is important for protection against infection. The prM protein present in the virion is a good inductor of immune response. prM antibodies are highly cross-reactive between all four serotypes and neutralisation ability ranged from poor to moderate. Further prM antibodies were shown to enhance DENV infection in FcR bearing cells in vitro, over a large range of concentrations. NS1 is a highly immunogenic protein that has been shown to induce protection against DENV in both natural infection and experimental models (Cedillo-Barrón et al. 2014) (Wahala and de Silva 2011).

1.5.2.1 Antibody dependent enhancement (ADE) of dengue virus infection

The Fc receptor- (FcR) dependent mechanism of ADE is widely used by many viruses in ADE infection to gain entry into host cells (Takada and Kawaoka 2003). Halstead and colleagues propose that FcR-bearing cells may bind to the Fc portion of an antibody which is bound to a virus, facilitating entry of the virus into the cell (Figure 1.12). This mechanism of entry for DENV also increases the chances of the virus infecting normally unsusceptible cells (Halstead et al. 1977). Complement-mediated ADE has been postulated as another mechanism of flavivirus ADE for non FcR bearing cells, in which complement receptors play a major role (Cardosa et al. 1983).

An association between severe dengue pathogenesis and ADE has been suggested by in-vitro, in-vivo and epidemiological studies. Several epidemiological studies have reported that individuals with prior dengue infection are likely to develop severe DHF/DSS in a secondary heterotypic strain infection (Halstead and O’Rourke 1977; Morens and Halstead 1990). Additionally, the presence of maternal DENV antibodies in infants born
to dengue-immune mothers have resulted in more severe dengue pathogenesis during their primary infection (Kliks et al. 1988).

ADE has been best described in in-vitro systems. Human monocytes, erythroleukemia cells (K562) and primary human DCs have been shown to mediate DENV ADE infection through an FcR-dependent pathway (Littaua et al. 1990; Sun et al. 2011). Human monocytes and K562 cells express Fc gamma R I and R II receptors which are used by DENV-antibody complex to attach to the host cells (Littaua et al. 1990). Mature monocyte-derived DCs and human cell lines bearing FcRs were also reported to support ADE infection (Boonnak et al. 2008). Dejnirattisai et al. showed serological response during DENV infection using human monoclonal antibodies. Anti-prM antibodies displayed partial virus neutralisation capacity and substantial cross-reactivity between serotypes. This response of prM leaves DENV susceptible to ADE infection (Dejnirattisai et al. 2010).

Furthermore, ADE infection was observed in an in-vivo study where a nonlethal illness in mice turned into a fatal disease resembling human DHF/DSS. In these mice, DENV-specific antibodies promoted massive infection of LSECs, resulting in increased severity of dengue disease (Zellweger et al. 2010).
1.6 Hypothesis and Aims

In natural infection, DENV encounters skin Langerhans cells in the dermal space which serve as the initial site of virus replication. Infected DCs are trafficked to lymph nodes where additional viral replication occurs, resulting in a primary viremia. The virus is then disseminated throughout the body causing a systemic infection, and replication occurs in selected organs, contributing to a secondary viremia (Espada-Murao and Morita 2011). Endothelial cells may serve as targets of viral replication following primary or secondary viremia. The onset of vascular leakage from increased micro vessel endothelial permeability is one of the life-threatening complications in DHF/DSS, of which the pathogenic mechanisms are still unclear. It is necessary to understand the underlying mechanisms of vascular leakage in order to identify better strategies for controlling DHF/DSS.

This study hypothesises that:

1. DENV infection of human microvascular endothelial cells, from organs that show natural tropism to DENV infection, leads to increased cell permeability.
2. DENV infection of endothelial cells induces the release of immune modulators which may contribute to vascular leakage, as seen in DHF/DSS.
3. DENV infection of endothelial cells generates a differential host gene expression that may contribute to determining the disease outcome.

To address these hypotheses we aim to:

1. Identify physiologically relevant *in vitro* microvascular endothelial cell models in order to study DENV infection;
a. Examine permissiveness of DENV in microvascular endothelial cells derived from human liver and brain;
b. Determine growth kinetics of DENV in endothelial cells;
c. Examine DENV entry to endothelial cells through antibody dependent enhancement;
d. Determine DENV-induced apoptosis in endothelial cells.

2. Characterise the soluble immunomediators induced by DENV infection;
   a. Assess cytokine profiles secreted by endothelial cells;
   b. Evaluate chemokine production by endothelial cells;
   c. Study growth factors secreted by endothelial cells.

3. Evaluate DENV-induced permeability changes in endothelial cells;
   a. Assess expression patterns of cell adhesion molecules in endothelial cells;
   b. Determine permeability alterations of human liver and brain microvascular endothelial monolayers by endothelial supernatants;
   c. Determine permeability alterations of human liver and brain microvascular endothelial monolayers by macrophage supernatants.

4. Characterise host gene response in endothelial cells to DENV infection;
   a. Examine the differential gene expression induced by DENV infection in endothelial cells;
   b. Determine host cell functional pathways affected by DENV infection;
   c. Identification of potential antiviral target genes for DENV infection.
Figure 1.1 Structure of the DENV virion

The nucleocapsid comprising multiple copies of capsid protein and a single stranded RNA genome surrounded by a lipid bilayer-envelope. The envelope is made of 180 copies from each of the two structural proteins: envelope glycoprotein and membrane protein.
Figure 1.2 DENV RNA genome

DENV single-stranded RNA molecule with a 10.7 kilobase length. The single open reading frame (ORF) of the DENV genome is flanked by a capped 5’ untranslated region (UTR) and a nonpolyadenylated 3’ UTR. The viral and cellular proteases interact with UTR and translate ORF to produce a single polyprotein containing three structural (C, prM and E) and seven non-structural (NS; NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) proteins.
Figure 1.3 DENV mosquito vector

DENV can transmit to humans through a bite of an infected female mosquito of (A) *Aedes aegypti* or (B) *Aedes albopictus* species. *Aedes aegypti* is considered as the primary vector of DENV infection. These mosquitoes have a worldwide distribution while most prevalent in tropical and subtropical regions of the world.

Source: Figure provided by Copyright Clearance Center with the permission of publisher Elsevier (Burt et al. 2012)
Figure 1.4 Life cycle of DENV

(1) DENV is transmitted to humans through a bite of an infectious mosquito. (2) DENV will enter into a wide range of cells through cellular receptors for replication. (3) DENV dissemble in the host cell cytoplasm and release genome. (4) Genome encodes a polyprotein and translocate to endoplasmic reticulum (ER) for processing. (5) The assembly of synthesised viral proteins with new viral RNA occurs in the lumen of the ER. (6) The new virions are released from the ER by budding into the Golgi apparatus and structural transitions take place in the trans-Golgi network. (7) Subsequently nucleocapsids are released into cytoplasm.
Figure 1.5 Areas at risk of DENV transmission, 2014

The integrated map indicate the relative amount of dengue globally. The World Health Organization has estimated 2.5 billion people are at risk of dengue infection in over 100 endemic countries and new areas where DENV can be transmitted. DENV is most common in urban and suburban areas in the Americas, South-East Asia, the Eastern Mediterranean and the Western Pacific and in rural areas in Africa.

Source: Figure provided by Copyright Clearance Center with the permission of publisher Elsevier (Guzman and Harris 2015)
Figure 1.6 DENV infection notifications by place of acquisition in Australia

The place of DENV acquisition before 1999 was unknown for most cases. Between 1991 to 1998 three locally acquired DENV outbreaks occurred in Northern Queensland. Since 2008 the number of imported dengue cases has increased and the presence of vector mosquito in urban areas of Queensland there is a risk of getting DENV infection endemic.

Source: (Knope et al. 2013)
Figure 1.7 Time course of clinical signs and symptoms of dengue infection

DENV causes a broad spectrum of illnesses ranging from a mild DF to severe DHF/DSS. DF is accompanied by retro-orbital pain, severe headache, severe muscle aches, bone and joint pains, abdominal pain, skin rash and nausea. Severe DHF is accompanied with haemorrhagic manifestations, such as coagulopathy, thrombocytopenia and increased vascular fragility and permeability.

Source: Figure modified from (Whitehead et al. 2007)
Figure 1.8 Clinical manifestations of DENV infection

DENV affects infants, young children and adults and causes a broad spectrum of illnesses ranging from a mild febrile illness to the most severe haemorrhagic fever. (A) Typical petechial rash in an infant with DF. (B) A minor bleeding around injection sites, a very common feature in dengue. (C) A hematoma in a patient with DHF. (D) Characteristic diffuse macular rash that appears after recovery from the acute illness in an adult patient.

Source: Reproduced with permission from (Simmons et al. 2012), Copyright Massachusetts Medical Society.
VE-cadherin is a strictly endothelial specific adhesion molecule responsible for barrier function. It binds with the adjacent cells VE-cadherin extracellular domains and adhere neighbouring cells together. The p120-catenin, β-catenin, γ-catenin and α-catenin connect cadherin complex to the actin cytoskeleton. Cell-cell biding is further facilitated by PECAM-1 and JAMS. Additionally JAMS binds with actin microfilaments via ZO-1 and α-catenin and stabilise adherens junction structure.

Source: Figure modified from (Yuan and Rigor 2010)
Figure 1.10 Structure of tight junctions

Tight junctions are considerably variable along different vascular beds and contribute to barrier function only at the brain, retina and testicles. Tight junctions are consist of interactions of claudin and occludin family members. Intracellularly tight junctions binds with actin microfilaments via ZO protein and α-catenin for stabilisation.

Source: Figure modified from (Yuan and Rigor 2010)
Figure 1.11 Host innate immune response to DENV infection

DENV RNA is recognised by pattern recognition receptors TLR-3 and phosphorylate TRIF to interact with TRAF3 and TRAF6. Interaction of with Ikk1 phosphorylate IRF3. TRIF induced TRAF6 and initiate IKK1/I KK2 dephosphorylation of IKB to activate NF-κB. Nuclear translocation of TRAF3 induced IRF3 and TRAF6 induced AP-1 and NF-κB produced type 1 IFN and chemokines. MDA5 and RIG signals through mitochondrial proteins to activate TRAF3 and TRAF6 to induce IFN-α/β production.

Source: Figure provided by Copyright Clearance Center with the permission of publisher Elsevier (Green et al. 2014)
Figure 1.12 Antibody dependent enhancement of DENV infection

ADE of infection occurs when pre-existing antibodies present in the body from a primary DENV infection, bind to an infecting DENV particle during a subsequent infection with a different DENV serotype. These non-neutralising heterotypic antibodies form DENV-antibody complex. It binds to FcR bearing cells via Fc portion of the antibody facilitating virus entry into the cell. This mechanism increases the chances of the virus infecting normally unsusceptible cells.
Table 1.1 Classification of the *Flaviviridae* family*

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Transmission</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Flavivirus</em></td>
<td>Dengue (DENV)</td>
<td>Mosquito-borne</td>
<td>Dengue Fever (DF)</td>
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<td></td>
<td></td>
<td></td>
<td>Haemorrhage</td>
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<tr>
<td></td>
<td>Yellow fever (YFV)</td>
<td>Mosquito-borne</td>
<td>Haemorrhage</td>
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<tr>
<td></td>
<td>Japanese encephalitis (JEV)</td>
<td>Mosquito-borne</td>
<td>Liver destruction</td>
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<td></td>
<td></td>
<td>Encephalitis</td>
</tr>
<tr>
<td></td>
<td>West Nile (WNV)</td>
<td>Mosquito-borne</td>
<td>Encephalitis</td>
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<td></td>
<td>Murray Valley Encephalitis</td>
<td>Mosquito-borne</td>
<td>Encephalitis</td>
</tr>
<tr>
<td></td>
<td>(MVEV)</td>
<td></td>
<td></td>
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<td></td>
<td>Tick-borne Encephalitis</td>
<td>Tick-borne</td>
<td>Encephalitis</td>
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<td>(TBEV)</td>
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<td><em>Hepacivirus</em></td>
<td>Hepatitis C</td>
<td>Parental</td>
<td>Hepatitis</td>
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<td></td>
<td></td>
<td>transfusion</td>
<td>Liver cancerz</td>
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<td><em>Pestivirus</em></td>
<td>Bovine viral diarrhea virus 1</td>
<td>Contact</td>
<td>Fever</td>
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<td>Gastroenteritis</td>
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<td></td>
<td>Classical swine fever virus</td>
<td>Contact</td>
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<tr>
<td><em>Pegivirus</em></td>
<td>Pegivirus A</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pegivirus B</td>
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*Updated from ICTV database*
<table>
<thead>
<tr>
<th>Genotype/Cluster</th>
<th>Distribution of respective isolates</th>
</tr>
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<tbody>
<tr>
<td>American genotype</td>
<td>Latin America, including Mexico, and older isolates from India, the Caribbean, and the Pacific Islands</td>
</tr>
<tr>
<td>Asian genotype 1</td>
<td>Thailand and Malaysia</td>
</tr>
<tr>
<td>Asian genotype 2</td>
<td>China, Philippines, Sri Lanka, Taiwan, and Vietnam</td>
</tr>
<tr>
<td>American/Asian genotype</td>
<td>China, Thailand, Vietnam, Brazil, Venezuela, and the Caribbean.</td>
</tr>
<tr>
<td>Cosmopolitan genotype</td>
<td>Australia, the Pacific Islands, Southeast Asia, the Indian Sub-continent, the Middle East, Africa, and Mexico</td>
</tr>
</tbody>
</table>

* Source: (Loroño-Pino et al. 2004)
## Table 1.3 DENV protein functions

<table>
<thead>
<tr>
<th>Protein</th>
<th>Role</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>Formation of nucleocapsid, RNA binding</td>
<td>(Alcaraz-Estrada et al. 2010; Leopoldo et al. 2011)</td>
</tr>
<tr>
<td>PrM</td>
<td>Stabilise E protein</td>
<td>(Alcaraz-Estrada et al. 2010; Leopoldo et al. 2011)</td>
</tr>
<tr>
<td>E</td>
<td>Virion assembly, Receptor binding, Membrane fusion</td>
<td>(Alcaraz-Estrada et al. 2010; Leopoldo et al. 2011)</td>
</tr>
<tr>
<td>NS1</td>
<td>Cofactor for viral RNA replication</td>
<td>(Panyasrivanit et al. 2009) (Mackenzie et al. 1996)</td>
</tr>
<tr>
<td>NS2a</td>
<td>Involve in viral RNA replication, Inhibition of IFN α/β signalling, Virus assembly</td>
<td>(Gubler et al. 2014)</td>
</tr>
<tr>
<td>NS2b</td>
<td>Cofactor for NS3 proteolytic activity</td>
<td>(Gubler et al. 2014)</td>
</tr>
<tr>
<td>NS3</td>
<td>Proteolytic processing</td>
<td>(Bartenschlager and Miller 2008)</td>
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<tr>
<td>NS4a</td>
<td>Function in RNA replication, induction of membrane alterations</td>
<td>(Gubler et al. 2014)</td>
</tr>
<tr>
<td>NS4b</td>
<td>Dissociation of NS3 helicase from single stranded RNA, Inhibition of IFN α/β signalling</td>
<td>(Gubler et al. 2014)</td>
</tr>
<tr>
<td>NS5</td>
<td>RNA dependent RNA polymerase, STAT-2 binding and degradation</td>
<td>(Bartenschlager and Miller 2008)</td>
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</tbody>
</table>
Table 1.4 Dengue virus receptors in mammalian cells

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Cell/Tissue expression</th>
<th>Serotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparan sulphate</td>
<td>Vero cells, BHK-21 cells, SW-13 cells</td>
<td>DENV1 - DENV4</td>
<td>(Chen et al. 1997; Lee et al. 2006a)</td>
</tr>
<tr>
<td>nLC4Cer</td>
<td>Vero cells, BHK-21 cells, K562 cells</td>
<td>DENV1 - DENV4</td>
<td>(Aoki 2006)</td>
</tr>
<tr>
<td>DC-SIGN / L-SIGN</td>
<td>Dendritic cells, Macrophages</td>
<td>DENV1 – DENV4</td>
<td>(Navarro-Sanchez et al. 2003; Pokidyshova et al. 2006; Tassaneetrithep et al. 2003)</td>
</tr>
<tr>
<td>Mannose receptor</td>
<td>Macrophages</td>
<td>DENV1 – DENV4</td>
<td>(Miller et al. 2008)</td>
</tr>
<tr>
<td>HSP70/HSP90</td>
<td>HepG2 cells, SH-SY5Y cells, Macrophage</td>
<td>DENV2</td>
<td>(Reyes-del Valle et al. 2005)</td>
</tr>
<tr>
<td>Laminin receptor</td>
<td>PS clone D cells, HepG2 cells</td>
<td>DENV1 – DENV3</td>
<td>(Tio et al. 2005)</td>
</tr>
<tr>
<td>GRP78</td>
<td>HepG2 cells</td>
<td>DENV2</td>
<td>(Cabrera-Hernandez et al. 2007; Jindadamrongwech et al. 2004; Upanan et al. 2008)</td>
</tr>
<tr>
<td>CD-14 associated protein</td>
<td>Monocytes, Macrophages</td>
<td>DENV2</td>
<td>(Chen et al. 1999)</td>
</tr>
</tbody>
</table>
Chapter II

Materials and Methods
2.1 Materials

2.1.1 Tissue culture reagents

The following tissue culture reagents were used for the propagation and maintenance of cell lines during the project.

   i. Foetal Calf Serum (FCS)

FCS was heat inactivated prior to use by heating at 56°C for 60 minutes and aliquots were stored at -20°C.

   ii. Tissue Culture Media

Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, Aldrich) was used for the propagation of baby hamster kidney (BHK)-21, LSEC and C6/36 cells. Endothelial Basal Medium-2 (EBM-2) (Lonza, CC-3156) was used for hCMEC/D3 and OptiMEM (Sigma, Aldrich) for Vero cells, propagation respectively. RPMI-1640 medium (Sigma, Aldrich) was used for the maintenance of THP-1 cells, monocytes and differentiation of monocyte derived macrophages. Leibovitz's L-15 (Gibco/Invitrogen, 11415-064) was used for C6/36 cell propagation without CO₂ conditions (see appendix for media). All media were supplemented with 2mM L-glutamine (L-glut, Sigma-Aldrich) and maintenance media contained 1% v/v penicillin/streptomycin, additionally (Sigma-Aldrich).

   iii. Cell lines

Vero cells obtained from Sigma-Aldrich (ATCC cat no: CCL-81) was maintained in Opti-MEM complete growth medium. DENV infection of the cell line was performed in corresponding maintenance media containing 2% heat inactivated FCS (HIFCS).
C6/36 cells, obtained from Dr Sylvie Alonso, National University of Singapore were grown in Leibovitz's L-15 medium supplemented with 10% HIFCS. Virus infection of cells was performed in maintenance medium. C6/36 cells obtained from Sigma-Aldrich (cat no: CRL-1660) were maintained in DMEM complete medium and virus propagation was done with DMEM maintenance medium.

THP-1 cells obtained from Sigma-Aldrich (ATCC cat no: TIB-202) was maintained in RPMI-1640 complete growth medium. DENV infection of THP-1 cells was performed in corresponding maintenance media containing 2% HIFCS.

hCMEC/D3 and LSEC cells were obtained from Prof. Georges Grau, Sydney University. hCMEC/D3 and LSEC cells were grown in complete growth media of EBM-2 and DMEM respectively. LSEC and hCMEC/D3 lines were used at constant passage number ± 1 for all experiments, that is passage 22 ±1 for LSEC and passage 30 ± 1 for hCMEC/D3. Virus infection was performed in respective maintenance media.

All mammalian cells were cultured at 37°C with 5% CO₂ and C6/36 cells at 28°C without CO₂ (Table 2.1).

### 2.1.2 Virus Isolates

DENV 2 strain D2Y98P, a virulent clinical isolate was provided by Dr. Sylvie Alonso. D2Y98P, derived from a 1998 DENV 2 Singapore human isolate has been exclusively passaged in *Aedes albopictus* C6/36 cells for 20 rounds. Virus was propagated in C6/36 cells at 28 °C, without CO₂. Virus titres were determined by plaque assay as described in sections 2.2.2.
2.1.3 Antibodies

Primary and secondary antibodies used in infection assays are listed in Table 2.2.

2.2 Methods

2.2.1 Cells maintenance and culture procedure

Frozen cells which were stored in liquid N$_2$ were rapidly thawed in 37 °C water bath and transferred immediately to a centrifuge tube containing 5 millilitres (ml) of growth medium. Cells were centrifuged at 200 x g for 5 minutes at room temperature. Supernatant was discarded and the pellet was gently resuspended in 5 ml of growth medium and transferred into culture flasks. Cells were incubated at optimal temperature for cell line in appropriate CO$_2$ conditions until they reached confluence to sub culture.

All cell cultures were maintained according to the standard cell culture methods. Briefly, cells grown to 80-90% confluency were subcultured as follows. The growth medium was discarded and monolayers were washed with 10 ml of PBS. Then 1 ml of Trypsin-EDTA solution (see appendix) was added to T75 flask and incubated at 37 °C for 5 minutes, to facilitate dispersal of monolayer. Dislodged cells from the culture flask were added to a tube containing 5 ml of media and centrifuged at 200 x g for 5 minutes at 22 °C. The resulting pellet was resuspended in growth medium and gently aspirated by pipetting. The cell suspension was aliquot at an appropriate subcultivation ratio to new culture flasks and incubated at 37°C or 28 °C.
2.2.2 Plaque assay

Plaque assays were carried in Vero cells. A density of 2.2x10^5 cells in 500 μL were seeded in wells of a sterile 12 well plate. The plate was gently tapped to disperse cells evenly and incubated at 37 ºC with 5% CO₂ overnight. Serial dilutions of virus stocks were prepared in serum-free Opti-MEM and stored on ice. Monolayers were washed with PBS three times and infected with 200 μL of virus dilution and incubated at 37ºC, in CO₂ atmosphere for two hrs with rocking at 30 minute intervals. Each dilution was tested in triplicate wells. The virus inoculum was removed and overlaid with 1 ml of 1% methyl cellulose (Sigma-Aldrich, see appendix for reagent preparation) in 1X Opti-MEM supplemented with 2% FCS. After five days incubation, monolayers were fixed with 3.7% formaldehyde (Sigma, Aldrich) in crystal violet (Sigma, Aldrich, see appendix for reagent preparation) and incubated overnight at room temperature. Monolayers were then washed thoroughly with water to remove the overlay medium. After plates were dried, the plaques were counted visually and the viral titre determined (PFU/ml).

2.2.3 Infection of LSECs

LSEC cells were seeded in 24 well plates at a density of 8 x 10^4 cells in 500 μL per well for 24 hrs at 37 ºC. After incubation monolayers were washed with PBS three times and infected with 100 μl of DENV at a MOI of 1, 5 and 10. The infected plates were incubated for two hrs with rocking at 30 minute intervals. After incubation, virus inoculum was removed from the wells and washed with PBS three times. Then 1 ml of DMEM maintenance medium was added to wells and incubated at 37 ºC. As described in section 2.2.2, supernatants were collected at 24 hour time points for five consecutive days and titrate virus by plaque assay.
2.2.4 Infection of hCMEC/D3s

The hCMEC/D3 cells were seeded in 24 well plates pre-coated with rat tail collagen (Cultrex) at a density of $8 \times 10^4$ cells/well. After 24 hrs incubation at 37 °C, monolayers were washed with PBS three times and infected with 100 μl of DENV at a MOI of 1, 5 and 10. The infected plates were incubated for two hrs with rocking at 30 minute intervals. After incubation, virus inoculum was removed from the wells and washed with PBS three times. Then 1 ml of EBM-2 maintenance medium was added to wells and incubated at 37 °C. At 24 hrs time points for five consecutive days, the supernatant were collected for determining viral titre.

2.2.5 Monocyte isolation from human blood and infection of monocyte derived macrophages

Human monocyte derived macrophages was cultured as previously described (Chang et al. 2004; Pirhonen et al. 1999). Firstly peripheral blood mononuclear cells (PBMCs) were isolated from anti-coagulated buffy coats (Blood Bank, Brisbane) by density gradient centrifugation. Buffy coat was diluted with 2-4X times the volume of buffer containing PBS, pH 7.2 and 2 mM EDTA (Sigma-Aldrich). Diluted blood suspension was layered above Ficoll-Paque (Sigma-Aldrich) at 7:3 ratios in 50 ml conical tube and centrifuged in a swinging-bucket rotor without brakes at 400Xg for 40 minutes at 20 °C. Carefully the mononuclear cell interphase was transferred to a new tube and washed with buffer by centrifuging at 200 Xg for 15 minutes at 20 °C. To remove any remaining red blood cells lysis buffer (Sigma-Aldrich) was added to the cell pellet and incubate for 5 minutes at rtp (room temperature) and centrifuged at 300 Xg for 10 minutes. Supernatant was pipette
off and continue washing once. Meanwhile cell count was performed to determine total PBMCs. Cell pellet was resuspended in 30 μl of buffer per 10^7 total cells to performed magnetic labelling. Magnetic, labelling and separation was performed using monocyte isolation kit II (Miltenyi Biotech), according to supplier’s instructions. Enriched monocyte fraction was quantified with haemocytometer. To evaluate the monocyte purity an aliquot of the cell faction was stained with CD14-FITC (Miltenyi Biotec) and analysed by flow cytometry. Purified cells were seeded in 24 plates at a density of 2 x 10^5 cells/well and cultured with complete RPMI-1640 medium supplemented with 10 ng/mL human GM-CSF (Miltenyi Biotec) and 10% human AB serum (Sigma-Aldrich), for 7 days at 37°C in 5% CO_2. Medium was supplemented every 2 days. After 7 days cells differentiated into macrophages were identified by their typical morphology and CD14 expression by flow-cytometric analysis. Adherent cells were washed with PBS three times and infected with 100 μl of DENV at a MOI of 1. The infected plates were incubated for two hrs with rocking at 30 minute intervals. After incubation, virus inoculum was removed from the wells and washed with PBS three times. Then 1 ml of RPMI supplemented with 10% human AB serum was added to wells and incubated at 37 °C. At 24 hrs time points for five consecutive days, the supernatant were collected for determining viral titre.
2.2.6 Flow cytometry analysis

i. Detection of cell-associated DENV antigen in endothelial cells

This experiment was performed to determine infectivity of endothelial cells by DENV. LSECs and hCMEC/D3 cells were seeded in 24 well plates separately, at a density of 8 x 10⁴ cells/well. After 24 hour incubation at 37 °C, monolayers were washed with PBS three times and infected with 100 μl of DENV at a MOI of 1. The infected plates were incubated for two hrs with rocking at 30 minute intervals. After incubation, virus inoculum was removed from the wells and washed with PBS three times. Then 1 ml of maintenance medium was added to the wells and incubated at 37 °C. At 24 hour time points for four days, the cells were harvested for fluorescence-activated cell sorting (FACS) analysis. BD Cytofix/Cytoperm™ fixation/permeabilization kit was used to determine cell associated DENV in all FACS experiments. The medium from the wells was removed and 1 ml of PBS supplemented with 2% FCS was added. Then the cells were harvested by gentle scraping and transferred to FACS tubes. Cells were washed with 1 X BD Perm/Wash buffer, provided in the BD cytofix/Cytoperm kit, by centrifuging at 1200 g for five minutes at 4 °C. Next cells were fixed and made permeable by adding 250 μl of fixation/permeabilization solution (provided in BD cytofix/Cytoperm kit), to tube, followed by incubation for 20 minutes at 4 °C. Cells were then washed twice with 1 X Perm/Wash buffer as described previously.

To reduce non-specific binding of antibodies, cells were pre-blocked by incubating with 2% (w/v) BSA in BD Perm/Wash buffer and incubated at 37 °C for 1 hour. Incubated cells were washed with 1 X BD Perm/Wash buffer, by centrifuging at 1200 g for five minutes at 4 °C. Permeabilised cells were stained with primary antibody 3H5 at 1:100
antibody dilution in BD Perm/Wash buffer. To each tube, 50 μl of antibody dilution was added and incubated for 30 minutes at 4 °C in the dark. Incubated cells were washed twice as previously described. Next the secondary antibody, Alexa fluor 488 goat anti-mouse IgG was diluted to 1:100 with BD Perm/Wash buffer and 50 μl was added to each well. A separate tube of permeabilised cells was incubated with secondary antibody only to use as a control. Then cells were incubated at 4 °C for 30 minutes in the dark. Incubated cells were washed twice with BD Perm/Wash buffer. Finally cells were resuspended in 500 μl of PBS supplemented with 2 % FCS for flow cytometry analysis.

ii. Detection of cell-surface antigens

To measure cell surface antigens (FcγRII/CD32, FcγRI/CD64, CD14 and CD31) on endothelial cells or PBMCs, cells were prepared as follows. Adherent LSEC and hCMEC/D3 monolayers reaching 80% confluence were detached by scraping. Cells in suspension (PBMCs) was collected to a centrifuge tube. Cells were washed twice with FACS buffer (0.5% BSA in 1xPBS) by centrifuging at 500 x g for 5 minutes at 4 °C. A cell count was performed and resuspended cells to a final concentration of 4 x 10⁶ cells/mL. Then 25 μl of cells (1 x 10⁵ cells) were transferred to a 5 ml FACS tube and added 10 μl of conjugated antibody ( PE/APC conjugated anti-CD32/64 for FcγRII/CD32, FcγRI/CD64, FITC conjugated anti-CD31 or APC conjugated anti-CD31) for staining. Cells were incubated for 45 minutes at 2-8 °C followed by washing cells twice in 1 ml of the FACS buffer. Finally cells were resuspend in 400 μl of propidium iodide (PI) buffer (1 μl PI in 1ml of FACS buffer). Samples analysed using FACS CyAn ADP analyser (Beckman Coulter, Inc.).
2.2.7 Endothelial cell fixation and staining for immunofluorescence assays

Endothelial cells were grown on sterile Cultrex rat collagen I (Trevigen) treated coverslip. Coverslips were immersed in 150 µg/mL rat collagen solution for 1 hour at 37 °C, then washed three times with PBS. Approximately 8 x 10⁴ cells were seeded onto the rat collagen treated coverslips in 24-well plates. After an overnight incubation cells were infected with DENV at a MOI of 1. At 24 hours post infection (hpi) cells were washed three times with phosphate buffered saline (PBS) and fixed in 3.6% formaldehyde (Sigma, Aldrich) in PBS for 20 minutes at 20-24 °C. Fixed cells were washed three times in PBS and permeabilised using 0.5% (v/v) Triton X-100 in PBS for 10 minutes at 20-24 °C and washed again three times in PBS. Cells were blocked in 1% (w/v) bovine serum albumin (BSA) in PBS and incubated at 37 °C for 1 hour. Primary antibodies were diluted accordingly in 1% (w/v) BSA in PBS and incubated with cells for 1 hour at 37 °C followed by three washed in PBS. Secondary antibody was diluted accordingly in 1% BSA in PBS and incubated with cells for 1 hour at 37 °C and washed three times in PBS and once with distilled water and mounted onto glass slides using ProLong gold antifade reagent with DAPI (Life Technologies). Staining was visualised under fluorescence microscope.

2.2.8 Annexin V-PE binding assay

Endothelial cells grown in 24 well plates (8 x 10⁴ cells/well) were incubated with DENV at MOI 1 and were harvested at indicated time points to determine the apoptotic cells using the PE annexin V apoptosis detection kit I (BD Pharmingen) according to the manufacture’s protocol. Briefly, 1x10⁶ endothelial cells were washed twice with cold
PBS and resuspended in binding buffer to stain with Annexin V-PE and 7-AAD stains (BD Pharmingen). After 15 minutes incubation samples were analysed by flow cytometry.

2.2.9 In situ end-labelling of DNA fragments by TUNEL assay

TUNEL assay was performed using TACS 2 TDT-DAB In Situ Apoptosis Detection Kit (Trevigen) according to the supplier’s instruction. Briefly, LSECs seeded directly on sterile microscopic slides. The cell monolayers were fixed with formaldehyde and treated with Proteinase K (Trevigen) to permeabilise cells followed by quenching endogenous peroxidase activity. The cells were covered with labelling reaction mixture and proceed to streptavidin-horseradish peroxidase binding. The monolayers were finally counterstained with methyl green (Trevigen) and washed sequentially by dipping ten times each in deionized water, 70% ethanol, 95% ethanol, 100% ethanol and xylene, in 2 changes. The development of brown colour in nuclei of apoptotic cells was monitored under light microscope.

2.2.10 Human cytokine, chemokine and growth factors quantitation by multiplex assay

Endothelial cells were seeded in 24 well plates at a density of 8x10^4 cells/well and incubated overnight. Cells were infected with DENV at MOI 1 on the following day and supernatants were collected at 24 and 48 hpi to quantify cytokines, chemokines and growth factors. Commercially available human 27-plex multiplex panel (Bio-Rad) was used according to the manufacturer’s instructions to analysed soluble factors in culture
supernatants. Standard curves for each cytokine were generated by using the reference cytokine concentrations supplied by the manufacturer.

2.2.11 Total RNA Isolation

i. Total RNA extraction by Trizol method

Total RNA was extracted from confluent endothelial cell monolayers grown in 24 well plates at 24 hour time intervals. At each time point cell monolayers was homogenised by adding 1 ml of TRIzol reagent and passing the cell lysate few times through a pipette. At this point samples can also be stored at -20°C for short term or at -80°C for long term storage. For phase separation 100 μl of chloroform was added to 1 ml of TRIzol reagent, mixed well and incubated for 10 minutes at rtp. Then the sample was centrifuged at 12 000 x g for 15 minutes at 4 °C. This centrifugation will separate sample in to 3 phases; phenol-chloroform phase in the bottom, an interphase and a colourless aqueous phase at the top containing RNA. Top aqueous phase was carefully transferred to a new clean tube to proceed with RNA precipitation. To precipitate RNA 500 μl of isopropyl alcohol was added and vortex for 10 seconds followed by 10 minutes incubation at rtp. Centrifuged the sample at 12 000 x g for 10 minutes at 4 °C and discard the supernatant leaving the pellet formed in the bottom and side of the tube. Then to wash RNA 1 ml of 75% ethanol was added and vortex to mix well. Centrifuged the samples at 7500 x g for 5 minutes at 4 °C and discard the supernatant. Briefly air dry the RNA pellet before dissolving in 100 μl of ultrapure water.
ii. **By QIAGEN RNeasy mini spin columns**

To extract total RNA from endothelial cells to use in microarray analysis for host gene studies, spin technology was used as per manufactures guidelines. Briefly, at each time point aspirate the supernatant from each well and washed cell monolayer with PBS for twice. Detached cells by adding 2.5% trypsin and transferred all to a tube. Centrifuged at 300 g x for 5 minutes and aspirate supernatant completely. Flicked the tube to loosen the pellet and disrupt cells by adding RLT buffer. Vortex the sample for 1 minute to homogenise and centrifuged the lysate for 3 minutes at maximum speed. To the supernatant 1 volume of 70% ethanol was added and immediately proceeded to next step. Complete lysate was transferred to RNeasy mini spin column and centrifuged for 15 seconds at 8000 x g. At each following step flow through was discarded and added RW1 buffer, RPE buffer separately and spin at same conditions as above. Another washing with RPE buffer was done for 2 minutes and finally to dry the membrane spin column was spin without any buffer. To elute the RNA, 50 μl of RNase free water was added and centrifuged for 1 minute at 8000 x g. RNA content was quantified using Nanodrop. A DNase digestion was not required during RNA purification since the silica-gel–membrane and spin-column technology used in QIAGEN RNeasy mini spin columns efficiently removes the majority of the DNA without DNase treatment.
2.2.12 cDNA synthesis

To proceed with cDNA synthesis the total RNA extracted was quantified using NanoDrop spectrophotometer. The maximum amount of total template that can be synthesised for the sample set was calculated. In all experiments initially 1000ng of template in 20 μl of mixture was synthesised and diluted 1:5 to obtain a final concentration of 10ng/μl of template. Template-primer master mix was prepared as shown in table 2.3.

All components were mixed gently and centrifuged to collect all components to the bottom of the tube. Tubes were then placed in the block cycler to denature the template under 70 °C for 10 minutes. Reverse transcription mix was prepared as shown in table 2.4 and 10μl was aliquot in to tubes after the denaturing has been completed.

Tubes were then placed in a thermal cycler and run at the following reaction conditions:

- Cycle 1: 10 min, 25 °C
- Cycle 2: 50 min, 37 °C
- Cycle 3: 10 min, 90 °C
- Cycle 4: Infinity, 4 °C

2.2.13 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

The CFX96™ Real-Time PCR Detection System (Bio-Rad) was used qRT-PCR analysis. To determine mRNA expression a master mix was prepared by adding the components into a 1.5ml microcentrifuge tube as listed in table 2.5. Appropriate forward and reverse primers were used to quantify cDNA in samples and hypoxanthine-guanine
phosphoribosyltransferase (HPRT) primers measures HPRT cDNA levels to normalise against (See Appendix).

Aliquot 19 µl of mastermix into each well on the 96-well plate, and pipette 5 µl of the synthesised cDNA into each well. Plate was sealed and spin down and run the PCR machine using a standard 3-step melt program, cycling parameters are as follows.

\[
\begin{align*}
\text{Denaturation} & : 95 ^\circ C \text{ for 15 min} \\
\text{Denaturation} & : 94 ^\circ C \text{ for 15 s} \\
\text{Annealing} & : 55 ^\circ C \text{ for 30 s} \\
\text{Extension} & : 72 ^\circ C \text{ for 30 s}
\end{align*}
\]

40 cycles

Data was acquired during extension step of each cycle. The cycling ended with a temperature gradient for melting curve analysis and only primer sets that produced a single melting peak were used for further analysis. With HPRT as an internal control, quantitative analysis was performed using the comparative C\(T\) method.

2.2.14 Endothelial cell monolayer permeability analysis

Eight well electrode arrays specific for (8W1E, Applied Biophysics) transendothelial electrical resistance (TEER) measurements were pre-treated with L-cysteine (10 mM) for 15 minutes at room temperature and washed twice in sterile water. Arrays to seed hCMEC/D3s were additionally coated with 0.3% collagen for 1 hour at 37 °C. Endothelial cells were seeded (LSECs: \(4 \times 10^4\) cells/well or hCMEC/D3s: \(4.5 \times 10^4\)) in coated arrays and loaded into the electrical cell-substrate impedance sensing (ECIS) morphological biosensor (Applied Biophysics) at 37°C. After electrical resistance of the endothelial cell monolayer, which is inversely proportional to permeability reached a steady-state,
experiments were started. LSEC monolayers were incubated with 200 µl of supernatant from 48 hpi mock control and DENV-infected LSECs. Similarly hCMEC/D3s monolayers were incubated with 200 µl supernatant from 48 hpi mock control and DENV-infected hCMEC/D3 supernatants. Additionally LSECs and hCMEC/D3 monolayers were also incubated with 200 µl of 96 hpi mock control and DENV-infected monocyte derived macrophage supernatants. Impedance readings of the endothelial cell monolayer were taken at 5 minute intervals for 24 hours. The ratio of the resistance at each time point to the resistance at the onset in that cycle was calculated for each electrode. All experiments were independently performed two times in triplicates.

2.2.15 Host cell gene expression analysis by microarray

The quality and quantity of human total RNA isolated from endothelial cells was ascertained on the Agilent Bioanalyser 2100 using the NanoChip protocol. A total of 500 ng of total RNA was then prepared for hybridisation to the Illumina humanHT 12 Expression Beadchip by preparing a probe cocktail (cRNA at 0.05µg/µl) that includes GEX-HYB Hybridisation Buffer (supplied with the beadchip). A total hybridisation volume of 15µl was prepared for each sample and 15µl loaded into a single array on the Illumina humanHT 12 Expression Beadchip. A total of 12 different labelled samples of mock control and DENV-infected LSECs and hCMEC/D3s were loaded into beadchip. The chip was then hybridised at 58°C for 16 hours in an oven with a rocking platform.

After hybridisation, the chip was washed using the appropriate protocols as outlined in the Illumina manual. Upon completion of the washing, the chips were then coupled with
Cy3 and scanned in the Illumina iScan Reader. The scanner operating software, GenomeStudio, converts the signal on the array into a TXT file for analysis.

2.2.16 Statistics

All statistical analysis was done with GraphPad Prism, version 5.0 (GraphPad Software, San Diego, CA). Data was analysed by Student’s t test and two-way ANOVA with Bonferroni posttest. A one-tailed $P$ value of $< 0.01$ was considered significant.
### Table 2.1 Details of cell lines used in the study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Morphology</th>
<th>ATCC® catalogue no</th>
<th>Tissue culture media</th>
</tr>
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<tbody>
<tr>
<td>Vero</td>
<td>African green monkey kidney</td>
<td>Epithelial</td>
<td>CCL-81</td>
<td>Opti-MEM</td>
</tr>
<tr>
<td>BHK-21</td>
<td>Baby-hamster kidney</td>
<td>Fibroblast</td>
<td>CCL-10</td>
<td>DMEM</td>
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<tr>
<td>C6/36</td>
<td><em>Aedes</em> albopictus larvae</td>
<td>Epithelial</td>
<td>CRL-1660</td>
<td>DMEM or L-15</td>
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<tr>
<td>hCMEC/D3</td>
<td>Human brain micro vessels</td>
<td>Endothelial</td>
<td>(Weksl et al. 2005)</td>
<td>EBM-2</td>
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<tr>
<td>LSEC</td>
<td>Liver sinusoidal</td>
<td>Endothelial</td>
<td>(Peyrefitte et al. 2006)</td>
<td>DMEM</td>
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Table 2.2 Antibodies used in this study

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<th>Product name</th>
<th>Species of origin</th>
<th>Description</th>
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<tr>
<td>3H5</td>
<td>Mouse</td>
<td>Specificity: DENV 2 serotype</td>
<td>TropBio, AU</td>
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<td></td>
<td></td>
<td>Isotype: IgG 1</td>
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<tr>
<td></td>
<td></td>
<td>E protein</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>Goat</td>
<td>Enzyme: Horseradish peroxidase (HRP)</td>
<td>Dako, Denmark</td>
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<tr>
<td>Mouse IgG (H+L)</td>
<td>Goat</td>
<td>Flurophore Alexa Fluor 488</td>
<td>Invitrogen, AU</td>
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<td>CD64-APC</td>
<td>Mouse</td>
<td>Isotype IgG1 kappa</td>
<td>R&amp;D Systems</td>
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<td></td>
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<td>Reactivity: Human</td>
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<td>CD32-PE</td>
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<td>CD14-FITC</td>
<td>Mouse</td>
<td>Isotype IgG2a</td>
<td>Miltenyi Biotec</td>
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<td></td>
<td></td>
<td>Reactivity: human, non-human primate</td>
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Table 2.3 cDNA template-primer master mix preparation

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<th>Component</th>
<th>Volume (μl)</th>
<th>Final Concentration</th>
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<tr>
<td>10 mM dNTP (Sigma–D7295)</td>
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<td>0.5 μM</td>
</tr>
<tr>
<td>Random nanamers (Sigma-R7647)</td>
<td>1</td>
<td>2.5 μM</td>
</tr>
<tr>
<td>Total RNA</td>
<td>8</td>
<td>10 ng/μl</td>
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<tr>
<td>Total volume</td>
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Table 2.4 Reverse transcription mix preparation

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<tr>
<td>10 x M-MLV Reverse Transcriptase Buffer</td>
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<td>1x</td>
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<tr>
<td>(Sigma- M1302)</td>
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<td></td>
</tr>
<tr>
<td>M-MLV Reverse Transcriptase</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>(Sigma-M1302)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribonuclease Inhibitor human</td>
<td>0.5</td>
<td>40 units/μl</td>
</tr>
<tr>
<td>(Sigma-R2520)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ultrapure water</td>
<td>6.5</td>
<td>-</td>
</tr>
<tr>
<td>Total volume</td>
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## Table 2.5 qRT-PCR master mix preparation

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<tr>
<td>FastStart SYBR Green Mastermix (Roche)</td>
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<td>1x</td>
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<tr>
<td>Primers (3µM)</td>
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<td>300 nM</td>
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<tr>
<td>RNase, DNase-free water</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>cDNA template</td>
<td>5</td>
<td>10 ng/µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>25</td>
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Chapter III

Human endothelial cells as experimental models to study dengue infection
3.1 Introduction

The absence of a suitable animal model to recapitulate features of DENV infection has impeded characterisation of the mechanisms underlying disease pathogenesis. The hallmark feature of DHF/DSS is vascular leakage of the endothelium. The relationship between dengue infection and the endothelium has been investigated in vitro and in vivo experiments. Nevertheless, the mechanisms by which dengue infection leads to increased vascular permeability remain unclear.

*In vitro* cell culture models of DENV infection have been used since late 1990s to investigate mechanisms of DENV induced endothelium damage each with limitations. The Human umbilical vein endothelial cell (HUVEC) model used by Anderson *et al.* was designed to investigate monolayer activation by culture supernatants from DENV-infected peripheral monocytes but not for endothelial barrier function studies (Anderson *et al.* 1997). In 1998 Bonner and O’Sullivan used endothelium cell of vessel 304 (ECV304) monolayers as a model system to study DSS. They reported that ECV304 cell line was more stable and able to obtain reproducible data compared to HUVECs and allowed for more experimental manipulations (Bonner and O’Sullivan 1998). ECV304 cells were later identified as a human urinary bladder carcinoma T24/83 derived cell line and therefore considered as an inappropriate cell line for endothelial studies (Brown *et al.* 2000). Jacobs and Levin optimise morphological and functional characteristics of HUVECs as an endothelial barrier model. They cultured HUVECs in human serum to resemble morphologically microvascular endothelium and permeability was assessed by transendothelial flux of albumin instead of using a labelled tracer molecule (Jacobs and Levin 2002). Since then HUVECs model has been used to study DENV infection. In a
recent study on cellular response of endothelial cells to DENV, >80% of HUVECs were shown to be infected with DENV (Dalrymple and Mackow 2012) which is inconsistent with previous studies showing HUVECs were less permissive to DENV (Arevalo et al. 2009; Avirutnan et al. 1998; Diamond et al. 2000). Although HUVECs can be infected with DENV, depending on culture conditions, the transendothelial barrier function of cultured HUVEC monolayers appeared to fluctuate (Talavera et al. 2004).

During DENV infection of humans the natural targets of DENV would be microvascular endothelial cells from different vascular territories where plasma leakage occur as a result of permeability increase (Bethell et al. 2001; Halstead 2002). Both ECV304 and HUVEC models are large vessels derived endothelial cells which exhibit different functional and phenotypic properties to microvascular territories (Peyrefitte et al. 2006). In this context endothelial cells derived from macrovascular territories i.e. HUVEC and ECV304, may not be an optimal model to study pathophysiology of vascular leakage seen during severe DENV infection.

Infection of cells by DENV can occur through direct or antibody dependent enhancement (ADE) -which has been suggested to contribute to the progression and severity of DENV infection (Guzman et al. 2013). Severe disease accompanies infection of Fcγ-receptor (FcγR) bearing cells by facilitating virus entry (extrinsic ADE) and modulate innate and adaptive immune mechanisms to enhance virus replication (intrinsic ADE) (Halstead et al. 2010). The literature on the presence of FcγRs on endothelial cells is questioned. Several groups have reported presence of FcγRs on endothelial cells including human aortic endothelial cells, dermal microvascular endothelial cells and HUVECs (Devaraj et al. 2005; Gröger et al. 1996; Liang et al. 2006). However Arevalo et al showed HUVECs
do not support ADE of DENV infection as they lack FcγR (Arevalo et al. 2009), indicating again HUVECs may not be an optimal *in vitro* model to study DENV pathogenesis due to the variances reported by different groups.

*In vitro* cell culture models are widely used to study molecular events that occur after DENV infection including apoptosis. Programmed cell death, or apoptosis, is a homeostatic mechanism resulting in the destruction of potentially harmful cells from tissues. Following *flaviviruses* infection, host cells may activate different apoptotic pathways. Moreover, DENV induced apoptotic cell death has been shown in both *in vitro* and *in vivo* models (Liu et al. 2014; Long et al. 2013; Martins Sde et al. 2012). In HUVEC model, Long *et al.* showed DENV induced apoptosis of HUVECs through an interferon-inducible gene (Long et al. 2013), while Arevalo *et al.* demonstrated that direct DENV infection did not increase HUVEC apoptosis (Arevalo et al. 2009). Although previous studies have reported DENV has directly caused apoptosis of endothelial cells, which may lead to disruption of endothelial barrier leading to transient vascular leakage (Avirutnan et al. 1998). The relative importance of apoptosis in the pathogenesis of DENV infection remains undefined and a suitable *in vitro* endothelial cell culture model is required for further analysis.

Thus, future studies on DENV induced vascular leakage require an appropriate cell line derived from a human microvascular endothelium to produce a more physiologically relevant model. This chapter describes two microvascular endothelial cell lines, Liver sinusoidal endothelial cells (LSECs) and human cerebral microvascular endothelial cells (hCMEC/D3s) as potential models to study dengue infection.
The LSEC line, derived from human liver microvasculature, is reported to retain its endothelial properties of primary liver sinusoidal endothelial cells through a high number of \textit{in vitro} passages (Peyrefitte et al. 2006). During severe DHF condition, infection of liver may cause mild to moderate liver dysfunction or even acute liver failure leading to hepatic encephalopathy (Lima Mda et al. 2011; Nguyen et al. 1997). As liver is a natural target of DENV these cells offer an excellent \textit{in vitro} model with close similarity to the tissue that seems to be a main target of DENV.

The hCMEC/D3 cell line derived from human brain microvasculature has been extensively used in human blood-brain barrier model. DENV-associated encephalopathies, including encephalitis, cerebral edema, acute disseminated encephalomyelitis and even Guillian Barré syndrome have been shown to be highly prevalent in fatal dengue cases however the breakdown of blood-brain barrier has not been widely studied (Miagostovich et al. 1997; Sam et al. 2013; Solomon et al. 2000; Verma et al. 2014). As brain is a natural target of DENV (Miagostovich et al. 1997; Ramos et al. 1998) we can rely on the use of cultured endothelial cells from brain endothelium to elucidate molecular mechanisms of DENV infection.

In this study cellular response of microvascular endothelial cells from liver and brain to a non-mouse adapted D2Y98P clinical isolate of DENV were examined. Cellular responses of LSECs and hCMEC/D3 against DENV will help to elucidate its suitability as a physiologically relevant \textit{in vitro} experimental model that compare to clinical disease of DENV infection. This chapter describes the importance of ADE infection of LSECs and hCMEC/D3s by D2Y98P-antibody immune complex. Furthermore, for the first time
D2Y98P strain was analysed for its ability to induced apoptosis in LSECs and hCMEC/D3 cells.

3.2 Results

3.2.1 Endothelial cell morphology and expression of endothelial cell markers

The morphology and expression of endothelial cell markers of LSEC and hCMEC/D3 cells was determined by microscopy and flow cytometry. To examine LSECs and hCMEC/D3 cells morphology cell were seeded in 24 well plates and confluent monolayers were examined under light microscope, 10X objective. Both LSECs and hCMEC/D3s exhibited a “cobblestone-like” monolayer typical of the morphology of endothelial cells (Ades et al. 1992) (Figure 3.1.A).

Both LSEC and hCMEC/D3 endothelial cell lines were analysed for the presence of endothelial cell-specific marker CD31 expression by flow cytometry. CD31 also known as PECAM-1 (platelet endothelial cell adhesion molecule 1) has been used as an endothelial marker and also shown to be continuously expressed in liver sinusoidal endothelial cells from patient liver (Huang et al. 2014; Pusztaszeri et al. 2006). Endothelial cells were seeded in 24 well plates. Confluent monolayers were stained with anti-CD31-FITC conjugated antibody or with a relevant isotype control mouse IgG1-FITC antibody and analyse by flow cytometry. Both cell lines: LSECs and hCMEC/D3, stained positive for CD31 surface marker expression confirming its endothelial cell phenotype (Figure 3.1.B).
3.2.2 Infectivity of human microvascular endothelial cells by DENV

DENV infectivity of LSECs and hCMEC/D3s, as well as its effects on endothelial cell responses and functions remain to be determined. Firstly in order to determine DENV infection of human LSECs and hCMEC/D3s, cells were seeded in 24 well plates and incubated for 24 hrs. On the following day cell monolayers were infected with DENV at MOI 1. At each time point cell monolayers and supernatants were collected and stained with 3H5 primary antibody followed by Alexa Fluor 488 secondary antibody and analysed for DENV E protein positive endothelial cells.

In LSECs, DENV antigens have detected in approximately 2% of the total cell population at 0 hpi. This suggest a possibility of attachment by being positive for DENV antigens as each DENV-infected sample was compared with mock control sample (Figure 3.2). By 24 hpi the DENV antigen was detected in 12% of LSEC population and by 48 hpi the infectivity level was decreased to 11%. The highest level of infectivity was reached at 24 hpi and infectivity level was maintained around 10%-12% until 72 hpi.

Comparatively in all three independent experiments a higher percentage of hCMEC/D3 cells (9%) were detected positive for DENV E protein at 0 hpi, compared to mock control samples (figure 3.2). By 24 hpi the infectivity level was decreased to 6%. Despite increase of DENV antigen positive cells at 0 hpi, the DENV positive cells at 48 and 72 hpi was greatly reduced up to 0.5% overtime.

Further appearance of DENV envelope protein on endothelial cells was detected by immunofluorescence assay (IFA) (Figure 3.3). Cells seeded on coverslips were incubated overnight and infected with DENV at MOI 1 on the following day. After 24 hours cell monolayers were stained for DENV E protein with 3H5 primary and Alexa Fluor 488
secondary antibodies. Cell nuclei were stained with DAPI for visualisation. Compared to mock control some of the DENV-infected LSECs were positively stained for DENV E protein. In hCMEC/D3 cells fewer cells were positively stained for virus compared to LSECs, consistent with the observation seen by flow cytometry analysis suggesting low level of infectivity.

Together these data implicates that DENV can attach to both LSECs and hCMEC/D3 cells as DENV E protein was detected at 0 hpi in DENV-infected cells compared to mock control cells by flow cytometry analysis. In LSECs an increase in the DENV-infected cell population suggests that virus may be replicating in these cells. Conversely a decrease in DENV positive hCMEC/D3 cells may be due to the absence of DENV replication in hCMEC/D3s, even though DENV antigens were detected in a higher percentage of the cell population at 0 hpi compared to LSECs. However, further analysis is required to confirm the ability of DENV to replicate in these cell lines.

3.2.3 Single-step growth kinetics of DENV in human endothelial cells

To determine the capability of LSEC and hCMEC/D3 cells to produce infectious progeny virions, the DENV replication in a one-step multiplication cycle was studied. Endothelial cells were seeded in 24 well plates and infected with DENV at a MOI of 1, 5 and 10. At 12 hour post infection intervals total virus titre was determined by plaque assay (Figure 3.4).

In LSECs, after initial absorption similar levels of DENV was detected in MOI 5 and 10 at 0 hpi whereas in MOI 1, higher level of DENV was detected compared to MOI 5 and
10. With all three MOI, virus titre decreased at 12 hpi. Thereafter the virus titre showed a rapid increase between 12 to 36 hpi, reaching a plateau between 36 and 60 hpi. Peak virus titres were obtained at 60 hpi for both MOI 1 and 10. Thus the eclipse phase was approximately up to 12 hours and a single replication cycle appeared to be 36-48 hours. Together, this data shows that DENV D2Y98P strain productively replicate in human LSECs.

In one-step growth curve for D2Y98P DENV strain in hCMEC/D3 cells, after 2 hours of virus absorption, cells infected with MOI 1 and 5 were detected with similar virus titres and a low titre for MOI 10. Likewise in LSECs at 12 hpi viral titres were reduced and a slight increase was detected between 12 to 24 hpi. After 24 hpi viral titres recovered in all MOI of infection was started to decrease. An eclipse phase up to 12 hpi was detected and viral titre has increased by 24 hpi showing that there is low level of virus replication in hCMEC/D3 cells. Although viral titres obtained at 24 hpi are lower than viral titre obtained at 0 hpi. Decrease of viral titre after 24 hpi in all MOIs showed that DENV replication in hCMEC/D3s is limited.

3.2.4 Antibody dependent enhancement of DENV infection in LSECs and hCMEC/D3s

Antibody dependent enhancement (ADE) has been put forward as a method of DENV infection of certain cells, mainly during secondary infections (Arevalo et al. 2009), which has been linked to severe disease manifestations. The presence of FcγRs on cells and sub-neutralising antibodies of DENV are necessary for ADE infection. The flavivirus group-reactive 4G2 monoclonal antibody has been widely used to induce ADE of DENV
infection *in vitro* (Boonnak et al. 2008; Puerta-Guardo et al. 2010). Expression of Fcγ receptors on LSECs and hCMEC/D3s has not been reported previously. Further we examined DENV uptake in LSECs and hCMEC/D3s could be enhanced by the presence of a 4G2 mAb-virus complex.

In ADE infection DENV require the formation of DENV-4G2 antibody complex which then attaches, via the Fc portion of the antibody, to the endothelial cell FcγR. Hence presence of subneutralising concentrations of monoclonal antibody 4G2 should enhance DENV attachment to endothelial cells if cells express FcγRs.

In order to determine whether LSECs and hCMEC/D3s are susceptible to ADE of DENV infection, cells were seeded in 24 well plates. 24 hours later DENV was co-incubated with serially diluted mAb 4G2 for 30 minutes at 37 °C allowing the formation of DENV-4G2 immune complex. Following incubation endothelial monolayers were infected with the antibody DENV mixture at MOI 1 and incubated for 2 hours. After virus attachment monolayers were thoroughly washed three times to remove unbound virus and cell monolayers and supernatant were collected to determine virus attachment at 0 hpi and replication at 24, 48 and 72 hpi by plaque assay.

Infection of endothelial cells with 4G2-DENV immune complex was compared with DENV only control. Figure 3.5 depict attachment of 4G2-DENV complex to endothelial cells at 0 hpi. The mAb 4G2 diluted in $10^{-1}$ has neutralised most of the DENV decreasing the free virus levels to attach with endothelial cells and it resulted in a low virus titre in both cell types. Comparatively 4G2 mAb dilutions $10^{-2}$ and $10^{-3}$ have sub-neutralised DENV and higher dilutions $10^{-4}$, $10^{-5}$ and $10^{-6}$ have not shown any difference to virus only control group. Even though neutralisation of the DENV infection at higher antibody
concentrations and a gradual increase of infection at lower concentrations were seen, an increase of viral titre as per with ADE phenomenon was not seen. Further plaque assay was performed at 24, 48 and 72 hpi to titrate virus yield (Figure 3.6). In both cell lines LSEC and hCMEC/D3, at any of the given time points no significant increase of virus yield was obtained compared to the control that only contain DENV.

In FcγR bearing cells nearly all DENV-specific antibodies will enhance infection at some concentration of the antibody regardless of its neutralising ability (de Alwis et al. 2014). Taken together from the data we obtained here the absence of an enhanced viral titre in the presence of antibody-virus complex suggest that ADE mediated infection is not involved in infecting LSECs or hCMEC/D3s.

ADE is mediated by FcγRI and FcγRII receptors expressed on cells (Mady et al. 1991). Microvascular endothelial cells from different vascular beds have shown specific functional changes including heterogeneity in the expression of FcγRs. Expression of Fcγ receptors on LSECs and hCMEC/D3s has not been reported previously. In order to determine whether the absence of an ADE in LSECs and hCMEC/D3s following infection with antibody-virus complex, cells were evaluated for the presence of FcγRs by flow cytometry analysis.

As seen in Figure 3.7 both cell were found to not express FCγRI or FCγRII. THP-1 cells were used as the positive control since THP-1 cells express FcγR of the FcγRI and FcγRII classes (Debets et al. 1990). In addition, the two endothelial cell lines were stimulated by infecting with DENV and evaluated for appearance of FcγRs. The both cell lines do not express FcRI or FcRII receptors in neither normal nor stimulated conditions (data not
shown). Together this data shows that neither LSECs nor hCMEC/D3 support ADE infection as they lack FCγRI or FCγRII receptors.

3.2.5 DENV induced apoptosis on LSECs and hCMEC/D3s

Several previous studies have shown that DENV can induce apoptosis in cells (Long et al. 2013; Martins Sde et al. 2012). To determine if DENV can induce apoptosis in LSECs and hCMEC/D3 cells, DNA fragmentation and phosphatidylserine (PS) exposure were examined by TUNEL (Terminal dUTP Nick End-Labeling) assay and flow cytometry respectively.

In order to determine apoptosis by flow cytometry analysis, phycoerythrin (PE) labelled Annexin V that preferentially bind with PS was used. Under normal physiological conditions PS is located in the inner leaflet of plasma membrane. Upon initiation of apoptosis PS is translocated to outer leaflet of the plasma membrane and exposing it to the external environment to identify them as targets for engulfment by phagocytic (Leventis and Grinstein 2010). Once PS is on outer surface of the membrane fluorescently labelled Annexin V can be used for detection.

Use of the vital dye 7-AAD in conjunction with PE-Annexin V allowed to distinguish early apoptotic cells. In early stages of apoptosis, cells maintain an intact plasma membrane excluding 7-AAD viability dye to intercalate in DNA. Thus distinguishing early apoptotic cells will only stain with Annexin V but not with 7-AAD. Comparatively during end stage of apoptosis, cells lose plasma membrane integrity and allowed Annexin V to bind with PS in inner leaflet and will stain with both Annexin-V and 7-AAD.
In figure 3.8 the two stages of apoptosis are shown along with its total apoptosis population, which is early and end stage taken together. In DENV-infected early stage apoptotic LSEC population which was only positive for Annexin-V was significantly enhanced reaching 20% at 48 hpi. Consistently, DENV-infected late stage apoptotic LSEC population was ~35% at 48 hpi and as a total 55% of DENV-infected cell population is going through apoptosis and death. The percentage of mock control cell population undergoing apoptosis, both early and late stages were recorded nearly 5%. The hCMEC/D3 cells reported no significant change of apoptosis percentage compared to mock control samples.

To confirm DENV induced apoptosis, LSECs and hCMEC/D3s were further analysed using TUNEL assay. A hallmark of late stage apoptosis is genomic DNA fragmentation can be detected by TUNEL assay. In the TUNEL method DNA strand breaks in cells is identified using terminal deoxynucleotidyl transferase (TdT). The TdT mediate addition of labelled dUTPs to the cleaved ends of DNA and these labelled sites was detected by horse radish peroxidase conjugated to streptavidin and visualise by DAB. As in figure 3.9 DENV-infected LSECs at 48hpi stained dark brown compared to the mock control sample. The unlabelled negative control did not show any staining. All cell monolayers were counterstained with methyl green to visualise. As seen in figure 3.10 DENV-infected or mock control hCMEC/D3s at 48 hpi, did not show any positive staining compared to TACS nuclease treated positive control.

Together this data show DENV induced apoptosis in LSECs, which may be a mechanism involved in vascular endothelium damage through a direct effect of DENV or an indirect result of the DENV infection.
3.3 Discussion

DENV can infect human endothelial cells both *in vitro* and *in vivo* including primary cells and cell lines. Viral antigens have also been shown in vascular endothelium in the liver and brain in DENV patients (Jessie et al. 2004). However, the contribution of endothelial cells during DENV infection is not clearly understood. As infection of human microvascular endothelial cells from dengue targeted organs has been studied scarcely, it is important to gain further insight into the role of endothelial cells to DENV infection and their potential to contribute to severe DENV infection.

Dalrymple and Mackow (Dalrymple and Mackow 2012) showed ≥80% of primary human umbilical vein endothelial cells (HUVEC) were infectable with DENV and a rapid production between 12-24 hpi. However as HUVECs are macrovascular endothelial cells hence the results obtained may vary compared to microvascular endothelial cells as different vascular beds have specific functional changes (Chi et al. 2003; Jackson and Nguyen 1997; Peyrefitte et al. 2006; Talavera et al. 2004; Willam et al. 1999). A study conducted on global gene expression pattern of 53 different cultured endothelial cells revealed a distinct difference in gene expression profile of large vessel derived endothelial cells to microvascular endothelial cells. These differences may be related to structural and mechanical characteristics and its physiological roles (Chi et al. 2003). Over the years studies on *in vitro* endothelial cell models have provided increasing evidence that there are differences in antigenic structure and cellular function between cultured endothelial cells derived from microvascular background to macrovascular background (Chi et al. 2003; Jackson and Nguyen 1997; Peyrefitte et al. 2006; Talavera et al. 2004; Willam et al. 1999).
Infection of microvascular LSECs with DENV strain DEN2/H/IMTSSA-MART/98-703 has been previously shown by Christophe N. Peyrefitte et al. (Peyrefitte et al. 2006) and 7-11% of LSECs were infected around day 2 to day 7. This is in agreement with the levels obtained in this study. DENV infection in hCMEC/D3 cells has not been reported earlier. In this study higher levels of DENV antigens detected in hCMEC/D3 cells at 0 hpi were decreased throughout 24, 48 and 78 hpi, and is explanatory as DENV replication in hCMEC/D3 cells is decreasing over time. Although virus titres has increased by 24 hpi and is lower than tires obtained at 0 hpi, DENV infection of hCMEC/D3s is non-productive. In contrast, DENV productively infect human brain microvascular endothelial cells (HBMECs) and showed 19% of cells were infected at 48 hpi (da Conceição et al. 2013). This may possibly be due to the use of different viral strains and further experiments needed to confirm this. These results also implicate that endothelial cells may contribute to viremia during DENV infection. However the contribution to viremia by these two different cell types varied according to the source of endothelial origin.

DENV infect cells by entering into the cells through receptor mediated endocytosis or via ADE mediated pathway. The presence of FcγR is necessary for ADE infection of cells. This study shows that LSECs nor hCMEC/D3 do not support ADE infection as they lack FCγRI or FCγRII receptors. Thus infection of these cells by DENV was only via direct entry as per the absence of fc receptors.

Accumulated evidence has shown that DENV induced cell apoptosis, may be related to endothelium damage leading to transient vascular leakage (Lin et al. 2002). Anti-NS1 DENV antibody induced HMEC-1 cell apoptosis via a caspase dependent pathway. In
which HMEC-1 cells produced Nitric Oxide (NO) after exposure to anti-NS1 antibody. NO can upregulate p53 and Bax and down-regulate Bcl-2 and Bcl-xL which leads to caspase 3 activation (Lin et al. 2002). In another study sera from acute dengue patients induced apoptosis in HMEC-1 cells and a possible role of TNF-α was suggested (Cardier et al. 2005). Long et al. showed that X chromosome-linked inhibitor of apoptosis protein-associated factor 1 (XAF1) gene expression was upregulated in infected HUVECs. XAF1 negatively regulates the function of the X-linked inhibitor of apoptosis protein (XIAP), a member of the IAP family that exerts anti-apoptotic effects (Long et al. 2013). Although Arevalo et al. demonstrated that DENV do not induced apoptosis in HUVECs (Arevalo et al. 2009). Apart from in vitro investigations, endothelial cell apoptosis was also showed important in haemorrhage development in a haemorrhage mouse model, in which caspase mediated endothelial cell apoptosis was triggered by NF-κB activation (Lin et al. 2014). Herein two apoptosis assays were performed on DNA fragmentation and PS exposure to confirm apoptosis of LSECs. PS located in the inner leaflet of the plasma membrane translocate to the outer membrane as it undergoes apoptosis (Elmore 2007). The high affinity of PS to annexin V protein was used for the detection of apoptotic cells. DENV induced apoptosis in LSECs at 48hpi, caused 55% of the cell population to undergo apoptosis. DENV induced apoptosis of LSECs may be related in disruption of the endothelial barrier and lead to vascular leakage.

Taken together, the data presented in this chapter demonstrated that LSECs and hCMEC/D3s are permissive to DENV in varying degrees. Effects of DENV on endothelial cell responses and functions are to be determined and thus it will be useful to use both models concurrently as a cell model to investigate the role of the endothelium in DENV pathogenesis. As DENV productively infects LSECs, but not in hCMEC/D3, it is
important to study the effects of DENV on these two endothelial cell lines comparatively in order to assess potential therapeutic targets for DENV infection.
Figure 3.1 Endothelial cell morphology and expression of endothelial cell markers

(A) To examine LSECs and hCMEC/D3 cells morphology cell were seeded in 24 well plates and incubated at 37°C. After cells reached confluence, monolayers were examined under light microscope, 10X objective. (B) To examine CD31 expression, endothelial cells were stained with anti-CD31-FITC or FITC mouse IgG1 isotype control antibody and analysed by flow cytometry. Representative dot plots form three independent experiments are shown with isotype control dot plots on the left.
Figure 3.2 DENV infection of microvascular endothelial cell lines

LSECs and hCMEC/D3s, cells were seeded in 24 well plates and incubated for 24 hrs. On the following day cell monolayers were infected with DENV at MOI 1 and incubated 2 hours for virus attachment to endothelial cells. After incubation monolayers were washed with PBS to remove unbound virus and incubated with maintenance medium. At each time point cell monolayers were collected to determine appearance of viral antigens by flow cytometry analysis. Collected cell supernatants and monolayers were washed with wash buffer prior to fixation/permeabilisation of cells. Permeabilised cells were then stained with 3H5 primary antibody, specific for DENV envelope antigen followed by Alexa Fluor 488 secondary antibody and analysed for DENV E protein positive endothelial cells. Representative flow cytometry histograms of mock (grey) and DENV-infected (black) of LSECs and hCMEC/D3s are shown. All values represent the means +/- SD of experiments performed in triplicate.
Figure 3.3 DENV infection of LSECs and hCMEC/D3s determined by immunofluorescence staining

Endothelial cells seeded on coverslips were incubated overnight and infected with DENV at a MOI of 1 or treated with medium alone (mock) on the following day. At 24 hpi LSECs and hCMEC/D3s were fixed with formaldehyde (Sigma, Aldrich) and stained with primary antibody 3H5 and Alexa 488 secondary antibody to visualise DENV envelope protein (green) monolayers and nuclei were stained with DAPI (blue). Expression of DENV antigen was detected by fluorescence microscope (200X objective).
Figure 3.4 Replication of DENV in Microvascular endothelial cell lines LSECs & hCMEC/D3s

LSECs and hCMEC/D3s were seeded in 24 well plates and incubated at 37°C overnight. After 24 hours the monolayers of (A) LSECs and (B) hCMEC/D3s were inoculated with DENV at a MOI of 1, 5 or 10. Following absorption unbound virus was removed by washing thoroughly and incubated with media. At 12 hour intervals up to 72 hours cells and supernatants were collected and total virus was determined by plaque assay. Each data point is expressed as the mean ± SEM for 3 data points and is representative of 3 independent experiments.
Figure 3.5 Attachment of DENV-4G2 immune complex to LSECs and hCMEC/D3s

The endothelial cells (A) LSECs and (B) hCMEC/D3s were seeded in 24 well plates and incubated overnight. 24 hours later DENV was co-incubated with serially diluted mAb 4G2 for 30 minutes at 37 °C allowing the formation of DENV-4G2 immune complex. Following incubation endothelial monolayers were infected with the antibody DENV mixture at MOI 1 or DENV alone. After 2 hours of incubation monolayers were thoroughly washed three times to remove unbound virus and cell monolayers and supernatant were collected to determine virus attachment at 0 hpi.
Figure 3.6 ADE infection of LSECs and hCMEC/D3s

The endothelial cells (A) LSECs and (B) hCMEC/D3s were seeded in 24 well plates and incubated overnight. 24 hours later DENV was co-incubated with serially diluted mAb 4G2 for 30 minutes at 37 °C allowing the formation of DENV-4G2 immune complex. Following incubation endothelial monolayers were infected with the antibody DENV mixture at MOI 1 or DENV alone. After 2 hours of incubation monolayers were thoroughly washed three times to remove unbound virus and cell monolayers and supernatant were collected to determine virus titre at 0, 24, 48 and 72 hpi by plaque assay.
Figure 3.7 Expression of human FcγRII/CD32 & FcγRI/CD64 receptors on microvascular endothelial cell lines LSECs and hCMEC/D3s

Endothelial cell monolayers reaching 80% confluence were extracted to determined presence of Fcγ receptors on LSECs and hCMEC/D3s. Cells were stained with APC conjugated anti-CD64 for FcγRI, PE conjugated anti-CD32 for FcγRII or with relevant isotype controls, mouse IgG2A PE or mouse IgG1 APC and analysed by flow cytometry. THP-1 cells were used as positive control. Representative histograms from the experiment are shown.
FcγRI/CD64 receptor

Isotype control

LSEC cells

hCMEC/D3 cells

THP-1 cells

FcγRII/CD32 receptor
Figure 3.8 DENV induced apoptosis in LSECs but not in hCMEC/D3s

Endothelial cells were infected with DENV at a MOI of 1 or culture media alone (mock). At respective time points cells were harvested and stained with PE conjugated Annexin-V that binds to phosphatidylserine of the plasma membrane and with 7-AAD viability stain to distinguish between necrotic and apoptotic cells. Percentage of early and late stage apoptotic mock (solid line) and DENV (dashed line) infected cell population of LSECs and hCMEC/D3s was analysed by flow cytometry. Early and late stage apoptosis together has been considered as total apoptosis. Each data point represents the mean ± SEM from three independent experiments. ** P < 0.01 and *** P < 0.001, two-way ANOVA with Bonferroni posttest.
Figure 3.9 DENV induced apoptosis in LSECs

LSECs grown in slides were infected with DENV at MOI 1 and after 48 hpi apoptosis were assessed by TUNEL assay. In (A) unlabelled negative control (B) TACS nuclease treated positive control (C) mock control and (D) DENV-infected samples dark brown cell staining indicate TUNEL-positive apoptotic cells.
Figure 3.10 DENV induced apoptosis in hCMEC/D3s

hCMEC/D3s grown in slides were infected with DENV at MOI 1 and after 48 hpi apoptosis were assessed by TUNEL assay. In (A) unlabelled negative control (B) TACS nuclease treated positive control (C) mock control and (D) DENV-infected samples are shown. Dark brown cell staining indicate TUNEL-positive apoptotic cells.
Chapter IV

The characterisation of soluble mediators in human endothelial cells following dengue virus infection
4.1 Introduction

Infection with DENV can result in either mild dengue fever or severe clinical manifestations such as DHF/DSS. Characteristic features of DHF are increased capillary permeability without morphological damage to the capillary endothelium, thrombocytopenia, altered leucocyte functions, altered homeostasis and liver damage (Basu and Chaturvedi 2008). During the course of DHF, spontaneous bleeding accompanied with extensive plasma leakage in tissue spaces and various serous cavities including pleura, pericardium, and peritoneal cavities result in DSS. It appears around defervescence and viral clearance and lasts for about 48 hours (Basu and Chaturvedi 2008; Malavige et al. 2012). Although endothelial dysfunction and increased vascular permeability is known to contribute to the DHF/DSS clinical outcome, little is conclusively understood about the factors which result in severe clinical disease and the observed pathophysiology of DENV infection.

A complex interplay between host genetic, immunological and viral factors is considered to be associated with DHF/DSS (Herrero et al. 2013). Previous studies investigating host immunological factors have postulated that DENV induces a massive production of immune mediators by different cell types, causing endothelial dysfunction and altered vascular permeability (Malavige et al. 2012). Clinical data supports a role of host immune mediators such as MCP-1, VEGF, RANTES, IL-7, IL-12, PDGF, IL-10, CXCL10 and G-CSF, in severe disease pathogenesis (Malavige et al. 2012; Rathakrishnan et al. 2012). In dengue patients including infants, vascular leakage has appeared in parallel to high levels of proinflammatory and anti-inflammatory cytokines in the sera, suggesting an
association with vascular leakage in severe DENV infection (Malavige et al. 2012; Nguyen et al. 2004).

In relation to blood vessel physiology, endothelial cells are capable of producing many secretory products including interleukin (IL)-1, IL-6, CXCL8, IL-1 receptor antagonist (IL-1ra), monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1 alpha (MIP-1α), CCL5 also known as regulated on activation, normal T cell expressed and secreted (RANTES), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte-colony stimulating factor (GSF) (Appanna et al. 2012; Bozza et al. 2008; Dewberry et al. 2000; Peyrefitte et al. 2006; Rathakrishnan et al. 2012; Rollins et al. 1990; Sumpio et al. 2002; Talavera et al. 2004). Endothelial cells from different vascular beds i.e. Human umbilical vein endothelial cells (HUVEC) (Huang et al. 2000), Human dermal microvascular endothelial cells (HMEC-1) (Talavera et al. 2004) have been shown to secrete immune factors linked to enhanced vascular permeability. Production of CXCL8 by HMEC-1 monolayers infected with DENV was associated with modified cytoskeleton and tight junction arrangement thereby altering transendothelial permeability (Talavera et al. 2004). Similarly in a HUVEC culture model DENV infection induced IL-6 and CXCL8 production, which may contribute to the pathogenesis of DHF/DSS (Huang et al. 2000).

Valuable insights into DENV-host interactions provided by clinical and experimental observations in-vitro was further supported by in-vivo models. In an AG129 mice model in which the infected animals exhibit increased vascular permeability with fatal non-
neurological infection, TNF-α and other immune effector molecules were associated with the breakdown of the vascular integrity in different tissues (Shresta et al. 2006).

In LSECs, DENV-induced production of IL-6, CXCL8 and CCL5 has been shown previously (Peyrefitte et al. 2006). Data is limited on the secretion of cytokines, chemokines and growth factors by LSECs in response to DENV infection. There are no studies reported on production of soluble mediators by DENV-induced hCMEC/D3 cells. Despite extensive research, the source of this factors and synergy between immunomediators and vascular leakage is not fully understood. Having known that high levels of immunomediators underlie the progression of severe dengue infection and demonstrated in Chapter three that LSECs and hCMEC/D3s can be infected by DENV it is opted here to gain further insight on immunomediators secreted by DENV-infected LSECs and hCMEC/D3s.

4.2 Results

4.2.1 Cytokine profiles in DENV-infected endothelial cells

Cytokine profiles were analysed in LSECs and hCMEC/D3s after DENV infection. LSECs and hCMEC/D3s seeded in 24 well plates were infected with DENV at MOI 1. Supernatants from DENV-infected or mock control endothelial cells were collected at 24 and 48 hpi and infection was confirmed by plaque assay, followed by Bioplex immunoassay analysis.
i. **IL-6 expression during DENV infection**

In LSECs, IL-6 levels was monitored over time (figure 4.1A). At 24 hpi, IL-6 level in supernatants from DENV-infected cultures were 4-fold higher to over mock control (P < 0.0001). IL-6 levels in supernatants has further increased by 48 hpi and was significantly higher (P < 0.0001) in infected cell supernatants compared to mock control. Of note, IL-6 level increased over time though fold increase compared to mock control decreased from 4-fold to 2.5-fold from 24 hpi to 48 hpi.

In hCMEC/D3 cells a similar profile of IL-6 cytokine (figure 4.1B) was observed to that of LSECs IL-6 profile (figure 4.1A). Following DENV infection IL-6 levels were markedly elevated over the mock controls and the fold change exceeded 3.5-fold and 2.3-fold at 24 hpi and 48 hpi respectively. These IL-6 levels were significantly higher at both 24 hpi (P <0.0001) and 48 hpi (P = 0.0005) in DENV-infected cell supernatants compared to mock control cell supernatants. As seen with LSECs, fold increase of IL-6 levels decreased in hCMEC/D3s from 24 hpi to 48 hpi, over time.

ii. **IL-1β expression during DENV infection**

In LSECs, elevated levels of IL-1β was detected in DENV-infected cell supernatants compared to mock control at both time points (figure 4.2A). At 24 hpi, a 2.6-fold increase of IL-1β in infected cell supernatants over mock controls was significantly different (P < 0.0001). Significant increase of IL-1β at 48 hpi (P = 0.0002) was also 1.4-fold higher over the mock controls.

IL-1β protein expression in hCMEC/D3s cell supernatants (figure 4.2B) were similar to that of LSECs IL-1β profile (figure 4.2A). By 24 hpi, IL-1β production in DENV-infected
LSECs has increased in 3.3-fold over mock control with a significant difference (P = 0.0002). The IL-1β has further increased by 48 hpi which was significantly different (P < 0.0001) to mock controls over a 2.5-fold increase.

iii. **TNF-α expression during DENV infection**

In LSECs, an increase in TNF-α levels was detected in DENV-infected cell supernatants relative to mock control (figure 4.3A). At 24 hpi the increase was significantly higher (P = 0.0011, 1.5-fold increase). By 48 hpi TNF-α levels has increased in both DENV-infected and mock control although the difference between the groups were not significant.

Similarly TNF-α levels in hCMEC/D3s supernatants were higher in DENV-infected group compared to mock control. At 24 hpi TNF-α production by DENV-infected cells has increased in 3-fold over mock control with a significant difference (P < 0.0001). At 48 hpi TNF-α production has further increased in both groups wherein a 1.6-fold increase in TNF-α levels in supernatants from DENV-infected cells over mock control was significantly different (P = 0.0029).

4.2.2 **Chemokine expression in DENV-infected endothelial cells**

To analyse chemokine profiles in LSECs and hCMEC/D3s, cells seeded in 24 well plates were infected with DENV at MOI 1 and supernatants collected after 24 and 48 hpi and infection was confirmed by plaque assay, followed by Bioplex immunoassay analysis.
i. **CXCL8 expression during DENV infection**

DENV infection of LSECs resulted in the production of high levels of CXCL8 (figure 4.4A). At 24 hpi CXCL8 levels in supernatants from DENV-infected cells were 6.4-fold higher (P < 0.0001) compared to mock control. By 48 hpi the fold change has decreased to 2.6-fold over mock controls, although significantly higher levels (P < 0.0001) were detected in DENV-infected cultures.

Higher levels of CXCL8 in DENV-infected hCMEC/D3 supernatants were detected compared to mock controls at 24 and 48 hpi (Figure 4.4B). CXCL8 levels at 24 hpi were significantly different between the two groups (P < 0.0001, 22-fold increase). By 48 hpi CXCL8 protein production in DENV-infected hCMEC/D3s was 7-fold over mock control with a significant difference (P < 0.0001).

ii. **CCL4 expression during DENV infection**

DENV infection of LSECs induced CCL4 production (figure 4.5A). At 24 hpi CCL4 levels in supernatants from DENV-infected cells were 3.1-fold higher (P < 0.0001) over mock control. By 48 hpi the fold change has decreased to 1.8-fold compared to mock control, although significantly higher levels (P < 0.0001) was detected in DENV-infected cultures.

CCL4 protein expression in hCMEC/D3s cell supernatants (figure 4.5B) was significantly different (P = 0.0020) between DENV-infected and mock control groups at 24 hpi. The CCL4 has further increased by 48 hpi which was significantly different (P = 0.0003) to mock controls over a 2.7-fold increase.
iii. **CCL2 expression during DENV infection**

Significantly higher levels (P = 0.0014) of CCL2 was detected in supernatants of infected LSECs at 24 hpi (figure 4.6A) compared mock control. By 48 hpi CCL2 in both mock control and DENV-infected LSEC supernatants were increased and showed similar levels.

In hCMEC/D3s, CCL2 protein production in DENV-infected cultures were significantly higher (P = 0.0002) compared to mock control at 24 hpi (figure 4.6B). The CCL2 levels in supernatants from DENV-infected and mock control cultures have increased by 48 hpi without a significant difference between the groups.

iv. **CXCL10 expression during DENV infection**

In LSECs, CXCL10 levels was significantly higher (P < 0.0001, fold change = 3.9) in DENV-infected cell supernatants than the mock control at 24 hpi (figure 4.7A). By 48 hpi, the CXCL10 has further increased in both mock control and DENV-infected cultures, which was significantly different (P = 0.0146) to mock controls over a 1.5-fold increase.

Bioplex assay performed for hCMEC/D3 cell supernatants did not contain beads for CXCL10 thus its expression was not analysed.

v. **CCL11 expression during DENV infection**

In LSECs, CCL11 levels was significantly higher (P = 0.0024, fold change = 1.7) in DENV-infected cell supernatants than the mock control at 24 hpi (figure 4.7B). By 48
hpi, CCL11 has further increased in both mock control and DENV-infected cultures, although there is no significant different between groups.

Bioplex assay for hCMEC/D3 cell supernatants did not contain beads for CCL11 thus its expression was not analysed.

**vi. CCL3 expression during DENV infection**

As seen in Figure 4.8A, elevated levels of CCL3 was detected in DENV-infected LSECs compared to mock control at both 24 and 48 hpi. At 24 hpi the significant increase (P < 0.0001) of CCL3 in infected cells were 2.5-fold higher than the mock control. The fold change was 1.8 by 48 hpi although a significantly higher (P < 0.0001) CCL3 production was detected in DENV-infected cell supernatants.

Bioplex assay for hCMEC/D3 cell supernatants did not contain beads for CCL3, therefore its expression was not analysed.

**vii. CCL5 expression during DENV infection**

In LSECs, CCL5 production by mock control and DENV-infected cells were similar at both 24 and 48 hpi (Figure 4.8B). By 48 hpi, CCL5 levels increased in supernatants from both mock control and DENV-infected compared to 24 hpi.

Bioplex assay for hCMEC/D3 cell supernatants did not contain beads for CXCL10 thus its expression was not analysed.
4.2.3 Expression of growth factors in DENV-infected endothelial cells

In order to investigate the possible relationship between DENV infection and levels of growth factors, LSECs and hCMEC/D3 cell supernatants were analysed. Endothelial cells were seeded in 24 well plates and infected with DENV at MOI 1. Cell-free culture supernatants were collected at 24 and 48 hpi and infection was validated by plaque assay. Levels of growth factors in cell supernatants were analysed by Bioplex immunoassay.

i. G-CSF expression during DENV infection

The levels of G-CSF production by DENV-infected LSECs were higher compared to mock controls (figure 4.9A). At 24 hpi G-CSF level in DENV-infected cultures was significantly increase (P < 0.0001, 2.3-fold) compared to mock cultures. However at 48 hpi the difference between the groups were not significantly different.

DENV-infected hCMEC/D3s produced higher G-CSF levels compared to mock controls (figure 4.9B). At 24 hpi G-CSF levels from DENV-infected cells were 6.2-fold higher compared to mock control cultures (P < 0.0001). By 48 hpi G-CSF levels have increased in both groups and DENV-infected cell supernatants were significantly higher (P = 0.0007) over mock control over a 3.2-fold change.

ii. GM-CSF expression during DENV infection

GM-CSF expression by DENV-infected LSECs at 24 hpi was significantly higher (P = 0.0137) compared to mock controls (figure 4.10A). By 48 hpi, both mock and DENV-infected cell supernatants contained similar levels of GM-CSF.
GM-CSF expression by DENV-infected hCMEC/D3s was not significantly different at both time points (figure 4.10B). Although by 48 hpi GM-CSF in DENV-infected cell supernatants were 2.4-fold higher over mock control.

iii. bFGF expression during DENV infection

As shown in figure 4.11A, in LSECs higher levels of bFGF were detected in supernatants from DENV-infected compared to mock controls at 24 hpi (P = 0.0021, 1.4-fold increase). At by 48 hpi, bFGF levels has decreased in DENV-infected compared to mock control, with a significant decrease (P = 0.0115).

iv. PDGF-BB expression during DENV infection

In DENV-infected LSEC supernatants, PDGF-BB was significantly elevated at 24 hpi (P = 0.0002, 1.6-fold increase). By 48 hpi PDGF-BB levels has increased in both groups to similar levels (figure 4.11B).

v. VEGF expression during DENV infection

In LSECs, VEGF levels were similar between DENV-infected and mock controls at 24 hpi (figure 4.12). By 48 hpi VEGF production has increased in both groups with a significantly decrease (P = 0.0012) in DENV-infected cell supernatants compared to mock control.

Bioplex assay for hCMEC/D3 cell supernatants did not contain beads for VEGF thus its expression was not analysed.
4.3 Discussion

Mosquito borne dengue infection causes mild fever that may develop into potentially lethal severe DHF/DSS conditions. A hallmark feature of severe dengue is vascular leakage which is linked with over-production of soluble mediators in host in response to dengue infection (Appanna et al. 2012; Bozza et al. 2008; Rathakrishnan et al. 2012). As such, immunomediators secreted in response to dengue infection have been extensively studied in-vitro, in-vivo and clinically, however, little is understood about the pathophysiology of vascular endothelium damage. The focus of this chapter was to characterise soluble immune mediators in response to DENV in LSECs and hCMEC/D3s.

In this study, analysis of soluble mediator profiles were determined in DENV-infected and mock control culture supernatants at two time points using Bio-plex immunoassay. Both cell types were capable of producing many secretory products including cytokines, chemokines and growth factors. A potent inflammatory cytokine IL-1β has been previously shown to be secreted by ECs such as HUVECs, rat cerebral endothelial cells (RCECs) (Borish et al. 1992; de Vries et al. 1996; Sumpio et al. 2002) and we showed that both LSECs and hCMEC/D3 cells produce IL-1β in low concentrations. Interestingly Peyrefitte and others (Peyrefitte et al. 2006) has reported undetectable levels of IL-1β in both mock and infected LSEC supernatants. The differences observed may be due to the sensitivity of the two methods i.e. ELISA and Bioplex assay, used in quantification of the cytokine.

The over production of IL-6 and CXCL8 in response to DENV infection by endothelial cells such as HUVECs, LSECs and human brain microvascular endothelial cells (HBMECs) has been reported (Huang et al. 2000; Peyrefitte et al. 2006; da Conceição et
CXCL8 is responsible for leukocyte recruitment and for tight junction and cytoskeleton reorganisation of endothelial cells causing permeability changes in endothelial cells (Huang et al. 2000; Peyrefitte et al. 2006; Talavera et al. 2004). IL-6, a multifunctional cytokine which has a role in angiogenesis, cell growth and proliferation has been reported to increased permeability of endothelial cells such as HUVECs, RCECs (Desai et al. 2002; de Vries et al. 1996). Alteration of ultrastructural distribution of tight junctions and morphological changes in cell shape by IL-6 increased endothelial permeability in HUVECs (Desai et al. 2002). In this study we showed that DENV-infected LSECs and hCMEC/D3s produced high levels of IL-6 and CXCL8 compared to mock controls. Despite low level of infection of hCMEC/D3s, produced higher levels of IL-6 and CXCL8 than LSECs. Similar results has been reported in HIV infection. For example low level of HIV infection of astrocytes and human brain pericytes secreted higher levels of soluble mediators including IL-6, by inducing neighbouring uninfected cells through cell-cell communication (Nakagawa et al. 2012; Eugenin and Berman 2007). However, further analysis is required to explain higher IL-6 and CXCL8 production by hCMEC/D3s. Evidence from literature suggest that LSECs and hCMEC/D3s may possibly contribute to the cytokine induced endothelium damage by producing these potent inflammatory mediators to respond locally and thus further investigation will be needed to determine their role in permeability.

In dengue pathogenesis elevated levels of CCL3, CCL4 and CCL2 chemokines were correlated with clinical presentation of DHF (Basu and Chaturvedi 2008; Bozza et al. 2008; Lee et al. 2006b). The two forms of macrophage inflammatory protein (MIP) chemokine: CCL3 and CCL4 are chemotactic for eosinophils, T-lymphocytes, monocytes
and macrophages. Significantly higher levels of CCL3 (Sierra et al. 2010) and CCL4 (Bozza et al. 2008) in dengue patients may have a role in disease pathogenesis. Elevated levels of CCL3 and CCL4 may be involved in early recruitment of dengue specific cytotoxic T-cells to the site of infection. Excessive T-cell activation and cytokine production at the site of infection can cause plasma leakage. Another study showed a direct relationship of CCL3 and CCL4 with DENV replication (Spain-Santana et al. 2001). Our results also showed an early induction of CCL3 and CCL4 production which may involve in dengue disease pathogenesis.

CCL2 belonging to CC chemokine family recruits monocytes, memory T-cells and dendritic cells to inflammatory sites (Sierra et al. 2010). MCP-1 is a highly expressed chemokine during DHF/DSS in patients (Lee et al. 2006b; Rathakrishnan et al. 2012). In HUVEC monolayers CCL2, increase permeability by disrupting distribution of tight junction protein ZO-1 (Lee et al. 2006b). In mouse brain endothelial cells, CCL2 induced reorganisation of actin cytoskeleton and distribution of tight junction proteins ZO-1, ZO-2, occludin and claudin-5 which contributes to increased brain endothelial permeability (Stamatovic et al. 2003). We showed that both LSECs and hCMEC/D3 infected with DENV produced significantly elevated levels of CCL2 and possibly that it may affect endothelium permeability alterations.

CCL11 is characterised by its high chemotactic selectivity for eosinophils. A study on vascular leakage in the human coronary artery endothelial cells (HCAECs) showed CCL11 is associated with down regulation of tight junction proteins, increase of oxidative stress and activation of MAPK p38, Stat3, and NF-κB to increase endothelial permeability
We showed that DENV-infection of LSECs induced increase expression of CCL11 which may be affect LSEC permeability alterations.

CXC motif chemokine, CXCL10 is secreted by several cell types including endothelial cells. Dengue patients has elevated levels of CXCL10 and it may be responsible for vascular permeability, as being a potent inhibitor of angiogenesis (Ferreira et al. 2015; Rathakrishnan et al. 2012). Dengue patients with liver alterations such as increase aspartate aminotransferase (AST) and painful hepatomegaly showed higher circulating levels of CXCL10 (Ferreira et al. 2015). Ferreira et.al suggest an association of CXCL10 with plasma leakage and liver dysfunction in Brazilian children with DENV infection. A novel host defence role of CXCL10 was shown as it abolish dengue infection by competitive binding to heparan sulfate (Chen et al. 2006). The LSEC cell culture model used in this study agree with above observations as elevated levels of CXCL10 and DENV infection was observed.

In this chapter, the soluble mediator profiles secreted by LSECs and hCMEC/D3s following DENV infection revealed that this study data are with accordance to the clinical data reported from patient’s serum in literature (Kumar et al. 2012; Rathakrishnan et al. 2012). Differences in soluble factors detected in this study compared to previous in vitro endothelial cell models, may suggest the unequal susceptibility of endothelial cells depending on the vascular bed origins and emphasise the importance of selection of endothelial cell types from DENV naturally targeted endothelial origins. The factors which have been elevated during disease manifestations that may be associated with severe forms of the disease was seen to be elevated in LSECs and hCMEC/D3s models
we used. This explain the suitability of these models to investigate permeability alterations in endothelial cells in response to DENV infection.
Figure 4.1 Secretion of IL-6 by DENV-infected endothelial cells

Microvascular endothelial cell lines (A) LSECs and (B) hCMEC/D3s were seeded in 24 well plates and after 24 hours of incubation at 37 °C, monolayers were infected with DENV at MOI 1 or mock control with culture medium alone. At 24 and 48 hpi IL-6 level in cell-free supernatant from DENV-infected (n=6) or mock control (n=6) were analysed with Bio-plex immunoassay using Luminex technology (cut-off level ≥ 1.5 pg/ml). Data are presented as mean ± SEM. **P < 0.01 and *** P < 0.001, Student unpaired t-test.
(A) **IL-6**

![Graph showing IL-6 cytokine concentration over time with error bars indicating statistical significance.]

- **Cytokine Concentration (pg/ml)**
- **Hours post-infection (hpi)**
- **Mock control**
- **DENV-infected**

(B) **IL-6**

![Graph showing IL-6 cytokine concentration over time with error bars indicating statistical significance.]

- **Cytokine Concentration (pg/ml)**
- **Hours post-infection (hpi)**
- **Mock control**
- **DENV-infected**
Figure 4.2 Secretion of IL-1β by DENV-infected endothelial cells

(A) LSECs and (B) hCMEC/D3 cells were seeded in 24 well plates and after 24 hours of incubation monolayers were infected with DENV at MOI 1 or mock control with culture medium alone. After 2 hours incubation monolayers were washed with PBS and incubated with maintenance medium at 37 °C. At 24 and 48 hpi culture supernatant were collected to analyse IL-1β level in supernatants from DENV-infected (n=6) or mock control (n=6) by Bio-plex immunoassay using Luminex technology. Data are presented as mean ± SEM. **P < 0.01 and *** P < 0.001, Student unpaired t-test.
(A) IL-1β

Cytokine Concentration (pg/ml)

Hours post-infection (hpi)

Mock control
DENV-infected

*** ***

(B) IL-1β

Cytokine Concentration (pg/ml)

Hours post-infection (hpi)

Mock control
DENV-infected

*** ***
Figure 4.3 Secretion of TNF-α by DENV-infected endothelial cells

Endothelial cells (A) LSECs and (B) hCMEC/D3 cells were seeded in 24 well plates and after 24 hours of incubation monolayers were infected with DENV at MOI 1 or mock control with culture medium alone. After 2 hours incubation monolayers were washed with PBS and incubated with maintenance medium at 37 °C. At 24 and 48 hpi culture supernatant were collected to analyse TNF-α levels in supernatants from DENV-infected (n=6) or mock control (n=6) by Bio-plex immunoassay using Luminex technology (cutoff level ≥ 6.8 pg/ml). Data are presented as mean ± SEM. **P < 0.01 and *** P < 0.001, Student unpaired t-test.
Figure 4.4 Production of CXCL8 by DENV-infected endothelial cells

Endothelial cells (A) LSECs and (B) hCMEC/D3s were seeded in 24 well plates and incubated overnight. On the following day confluent monolayers were infected with DENV at MOI 1 or mock control with culture medium alone. At 24 and 48 hpi culture supernatant were collected to analyse CXCL8 levels in supernatants from DENV-infected (n=6) or mock control (n=6) by Bio-plex immunoassay using Luminex technology (cut-off level ≥ 1.6 pg/ml). Data are presented as mean ± SEM. **P < 0.01 and *** P < 0.001, Student unpaired t-test.
(A) CXCL8

Chemokine Concentration (pg/ml)

Hours post-infection (hpi)

Mock control

DENV-infected

(B) CXCL8

Chemokine Concentration (pg/ml)

Hours post-infection (hpi)
Figure 4.5 Production of CCL4 by DENV-infected endothelial cells

Endothelial cells (A) LSECs and (B) hCMEC/D3s were seeded in 24 well plates. After 24 hours cells were infected with DENV at MOI 1 or mock control with culture medium alone. Culture supernatant were collected to analyse CXCL8 levels in supernatants from DENV-infected (n=6) or mock control (n=6) by Bio-plex immunoassay using Luminex technology at 24 and 48 hpi (cut-off level ≥ 0.8 pg/ml). Data are presented as mean ± SEM. **P < 0.01 and *** P < 0.001, Student unpaired t-test.
Figure 4.6 Production of CCL2 by DENV-infected endothelial cells

Microvascular endothelial cell lines (A) LSECs and (B) hCMEC/D3s were seeded in 24 well plates and after 24 hours of incubation at 37 °C, monolayers were infected with DENV at MOI 1 or mock control with culture medium alone. At 24 and 48 hpi CCL2 level in cell-free supernatant from DENV-infected (n=6) or mock control (n=6) were analysed with Bio-plex immunoassay using Luminex technology (cut-off level ≥ 1.4 pg/ml). Data are presented as mean ± SEM. **P < 0.01 and *** P < 0.001, Student unpaired t-test.
(A) CCL2

Chemokine Concentration (pg/ml)

Hours post-infection (hpi)

Mock control
DENV-infected

(B) CCL2

Chemokine Concentration (pg/ml)

Hours post-infection (hpi)
**Figure 4.7 Production of CXCL10 and CCL11 by DENV-infected endothelial cells**

LSECs were seeded in 24 well plates. After 24 hours cells were infected with DENV at MOI 1 or mock control with culture medium alone. Culture supernatant were collected to analyse (A) CXCL10 and (B) CCL11 levels in supernatants from DENV-infected (n=6) or mock control (n=6) by Bio-plex immunoassay using Luminex technology at 24 and 48 hpi (cut-off level was ≥ 3.5 pg/ml for CXCL10 and ≥ 1.5 pg/ml for CCL11). Data are presented as mean ± SEM. **P < 0.01 and *** P < 0.001, Student unpaired t-test.
**Chemokine Concentration (pg/ml)**

**(A) CXCL10**

- **Hours post-infection (hpi):** 24, 48
- **Chemokine Concentration (pg/ml):**
  - 24 hpi: Mock control (light gray), DENV-infected (dark gray)
  - 48 hpi: Mock control, DENV-infected

**(B) CCL11**

- **Hours post-infection (hpi):** 24, 48
- **Chemokine Concentration (pg/ml):**
  - 24 hpi: Mock control, DENV-infected
  - 48 hpi: Mock control, DENV-infected

- **Significance Levels:**
  - ***: p < 0.001
  - **: p < 0.01
  - *: p < 0.05

- **Legend:**
  - Mock control
  - DENV-infected
Figure 4.8 Production of CCL3 and CCL5 by DENV-infected endothelial cells

LSECs were seeded in 24 well plates and after 24 hours cells were infected with DENV at MOI 1 or mock control with culture medium alone. Culture supernatant were collected to analyse (A) CCL3 and (B) CCL5 levels in supernatants from DENV-infected (n=6) or mock control (n=6) by Bio-plex immunoassay using Luminex technology at 24 and 48 hpi (cut-off level was ≥ 1.2 pg/ml for CCL3 and ≥ 2.4 pg/ml for CCL5). Data are presented as mean ± SEM. **P < 0.01 and *** P < 0.001, Student unpaired t-test.
Fig. 4.9 Production of G-CSF by DENV-infected endothelial cells

Endothelial cells (A) LSECs and (B) hCMEC/D3 cells were seeded in 24 well plates and after 24 hours of incubation monolayers were infected with DENV at MOI 1 or mock control with culture medium alone. After 2 hours incubation monolayers were washed with PBS and incubated with maintenance medium at 37 °C. At 24 and 48 hpi culture supernatant were collected to analyse G-CSF levels in supernatants from DENV-infected (n=6) or mock control (n=6) by Bio-plex immunoassay using Luminex technology (cut-off level ≥ 1.7 pg/ml). Data are presented as mean ± SEM. **P < 0.01 and *** P < 0.001, Student unpaired t-test.
(A) G-CSF

Growth factor concentration (pg/ml)

Hours post-infection (hpi)

Mock control
DENV-infected

(B) G-CSF

Growth factor concentration (pg/ml)

Hours post-infection (hpi)

Mock control
DENV-infected
Fig. 4.10 Production of GM-CSF by DENV-infected endothelial cells

Endothelial cells (A) LSECs and (B) hCMEC/D3 cells were seeded in 24 well plates and after 24 hours of incubation monolayers were infected with DENV at MOI 1 or mock control with culture medium alone. After 2 hours incubation monolayers were washed with PBS and incubated with maintenance medium at 37 °C. At 24 and 48 hpi culture supernatant were collected to analyse GM-CSF levels in supernatants from DENV-infected (n=6) or mock control (n=6) by Bio-plex immunoassay using Luminex technology (cut-off level ≥ 2.7 pg/ml). Data are presented as mean ± SEM. **P < 0.01 and *** P < 0.001, Student unpaired t-test.
(A) GM-CSF

Growth factor concentration (pg/ml)

Hours post-infection (hpi)

Mock control
DENV-infected

(B) GM-CSF

Growth factor concentration (pg/ml)

Hours post-infection (hpi)
**Fig. 4.11 Production of bFGF and PDGF-BB by DENV-infected endothelial cells**

LSECs were seeded in 24 well plates and after 24 hours of incubation monolayers were infected with DENV at MOI 1 or mock control with culture medium alone. After 2 hours incubation monolayers were washed with PBS and incubated with maintenance medium at 37 °C. At 24 and 48 hpi culture supernatant were collected to analyse (A) bFGF and (B) PDGF-BB levels in supernatants from DENV-infected (n=6) or mock control (n=6) by Bio-plex immunoassay using Luminex technology (cut-off level was ≥ 1.0 pg/ml for bFGF and ≥ 2.3 pg/ml for PDGF-BB). Data are presented as mean ± SEM. **P < 0.01 and *** P < 0.001, Student unpaired t-test.
(A) bFGF

![Graph showing bFGF levels over time.](image)

- **Significance:**
  - *: p < 0.05
  - **: p < 0.01
  - ***: p < 0.001

(B) PDGF-BB

![Graph showing PDGF-BB levels over time.](image)

- **Significance:**
  - ***: p < 0.001

**Axes:**
- Hours post-infection (hpi)
- Growth factor concentration (pg/ml)
Fig. 4.12 Production of VEGF by DENV-infected endothelial cells

LSECs were seeded in 24 well plates and after 24 hours of incubation monolayers were infected with DENV at MOI 1 or mock control with culture medium alone. After 2 hours incubation monolayers were washed with PBS and incubated with maintenance medium at 37 °C. At 24 and 48 hpi culture supernatant were collected to analyse VEGF levels in supernatants from DENV-infected (n=6) or mock control (n=6) by Bio-plex immunoassay using Luminex technology (cut-off level ≥ 2.5 pg/ml). Data are presented as mean ± SEM. **P < 0.01 and *** P < 0.001, Student unpaired t-test.
Chapter V

Permeability alterations of endothelial monolayers

after dengue virus infection
5.1 Introduction

In the human body a well-constructed and intricate network of blood vessels maintains the circulation of blood. These vessels are composed of three layers; the outer tunica adventitia, the tunica media and the innermost tunica intima layer, which is composed of endothelial cells. The capillary beds (microvessels) are lined only with a single layer of tunica intima whereas veins and arteries (macrovessels) have all three layers (Levick 2013). Microvessels serves as the major site for blood-tissue exchange by modulating fluid and metabolic homeostasis. The semipermeable property of the endothelium ensures efficient transport of nutrients and gasses in these microvessels and dysregulation of endothelial barrier function affects tissue viability and organ function (Yuan and Rigor 2010). Disruption of endothelial barrier function has been reported to occur during DENV infection contributing to plasma leakage in DHF.

Endothelial cells show differences in cell architecture depending on the tissue of origin as well as microvascular endothelium from different territories contributes differently to pathophysiological events during DENV infection. Although DENV induced permeability alterations in endothelial monolayers such as HUVEC and HMVEC-1 have been shown (Carr et al. 2003; Kelley et al. 2012), no permeability studies on microvessels such as LSEC and hCMEC/D3 monolayers have been reported. Permeability studies on LSEC and hCMEC/D3 monolayers will yield important information as these cells represent natural targets of DENV infection.

DENV infection of endothelial cells alters the specific expression patterns of cell adhesion molecules (CAMs) (Anderson et al. 1997; Peyrefitte et al. 2006). CAMs enable cell-to-cell and cell-to-matrix interactions. CAMs of the immunoglobulin superfamily,
such as vascular cell adhesion molecule 1 (VCAM-1; also known as CD106) and intercellular adhesion molecule 1 (ICAM-1; also known as CD54), regulates the adhesion of immune cells to the endothelium (Freemont and Hoyland 1996). Activation of endothelial cells by means of expression of CAMs in response to DENV infection has only been reported with LSECs (Peyrefitte et al. 2006) while the effect of DENV infection on CAM expression in hCMEC/D3 monolayer is not known.

Matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinase (TIMP) are also important proteins in regulating endothelial permeability. MMPs play an important role in the breakdown of the extracellular matrix. The MMP family consist of at least fifteen secreted and membrane-bound zinc-endopeptidases (Luplertlop and Misse 2008; Nagase and Woessner 1999). Growth factors, cytokines, hormones and cellular transformation regulate MMPs expression. Another study revealed cell-to-cell and cell-to-matrix interactions can also modulate MMP gene expression (Nagase and Woessner 1999). More importantly, regulation of MMP occurs naturally through inhibitor proteins known as TIMP. These endogenous regulators comprise a family of four inhibitors: TIMP-1, TIMP-2, TIMP-3 and TIMP-4. The balance between matrix formation and degradation is maintained by TIMPs. DENV infection can cause an imbalance of MMP/TIMP physiological equilibrium triggering vascular leakage in in vitro HUVECs model as well as in an in vivo BALB/c mice model (Luplertlop et al. 2006; Luplertlop and Misse 2008). The expression of MMPs and TIMPs in LSEC and hCMEC/D3 during DENV infection is not known.

DENV is known to preferentially infect peripheral blood monocytes which secrete high levels of immunomediators detected during DHF/DSS (Durbin et al. 2008). Also DENV-
infected primary human monocytes produced high levels of DHF/DSS associated immunomediators and the supernatants from DENV-infected monocytes/macrophages were shown to increase permeability in different endothelial cell monolayers including HUVECs and HMVECs (Carr et al. 2003; Kelley et al. 2012). Supernatants from DENV-infected monocyte-derived macrophages (MDMs), induce permeability alterations in HUVEC monolayers, in which the increase of permeability was not directly related with DENV infection of HUVECs or release of TNF-α from MDMs (Carr et al. 2003). In another study, permeability of HMVEC monolayers was increased by immunomediators secreted in the supernatants of DENV-infected monocytes. They suggest that primarily TNF-α induced phenotypic changes in HMVECs leading to increased monolayer permeability (Kelley et al. 2012). LSEC and hCMEC/D3 monolayer permeability after exposure to DENV-infected MDMs supernatants has not been reported.

As previously demonstrated in Chapters Three and Four, LSECs and hCMEC/D3s are permissive to DENV infection and infection results in the production of inflammatory cytokines, with elevated levels of IL-6, CXCL8, IL-1β, TNF-α, IP-10, CCL2 and CCL3. In this chapter we examined the activation and alteration of permeability of LSEC and hCMEC/D3 monolayers during DENV infection. The expression of ICAM-1, VCAM-1, MMP-2, MMP-9, TIMP-1 and TIMP-2 in LSECs and hCMEC/D3s after DENV infection were analysed and compared to mock control cells. Additionally, supernatants from LSECs and hCMEC/D3s were studied for their ability to alter endothelial permeability in homologous LSEC and hCMEC/D3 monolayers, respectively. We further investigated the effect of monocyte derived macrophage (MDM) supernatants on LSEC and hCMEC/D3 monolayers on endothelial permeability.
5.2 Results

5.2.1 ICAM-1 and VCAM-1 mRNA expression during DENV infection

Changes in ICAM-1 and VCAM-1 expression in endothelial cells infected with DENV were determined by real time PCR. The confluent monolayers of LSECs and hCMEC/D3s infected with DENV at MOI 1 or mock controls were collected at 24 and 48 hpi to determined ICAM-1 and VCAM-1 mRNA expression. At each time point total virus titre was determined by plaque assay to confirm infection.

Infection of LSECs with DENV increased ICAM-1 expression in DENV-infected cells compared to mock control (figure 5.1A). At 24 hpi, ICAM-1 expression in DENV-infected cells was 1.4 fold higher than the mock control (P= 0.012). By 48 hpi, a 1.2 fold higher expression of ICAM-1 was observed in DENV-infected LSECs compared to mock controls (P = 0.0165).

ICAM-1 mRNA expression in DENV-infected hCMEC/D3s increased significantly at 24 hpi (P = 0.0239) and 48 hpi (P = 0.0359) compared to mock control (figure 5.1B) being 1.3 fold higher at 24 hpi and 1.6 fold higher at 48 hpi.

Infection of LSECs with DENV induced expression of VCAM-1 compared to mock control at both 24hpi (P = 0.0005) and 48 hpi (P < 0.0001) (figure 5.2A). At both time points, the increase of VCAM expression in DENV-infected cells were 1.5-fold higher over mock controls.

DENV infection induced VCAM-1 expression in hCMEC/D3 cells (figure 5.2B). There was a significant increase (P = 0.0031, 2.3 fold increase) in VCAM-1 expression at 24 hpi in infected cells, which decreased at 48 hpi. However, compared to mock control,
VCAM-1 expression was still significantly higher ($P = 0.0094$) at 48 hpi. Taken together, the data indicate that DENV can activate LSECs and hCMEC/D3s, as indicated by increased expression of CAMs.

### 5.2.2 MMP and TIMP mRNA expression during DENV infection

In this study we investigated expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in LSECs and hCMEC/D3s after DENV infection, with mRNA expression being measured at 24 and 48 hpi by quantitative RT-PCR. At each time point total virus titre was determined by plaque assay to confirm infection.

MMP-2 mRNA expression was similar in DENV-infected and mock control LSECs at 24 hpi (figure 5.3A). By 48 hpi, MMP-2 mRNA expression in DENV-infected cells was decreased compared to mock control; however the observed decrease was not significant between infected and mock control cells.

In hCMEC/D3 cells, MMP-2 mRNA expression at 24 hpi was similar between DENV-infected and mock control groups (figure 5.3B). The MMP-2 expression in both groups increased by 48 hpi, but the difference was not significant between infected and mock control group.

MMP-9 mRNA expression increased 2-fold ($P = 0.0217$) in DENV-infected LSECs at 24 hpi (figure 5.4A). Over time the MMP-9 expression did not change in mock control, whereas it increased in DENV-infected cells. At 48 hpi, MMP-9 expression was increased by 1.9-fold over mock control ($P = 0.0153$).
Interestingly, no significant increase in MMP-9 expression in DENV-infected hCMEC/D3 cells was observed at 24 hpi or 48 hpi (figure 5.4B) with expression levels similar to mock control cells.

Similar levels of TIMP-1 mRNA expression were detected at 24 hpi in DENV-infected and mock control hCMEC/D3s (figure 5.5). At 48 hpi a slight increase in TIMP-1 mRNA expression in DENV-infected cells was detected, but this was not significantly different to mock control. No TIMP-1 mRNA expression was detected in DENV-infected or mock control LSECs in any of the three independent experiments performed (data not shown).

Expression of TIMP-2 in DENV-infected LSECs at 24 hpi showed no difference to mock control (figure 5.6A). At 48 hpi a slight non-significant decrease in TIMP-2 mRNA expression in infected LSECs compared to mock control was detected.

At 24 hpi DENV-infected and mock control hCMEC/D3 cells expressed similar mRNA levels of TIMP-2 without a significant difference (figure 5.6B). By 48 hpi a slight increase in TIMP-2 expression in both groups compared to 24 hpi was detected with no significant difference.

5.2.3 DENV infection of human MDMs

To study replication of DENV in MDMs, mononuclear cells were isolated from the buffy coat blood packs from healthy donors provided by the Australian Red Cross Blood Bank. Peripheral blood mononuclear cells (PBMCs) were purified and labelled with CD14-APC, a monocyte/macrophage marker to determine purity of enriched monocytes. Flow cytometry analysis showed that 95 % of the isolated PBMCs were CD14+ (Figure 5.7A).
Purified cells were seeded in 24 well plates and allowed to differentiate into macrophages. MDMs were infected with DENV at MOI 1 and incubated two hours for virus attachment followed by washing of cells with PBS to remove unbound virus.

At 24 hpi intervals, virus titres were determined by plaque assay up to 96 hours (Figure 5.7B). A virus titre of $2.3 \times 10^2$ PFU/ml at 0 hpi, slightly increasing to $5.3 \times 10^2$ PFU/ml by 24 hpi. The virus titres in samples collected after 24 hpi then began to decrease with titres of $1.5 \times 10^2$ PFU/ml and $4.2 \times 10^1$ PFU/ml at 48 hpi and 72 hpi, respectively. No virus was detected in samples collected at 96 hpi. MDM infection with DENV was non-cytopathic with no visible cytopathic effects.

5.2.4 DENV induced cytokine production in human MDMs

DENV-induced cytokine production in MDMs were analysed. MDMs seeded in 24 well plates were infected with DENV at MOI 1. Supernatants from DENV-infected or mock control MDMs were collected at 24 and 48 hpi, and analysed using a Bioplex immunoassay. At each time point total virus titre was determined by plaque assay to validate infection.

i. TNF-α expression during DENV infection

DENV-infected MDMs showed an overall trend of increased levels of TNF-α compared to mock controls at 24, 48 and 96 hpi (Figure 5.8A). The TNF-α level detected at 24 hpi in DENV-infected MDMs was not significantly different to mock controls. The level of TNF-α at 48 hpi was 1.5-fold over mock controls ($P = 0.0300$). TNF-α levels decreased
at 72 hpi in both groups with no significant difference between the groups. The levels of TNF-α at 96 hpi was significantly increased by 1.6-fold over mock control (P = 0.0024).

ii. **IL-6 expression during DENV infection**

Elevated levels of IL-6 was detected in supernatants from DENV-infected MDMs compared to mock control at all four time points (figure 5.8B). At 24 hpi level of IL-6 in infected cells was 5.2-fold higher than the mock control (P = 0.0111). The fold change decreased to 3.5-fold by 48 hpi, but was significantly different from mock control (P = 0.0383). The increased level of IL-6 at 72 hpi in DENV-infected cells was not significantly different to the mock control. However at 96 hpi IL-6 production by DENV-infected cells has significantly elevated by 4.5 fold over mock control (P = 0.0077).

iii. **IL-1β expression during DENV infection**

Higher levels of IL-1β in DENV-infected MDM supernatants were detected at 48, 72 and 96 hpi compared to mock controls (Figure 5.8C). IL-1β levels at 24 hpi were not significantly different between the two groups. By 48 hpi IL-1β production in DENV-infected MDMs significantly increased 2.8 fold over mock control (P = 0.0211). IL-1β protein levels in the supernatant further increased by 72 hpi, with a 3.8 fold increase in DENV-infected MDM. The continual increase in IL-1β level in DENV-infected cells was detected until 96 hpi. At 96 hpi, IL-1β levels was significantly higher (P = 0.0271, fold change = 3.4) in DENV-infected cells than the mock control.
Together these data showed that DENV induced MDMs to produce important proinflammatory cytokines such as TNF-α, IL-6 and IL-β. These factors are known to induce endothelial permeability in different endothelial cells such as HUVECs and HMVEC. Further investigation will be required to determine the ability of infected MDM supernatant to increase permeability on LSEC and hCMEC/D3 monolayers.

5.2.5 DENV induced chemokine production in human MDMs

Chemokine production by MDMs was detected by Bioplex assay kit, which contain beads for CXCL8, CCL4 (MIP-1β) and CCL2 (MCP-1). Supernatants from mock and DENV-infected MDMs were analysed at 24, 48, 72 and 96 hpi in three independent experiments carried out in triplicate. At each time point total virus titre was determined by plaque assay to validate infection, before Bioplex analysis.

i. CCL2 expression during DENV infection

Supernatants from DENV-infected MDMs contained higher levels of CCL2 compared to mock controls (figure 5.9A). At 24 hpi CCL2 levels in DENV-infected cultures was 3.4-fold greater than in the mock controls (P = 0.0055). The fold difference between infected and mock control cultures has decreased to 2.4 fold over 48 and 72 hpi. Although at both time points a significant increase in CCL2 in DENV-infected compared to mock control was detected (48 hpi: P = 0.0007 and 72 hpi: P = 0.0007). The highest CCL2 levels were detected at 96 hpi in DENV-infected MDM cultures with a 3.9-fold increase over mock control.
i. **CXCL8 expression during DENV infection**

The production of CXCL8 (IL-8) chemokine by MDMs is shown in figure 5.9B. CXCL8 production by DENV-infected MDMs was not significantly different to than in mock control MDMs. In both mock and DENV-infected cells, the highest level of CXCL8 was detected at 48 hpi, which gradually decreased overtime.

ii. **CCL4 expression during DENV infection**

As shown in figure 5.9C, CCL4 secretion from DENV-infected MDMs was highest at 24 hpi and decreased after that time point. The levels of CCL4 did not differ between mock control and DENV-infected groups.

Together these data shows that DENV-infected MDMs produced higher levels of CCL2 than mock control MDMs, while CCL4 and CXCL8 chemokines did not differ significantly between the two groups.

5.2.6 **Microvascular endothelial monolayer permeability changes by supernatants from DENV-infected cell cultures**

We examined permeability alterations of LSEC and hCMEC/D3 monolayers by DENV-infected endothelial cell supernatants. We further analysed human MDM supernatants for its ability to cause permeability changes in LSEC and hCMEC/D3 monolayers.

In order to determine permeability alterations, LSECs or hCMEC/D3s were seeded in electrode arrays connected to the ECIS morphological biosensor and monitored until
monolayers reached steady-state, indicative the formation of an intact monolayer (Figure 5.10).

Intact LSEC monolayers were treated with DENV-infected and mock control LSEC supernatants collected at 48 hpi. The hCMEC/D3 cell monolayers were treated with hCMEC/D3 cell supernatants collected at 48 hpi from DENV-infected and mock control cultures. In chapter four, LSEC and hCMEC/D3 cell supernatants from 48 hpi were shown to have higher cytokine and chemokine levels, and thus were selected for permeability studies. Separately both hCMEC/D3 and LSEC monolayers were also treated with DENV-infected and mock control MDM cell supernatants collected at 96 hpi. MDM cell supernatants from 96 hpi was selected as, i) elevated levels of TNF-α, IL-6 and IL-1β were detected in DENV infected MDM supernatants and increased levels of these factors were reported in DENV patient sera (Rathakrishnan et al. 2012), ii) in dengue patients DHF/DSS vascular leakage occurs after viral clearance and defervescence and this is comparable with our data as no progeny virus was detected in 96 hpi MDM supernatants (Appanna et al. 2012). All supernatants used in permeability assay was validated for DENV infection by plaque assay. Every 5 minutes for a 24 hours period impedance of endothelial monolayers was recorded. The ratio of resistance at each time point to the resistance at onset of that cycle was compared between DENV and mock control groups.

LSEC monolayers treated with DENV-infected and mock control LSEC supernatants showed a sharp decrease of relative resistance at the beginning of treatment (figure 5.11A). Initial decrease of relative resistance was recovered by both LSEC monolayers treated with supernatants from DENV-infected and mock cultures in a similar pattern.
Treatment of confluent hCMEC/D3 cell monolayers with DENV-infected and mock control hCMEC/D3 supernatants resulted in a slight decrease in relative resistance at the beginning of the treatment, which recovered over time (figure 5.11B). There was no difference in relative resistance observed between the DENV-infected and mock control cell supernatant treatments.

Initially treatment of LSEC monolayer with supernatant from DENV-infected or mock control 96 hpi MDM supernatant caused an abrupt decrease in relative resistance (figure 5.12A). The LSEC monolayer treated with mock control MDM supernatant recovered relative resistance after four hours of treatment. The relative resistance of LSEC monolayer treated with DENV-infected MDM supernatant was not increased 4-12 hours after treatment. After 12 hours of treatment an increase of relative resistance on LSEC monolayer treated with DENV-infected MDM supernatant was detected.

Treatment of hCMEC/D3 monolayer with mock control and DENV-infected MDM supernatant caused a small decrease in relative resistance when treatment was started (figure 5.12B). Then from 0 to 24 hours the relative resistance was increased in both monolayers treated with mock control and DENV-infected supernatants.
**5.3 Discussion**

Increased levels of immunomediators have been detected in the sera of dengue patients with severe vascular leakage complications. The different immunomediator profile patterns identified during different phases of DENV infection indicate a role of immunomediators in DENV pathogenesis (Bozza et al. 2008; Rathakrishnan et al. 2012). Supernatants from, DENV-infected monocyte/macrophages have been found to increase permeability in endothelial monolayers (Carr et al. 2003; Kelley et al. 2012), which may relate to vascular leakage. As discussed in Chapter 4, infection of LSECs and hCMEC/D3s resulted in production of proinflammatory and anti-inflammatory cytokines, chemokines and growth factors that might be important in pathophysiology of vascular endothelial damage. Thus we examined the permeability alterations of LSEC and hCMEC/D3 monolayers in response to immunomediators secreted by DENV-infected endothelial cells and MDMs.

In order to study permeability alterations of endothelial monolayers, we first determined the patterns of vascular specific mRNA expression of endothelial cells. CAMs are essential for the immune response as they mediate leukocyte endothelial transmigration. CAMs are expressed on the endothelium as well as in the serum of healthy individuals in soluble form. Elevated levels of soluble forms of ICAM-1 and VCAM-1 have been reported in plasma or serum of dengue patients (Azeredo et al. 2006b). In addition an association between soluble VCAM-1 and DHF has been reported (Murgue et al. 2001). Culture supernatants from DENV-infected monocytes can activate endothelial cells by increasing ICAM-1 and VCAM-1 mRNA expression (Anderson et al. 1997). Another study showed that DENV infection of different endothelial cell types resulted in specific
ICAM-1 expression patterns: infection up-regulated ICAM-1 expression in LSECs but down-regulated ICAM-1 expression in HMEC-1 (Peyrefitte et al. 2006). Our results show that ICAM-1 and VCAM-1 mRNA expression in DENV-infected LSECs and hCMEC/D3s was elevated compared to mock controls. Together, these results demonstrate that DENV infection can activate LSECs and hCMEC/D3s, as indicated by increased expression of ICAM-1 and VCAM-1. Bearing in mind the limitations of extrapolating to in vivo, the results suggest that in the liver and brain, endothelial cells could play a role in initiating or enhancing the inflammatory response, leading to vascular leakage.

MMPs are potent inducers of vascular permeability changes in the pathogenesis of DENV infection (Luplerdlop et al. 2006; Luplertlop and Misse 2008). Our results showed elevated MMP-9 expression in DENV-infected LSECs but not in hCMEC/D3s. MMP-2 was not elevated in either LSECs or hCMEC/D3s following DENV infection. Our results are in accordance with the findings that mild and severe dengue patients have elevated levels of MMP-9 but no significant differences in MMP-2 levels (van de Weg et al. 2014). West Nile Virus (WNV), which belongs to the same family as DENV, uses MMP-9 to facilitate entry into the brain by enhancing blood-brain barrier permeability (Wang et al. 2008). It is likely that absence of elevated MMP9 levels in hCMEC/D3s may therefore not facilitate permeability changes in hCMEC/D3 monolayers. Interestingly endothelial cells can produce TIMP-1 and TIMP-2, which are natural inhibitors of MMP-2 and MMP-9, respectively. Elevated levels of TIMP-1 and TIMP-2 has been shown in DENV-infected patients (van de Weg et al. 2014). Luplerdlop et al. also showed that DENV infection of HUVECs could induce the production of TIMP-1 and TIMP-2 (Luplertlop and Misse 2008). However, infection of either LSCEs or hCMEC/D3s did not result in
an elevation of TIMP production compared to mock controls. Therefore together these results show that the dynamic equilibrium between MMP/TIMP is disrupted by DENV infection of LSECs, as there was increased expression of MMP-9 but no change in the expression of its inhibitor-TIMP-2. This may contribute to increased LSEC monolayer permeability during DENV infection.

Supernatants from DENV-infected monocytes/macrophages have previously been shown to increase permeability of endothelial monolayers such as HUVECs and HMVEC-1 (Carr et al. 2003; Kelley et al. 2012). Neither LSECs nor hCMEC/D3s have been examined for DENV induced permeability alterations. To study permeability changes in LSEC and hCMEC/D3 monolayers induced by DENV-infected cell culture supernatants, we first characterised the cytokine/chemokine profile secreted by MDMs.

Elevated levels of TNF-α, IL-6 and IL-1β were detected in DENV-infected 96 hpi MDM supernatants compared to mock controls. Increased levels of these factors were reported in DENV patient sera and also in DENV-infected cell cultures of HMVECs (Kelley et al. 2012; Rathakrishnan et al. 2012). Permeability changes in HMVEC monolayers were shown to be induced by TNF-α secreted by DENV-infected THP-1 cells, in which antibody neutralisation of TNF-α was found to reduce permeability (Kelley et al. 2012). A separate study showed that TNF-α increased capillary permeability synergistically with IL-1γ/β and IFN-γ (Burke-Gaffney and Keenan 1993). In DENV-infected MDM supernatants, lower levels of IL-1β detected in early time points increased over time, and similarly in DENV patient sera, low IL-1β levels during febrile phase were peaked during defervescence (Rathakrishnan et al. 2012). Higher levels of IL-6 were detected at all four time points in DENV-infected MDM supernatants. IL-6 can increase vessel permeability.
and leukocyte recruitment and plays an important role in local inflammatory reactions (Huang et al. 2000). Moreover, IL-6 and IL-1β are associated with regulation of coagulation and fibrinolysis in DSS patients (Suharti et al. 2002). DENV infection of MDMs also induced CCL2 production. In human vascular endothelium, disruption of tight junctions leading to increased vascular permeability was partially dependent on CCL2 (Lee et al. 2006b). Together with the literature, our results highlight the importance of investigating the effects of these factors on permeability alterations in an endothelial cell culture model.

As DENV-infected endothelial cell and MDM supernatants contain soluble factors responsible for disruption of endothelial barrier function, permeability alterations in LSEC and hCMEC/D3 monolayers were further analysed. Treatment with DENV-infected MDM supernatant decreased resistance in LSEC monolayers, causing a transient increase in monolayer permeability. DENV-infected macrophage and monocyte supernatants have been previously shown to induce permeability changes in HUVECs and HMVECs (Carr et al., 2003; Kelley et al., 2012). During DHF/DSS, vascular leakage occurs after viral clearance and defervescence showing it is not a direct effect of DENV infection and is possibly due to vasoactive mediators produced during infection (Appanna et al., 2012; Patkar et al., 2013). Similarly, absence of progeny virus at 96 hpi and in UV-inactivated supernatants confirmed absence of an infection and indicated that permeability changes in LSEC monolayers by supernatants from 96 hpi DENV-infected MDMs, were not due to infection of endothelial cells. Increased permeability in LSECs may instead be mediated by permeabilising factors present in MDM supernatants.
The LSEC monolayers treated with DENV-infected LSEC supernatants did not show increased permeability of LSEC monolayer. In a HUVECs model, DENV infection alone did not cause an increase in permeability, whereas addition of TNF-α and DENV together did increase permeability in HUVECs monolayers (Dewi et al. 2004). In another study, effects of DENV infection was also shown not to affect the permeability in ECV304 monolayers over a 15-day period and showed no significant permeability increase (Bonner and O’Sullivan 1998). Similar results were also demonstrated in a HMVEC model, where no significant permeability changes were shown after 72 hours after infection; however, supernatants from DENV-infected THP-1 monocytes increased HMVEC monolayer permeability (Kelley et al. 2012). Our results are in accordance with these previous studies as immunomediators secreted in the supernatants of DENV-infected MDMs mediated increased permeability in LSEC monolayers, while supernatants from DENV-infected endothelial cells did not.

The factors that mediate increased permeability in endothelial monolayers are not clearly understood. In the HMVEC model permeability changes appear to be caused primarily by TNF-α and CXCL8 (Kelley et al. 2012), whereas permeability in HUVECs was not directly related to TNF-α release (Carr et al. 2003). Another study showed permeability in the HUVEC model is increased by addition of TNF-α to DENV-infected cells, while addition of TNF-α alone did not increase permeability (Dewi et al. 2004). DENV-infected LSEC and MDM culture supernatants contained significantly elevated levels of factors such as TNF-α, IL-6, IL-1β and CCL2. Therefore we suggest that transient leakage in LSEC monolayers is mediated by factors present in DENV-infected MDM supernatants, although permeability changes are not primarily caused by TNF-α, IL-6, IL-1β or CCL2. Further evidence from this study, suggests vasoactive factor/s specifically produce by the
monocytic cell lineage are primarily responsible for permeability increase in endothelial cells. Factors such as TNF-α, IL-6, IL-1β and CCL2, which can also be produced by endothelial cells, may have a synergistic role in determining severity of permeability, although they are not primarily responsible.

hCMEC/D3 monolayers were also investigated for permeability responsiveness to DENV infection, but DENV-infected supernatants from hCMEC/D3s or MDMs did not alter hCMEC/D3 monolayer permeability. Compared to other organ endothelia, brain endothelia have higher levels of tight junctions, which contributes to extremely low permeability of the endothelial barrier. The hCMEC/D3 cell line is reported to maintain most of the unique structural and biochemical properties of brain endothelium in vivo (Weksler et al. 2005). Additionally, liver sinusoidal capillaries are among the leakiest vessels in the body (Aird 2007; Daneman et al. 2010). Therefore the absence of permeability change in hCMEC/D3 monolayers in response to DENV-infected MDM supernatants (in contrast to LSEC monolayers) may be due to the highly specific structural organisation of brain endothelium.

This chapter focused on permeability changes in endothelial monolayers in response to DENV infection. The data showed that DENV infection could lead to activation of endothelial cells and enhanced expression of CAMs. Supernatants from DENV-infected endothelial cells did not alter monolayer permeability, while DENV-infected MDM supernatants caused a transient increase in monolayer permeability in LSECs but not hCMEC/D3s. This is the first study to show that human microvascular endothelial cells from liver can be induced to alter their permeability in response DENV infection.
Figure 5.1 Expression of ICAM-1 in DENV-infected endothelial cells

Microvascular endothelial cell lines (A) LSECs and (B) hCMEC/D3s were seeded in 24 well plates. After 24 hours cells were infected with DENV at MOI 1 or mock control with culture medium alone. At 24 and 48 hpi total RNA was extracted and cDNA synthesis performed. ICAM-1 mRNA expression levels in DENV-infected or mock control samples were analysed with Real Time PCR. Each data point is expressed as the mean ± SEM for 3 data points and is representative of 3 independent experiments. *P < 0.1 and ** P < 0.01, Student unpaired t-test.
Figure 5.2 Expression of VCAM-1 in DENV-infected endothelial cells

Microvascular endothelial cell lines (A) LSECs and (B) hCMEC/D3s were seeded in 24 well plates and after 24 hours infected with DENV at MOI 1 or mock control with culture medium alone. At 24 and 48 hpi total RNA was extracted and cDNA synthesis was performed. VCAM-1 mRNA expression level in DENV-infected or mock control samples were analysed with Real Time PCR. Each data point is expressed as the mean ± SEM for 3 data points and is representative of 3 independent experiments. **P < 0.01 and *** P < 0.001, Student unpaired t-test.
Figure 5.3 Expression of MMP-2 in DENV-infected endothelial cells

Endothelial cell lines (A) LSECs and (B) hCMEC/D3s were seeded in 24 well plates and after 24 hours infected with DENV at MOI 1 or mock control with culture medium alone. Total RNA was extracted at 24 and 48 hpi was followed by cDNA synthesis. MMP-2 mRNA expression level was analysed by Real Time PCR. Each data point is expressed as the mean ± SEM for 3 data points and is representative of 3 independent experiments. **P < 0.01 and *** P < 0.001, Student unpaired t-test.
Figure 5.4 Expression of MMP-9 in DENV-infected endothelial cells

Microvascular endothelial cell lines (A) LSECs and (B) hCMEC/D3s were seeded in 24 well plates and after 24 hours infected with DENV at MOI 1 or mock control with culture medium alone. At 24 and 48 hpi total RNA was extracted and cDNA synthesis performed. MMP-9 mRNA expression level in samples were analysed by Real Time PCR. Each data point is expressed as the mean ± SEM for 3 data points and is representative of 3 independent experiments. **P < 0.01 and *** P < 0.001, Student unpaired t-test.
Figure 5.5 Expression of TIMP-1 in hCMEC/D3 cells

Microvascular endothelial cell line hCMEC/D3s were seeded in 24 well plate and after 24 hours infected with DENV at MOI 1 or mock control with culture medium alone. At 24 and 48 hpi total RNA was extracted and cDNA synthesis was performed. TIMP-1 mRNA expression level in DENV-infected or mock control samples were analysed with Real Time PCR. Each data point is expressed as the mean ± SEM for 3 data points and is representative of 3 independent experiments. **P < 0.01 and *** P < 0.001, Student unpaired t-test.
Figure 5.6 Expression of TIMP-2 in dengue infected endothelial cells

Microvascular endothelial cell lines (A) LSECs and (B) hCMEC/D3s were seeded in 24 well plates and after 24 hours infected with DENV at MOI 1 or mock control with culture medium alone. Total RNA was extracted at 24 and 48 hpi followed by cDNA synthesis. TIMP-2 mRNA expression level was analysed by Real Time PCR. Each data point is expressed as the mean ± SEM for 3 data points and is representative of 3 independent experiments. **P < 0.01 and *** P < 0.001, Student unpaired t-test.
PBMCs cells were isolated from the buffy coat blood packs. (A) Isolated PBMCs were labelled with CD14-APC and analysed by flow cytometry to determine purification. (B) Purified cells were seeded in 24 well plates and infected with DENV at MOI 1 or mock control with medium alone. Total virus was determined in all samples at 24 hour intervals up to 96 hpi by plaque assay. Each data point expressed as the mean ±SEM for 3 data points.
Figure 5.8 Secretion of cytokines by DENV-infected endothelial MDMs

MDMs were seeded in 24 well plates and infected with DENV at MOI 1 or mock control with culture medium alone. At 24, 48, 72 and 96 hpi (A) TNF-α (B) IL-6 and (C) IL-1β level in cell-free supernatant from DENV-infected or mock were analysed with Bio-plex immunoassay using Luminex technology. Each data point is expressed as the mean ± SEM for 3 data points and is representative of 3 independent experiments. ** P< 0.01 and *** P < 0.001, two-way ANOVA with Bonferroni posttest.
Figure 5.9 Secretion of chemokines by DENV-infected MDMs

MDMs were seeded in 24 well plates and infected with DENV at MOI 1 or mock control with culture medium alone. At 24, 48, 72 and 96 hpi (A) CCL2 (B) CXCL8 and (C) CCL4 level in cell-free supernatant from DENV-infected or mock control samples were analysed with Bio-plex immunoassay using Luminex technology. Each data point is expressed as the mean ± SEM for 3 data points and is representative of 3 independent experiments. ** P< 0.01 and *** P< 0.001, two-way ANOVA with Bonferroni posttest.
(A) CCL2

Cytokeine Concentration (pg/ml)

Hours post-infection (hpi)

Mock control

DENV-infected

***

(B) CXCL8

Cytokeine Concentration (pg/ml)

Hours post-infection (hpi)

(C) CCL4

Cytokeine Concentration (pg/ml)

Hours post-infection (hpi)
Figure 5.10 Alteration of transendothelial electrical resistance by DENV cell supernatants

LSECs were seeded in electrode arrays connected to the ECIS morphological biosensor and impedance was measured every 5 minutes until monolayers reached steady-state. Once the monolayers reached a steady state it confirms the maximum membrane tightness. Treatments were started only after intact monolayer formation is confirmed. Bars represent the mean ± SEM of two independent experiments conducted in triplicate.
Figure 5.11 Alteration of transendothelial electrical resistance by DENV-infected endothelial cell supernatants

Endothelial cells (A) LSECs (B) hCMEC/D3s were grown in electrode arrays until maximum membrane tightness was achieved. Intact LSEC or hCMEC/D3 monolayers were treated with supernatants from 48 hpi mock control (black) or DENV-infected (red) LSECs or hCMEC/D3s, respectively. Before treatment, supernatant were UV-treated to inactivate DENV. The impedance of the monolayer was measured every 5 minutes for 24 hours and for each well impedance at time was normalised according to the impedance of the well at the beginning of treatment. The bars represent the mean± SEM of two independent experiments in triplicate.
(A) LSEC monolayer treated with 48 hpi LSEC supernatant

(B) hCMEC/D3 monolayer treated with 48 hpi hCMEC/D3 supernatant
Figure 5.12 Alteration of transendothelial electrical resistance by DENV-infected MDM supernatants

Endothelial cells (A) LSECs and (B) hCMEC/D3s were grown in electrode arrays until maximum membrane tightness was reached. The intact monolayers were treated with supernatants from mock control (black) or DENV-infected (red) MDMs. Before treatment, supernatant were UV-treated to inactivate DENV. The impedance of the monolayer was measured every 5 minutes for 24 hours and for each well impedance at time was normalised according to the impedance of the well at the beginning of treatment. The bars represent the mean± SEM of two independent experiments in triplicate.
Chapter VI

Host gene response in endothelial cells to dengue virus infection
6.1 Introduction

Studies on host cell gene expression changes during viral infections offer interesting insights into viral pathogenesis. The advent of DNA microarray technology has made it possible to investigate the expression of multiple genes of interest with a relatively small amount of sample, and thus has an increasingly important role in biomedical research. Most importantly, DNA microarray technology is able to provide relative measurements of transcriptional levels of thousands of genes simultaneously, allowing identification of differentially expressed genes that are functionally important. By not limiting analysis to a certain gene of interest, it has also permitted large-scale identification of functionally related gene sets and gene regulated pathways (Jenner and Young 2005).

Most studies on DENV infection have focused on disease pathogenesis, virulence and host immunological response while the molecular host response to DENV infection been less extensively studied. DENV replication within the infected host cell can alter the cellular gene expression profile and thus may provide additional information on DENV pathogenesis at the molecular level. With regard to DENV infection, gene expression studies have been carried out in DENV-infected HUVECs, HepG2 cells, peripheral blood monocyte-derived macrophages and ECV304 cells (Fink et al. 2007; Liew et al. 2006; Long et al. 2013; Moreno-Altamirano et al. 2004; Warke et al. 2003). Identification of differentially expressed host cellular genes during DENV infection has facilitated recognition of functionally important genes.
Genome-wide transcriptome analysis performed on DENV-infected HUVECs revealed interferon inducible genes were differentially expressed with prominent upregulation of XAF1. This study showed that XAF1 promoted apoptosis in endothelial cells in an XIAP dependent manner after DENV infection (Long et al. 2013). Another gene expression study on DENV-infected HUVECs demonstrated activation of broad functional responses including stress, defence, immune, wounding, inflammatory, and antiviral pathways. During DENV infection inflammatory response were mainly enriched by TNF-α, IL-1β, and IFN-α/β signalling pathways. This study suggested novel roles for antiviral proteins such as regulation of cell-cell interactions during DENV-infection by galectin-9 (Gal-9) and endothelial and smooth muscle cell-derived neuropilin-like protein (ESDN), and chemokine response by regulator of G protein signalling 2 (RGS2 (Warke et al. 2003)). In addition microarray analysis on ECV304 endothelial-like cells infected with DENV revealed upregulation of gene expression important in cell cycle, apoptosis, cellular receptors, transcriptional regulation, signal transduction, enzymes, protein transport, and cytoskeleton (Liew et al. 2006).

Studies on host gene expression in DENV-infected microvascular endothelial cells, particularly from natural target organs of DENV is limited. As demonstrated in earlier chapters, microvascular LSECs and hCMEC/D3s can be infected by DENV to varying degrees and infection caused cells are able to produce high levels of soluble immunomediators. Additionally, DENV infection caused transient endothelial permeability alterations in LSEC monolayer. Here, microarray technology will be used to analyse host gene expression in DENV-infected LSECs and hCMEC/D3 to gain further insight into the molecular events that occur during DENV infection.
Different types of microarrays have been developed over years such as spotted microarrays, *in situ* synthesised arrays: Affymetrix and NimbleGen, and bead-based microarray systems: Illumina (Gunderson et al. 2004). Bead-based microarray systems such as Illumina, have been developed with increased sensitivity and specificity (Nielsen et al. 2003). According to MicroArray Quality Control (Shi et al.) project, Illumina platform has better intrasite repeatability overall as well as high correlation values with comparison assays (Shi et al. 2006). Illumina platform microarray was thus selected for expression analysis on DENV-infected LSECs and hCMEC/D3s.

One of the core objectives of microarray analysis on DENV-infected LSECs and hCMEC/D3s was to identify genes whose transcript levels were altered compared to mock controls. Clustering analysis in which genes are divided into groups based on similarity followed the identification of genes of interest. Finally to extract biological information on why genes are differentially expressed and belonging to the same cluster, the biological features overrepresented in these groups were determined by Gene set enrichment analysis (GSEA). GSEA will interpret biological meaning, focusing on gene sets that share common biological function, chromosomal location or regulation (Subramanian et al. 2005).
6.2 Results

6.2.1 Quality control and normalisation of microarray data

The quality of purified total RNA from mock control and DENV-infected LSECs and hCMEC/D3s was assessed by Agilent Bioanalyzer electropherograms (figure 6.1A). The ratio between 28S and 18S ribosomal RNA was determined to accurately identify the quality of RNA for microarray analysis. The microarray quality control consortium uses a RNA Integrity Number (RIN) > 8.0 and 28S/18S ratio > 0.9 as quality criteria. All LSEC and hCMEC/D3 samples resulted in RINs > 9.7 and 28S/18S ratios > 2 and were thus considered suitable for microarray quality (table 6.1). The 28S and 18S ribosomal bands were also viewed as a gel image (figure 6.1B).

To ensure quality of microarray data was maintained at each step of the microarray experiment, quality assessment was an integral part of the analysis. The ‘arrayQualityMetrics’ from the Bioconductor project was used for quality assessment and outliers were detected by two different methods including between arrays comparison and array intensity distributions. Between arrays comparison was performed using ‘distances between arrays’ and array intensity distributions by boxplots of arrays.

Graphs of distances between arrays are shown in figure 6.2A for LSECs and hCMEC/D3s, in which a false colour heatmap was generated and colour scale cover the range of distances encountered in the dataset. Patterns in this plot indicate no clustering of the arrays, which may be due to biological or experimental factors. Outlier detection was performed for both LSECs and hCMEC/D3s where no outliers were detected. The signal intensity distributions of the arrays are summarised in the boxplot (figure 6.2B).
Each box corresponds to one array. Both boxplots for LSEC and hCMEC/D3s, showed a similar position and width for each box with no boxes found to vary in distribution from others, indicating no experimental errors. Applying set of filters to remove probes with weak signals, followed quality control. In LSECs 23170 probes and in hCMEC/D3s 21543 probes had an expression above 1 and only these probes were used for further analysis (table 6.2).

Normalisation of microarray data is important to make meaningful comparisons of differentially expressed genes and to retrieve reliable biological results from the analysis. Quantile normalisation method was used to normalise LSEC and hCMEC/D3 microarray data (figure 6.2C). In the Q-Q plot a straight diagonal line was detected confirming same distribution of probe intensities for each array in the data set.

### 6.2.2 Differentially expressed genes during DENV infection of endothelial cells

An overview of gene expression during DENV infection of LSECs is shown in the volcano plot on figure 6.3A, in which the association between the p-values and the log2 of the fold change is illustrated. Compared to mock control, DENV-infected LSECs had 193 up-regulated and 162 downregulated genes with an absolute fold change greater than two and a p-value less than 0.05.

In hCMEC/D3s, significantly differentially expressed genes were also identified through volcano plot filtering (figure 6.4A). In DENV-infected hCMEC/D3 cells 256 genes were significantly up-regulated and 186 genes downregulated compared to mock controls. The number of genes that have an absolute fold change greater than two and a p-value less than 0.05 was considered as a significant change.
Significantly differentially expressed genes filtered through volcano plots were used to generate heat maps. Heat map diagrams show the expression of the differentially expressed genes in all samples of DENV-infected compared to mock control, LSECs (figure 6.3B) and hCMEC/D3s (figure 6.4B). The fold change and p-value of several differentially expressed genes in DENV-infected cultures compared to mock controls are shown for LSECs (figure 6.5) and hCMEC/D3s (figure 6.6).

### 6.2.3 Clustering and classification of differentially expressed gene sets

Differentially expressed genes identified in section 6.2.2, were divided into clusters based on functional similarity. The functional enrichment analysis was performed by ClueGO, Cytoscape plug-in. The functionally grouped annotation network showed enrichment in three clusters of gene sets from 193 upregulated genes in DENV-infected LSECs compared to mock control, that were enriched for distinct functional categories (figure 6.7). The clusters were manually named as 1) interferon response cluster 2) virus response cluster and 3) endothelial cell migration cluster. Gene sets belonging to type I interferon-signalling pathway contributed mainly to the enrichment of interferon response cluster. Whereas gene sets belonging to regulation of viral genome replication, negative regulation of viral genome replication, viral life cycle, viral process and multi-organism process has equally contributed to virus response cluster. Involvement of the virus response cluster with the interferon response cluster suggests viral induction of the innate immune response. Furthermore, the enrichment map analysis showed regulation of viral genome replication gene set and negative regulation of viral genome replication gene set, are associated with negative regulation of viral life cycle gene set. Negative regulation of
endothelial cell migration gene set largely contributed to the third cluster, endothelial cell migration. Among the downregulated genes in DENV-infected LSECs, a single cluster was identified that was enriched with gene sets for biosynthetic process of peptides, amides and cellular modified amino acids. Most of the higher-expressed genes in DENV-infected LSECs were involved in type I interferon signalling pathway, response to type I interferon and cellular response to type I interferons.

To capture biological information associated with differentially expressed genes in DENV-infected hCMEC/D3s, ClueGO, a Cytoscape plugin was used for enrichment analysis (figure 6.8). The enrichment map visualises functionally coherent gene sets among these upregulated genes. Among these gene sets, 4 main clusters can be identified with various functional categories. Gene sets belonging to DNA modification, positive regulation of VEGF production, TLR1:TLR2 signalling pathway and negative regulation of organic acid transport contributed mainly to the enrichment of these four clusters. In addition, miscellaneous gene sets such as negative regulation of NF-κB transcription factor activity, transcription from RNA polymerase III promoter and collagen fibril organisation were also higher-expressed genes in DENV-infected hCMEC/D3s.

6.2.4 Extraction of biological information

GSEA analysis for ‘hallmark gene sets’ was performed to summarise and represent well defined biological states or processes and to display coherent expression. GSEA calculates an enrichment score to reflect the degree to which genes in a certain pathway are overrepresented at the top (upregulated) or bottom (downregulated) of the rank gene list. It focuses the analysis at the functionally related gene sets instead of single genes.
Gene sets associated with interferon were specifically induced in DENV-infected LSECs, in which the interferon alpha response gene set is the highest upregulated gene set followed by interferon gamma response gene set (figure 6.9). Other gene sets upregulated with a high enrichment score are inflammatory response, IL6 jak stat3 signalling, complement, TNF-α signalling via NF-κB and upregulation of Kras signalling. The lowest downregulated gene set was mitotic spindle.

In hCMEC/D3 cells allograft rejection gene set has the highest upregulated enrichment score (figure 6.10). In hCMEC/D3 cells IL6 jak stat3 signalling and complement gene sets were downregulated and which was found to be upregulated in LSECs. Glycolysis and downregulation of Kras signalling has the lowest downregulated enrichment scores.

Positively enriched interferon alpha response gene set was selected to create network figures which contain genes with increased expression upon DENV infection of LSECs (figure 6.11). The network topology on interferon alpha response in DENV-infected LSECs compared to mock controls, revealed interesting interactions among well characterised genes associated with IFN response. MX1, USP18, IFIT1, PLSCR1, IRF7, IFI44L and OAS1 are among the highly upregulated genes while IRF2 and CD47 were downregulated genes.

Functional network analysis showed that upregulated serine hydrolases such as PSMB8 and PSMB9, are predicted to interact with other serine hydrolases like PSME2, PSMB10, PSME1 and PSMA3, some of which also identified as differentially expressed, and non-serine hydrolase genes TAP1 and WARS.
6.3 Discussion

The pathogenesis of DENV infection is multifactorial, with disease severity depending on both viral and host factors. In patients with severe DSS, the underlying mechanism of vascular leakage is not clearly understood and host immune factors are considered as the most important contributing factor (Simmons et al. 2007). In previous chapters we showed that DENV infection of human microvascular LSECs and hCMEC/D3s increases cytokine production, apoptosis and transient permeability alterations, to varying degrees. Thus further analysis of endothelial cell gene expression during DENV infection will help to elucidate the underlying mechanisms of DENV infection. The development of microarray technology has allowed researchers to study gene expression changes on a much larger scale as genetic susceptibility has been observed to play an important role in several infectious diseases (Chapman and Hill 2012). Therefore, in this chapter DNA microarray technology was used to identify candidate genes differentially expressed during DENV infection and to extract biological information from enriched gene clusters.

DENV infection has induced differential gene expression in LSECs with 193 upregulated and 162 downregulated genes. A number of gene were identified as candidates to play an important role in DENV infection. These include OAS1, OAS2, OAS3, MX2 and XAF1 genes. The OAS gene family includes IFN-induced antiviral enzymes that are important in the antiviral action of IFN. We found higher expression of OAS1, OAS2 and OAS3 genes in DENV-infected LSECs. OAS family members become activated in the presence of double stranded or single stranded RNA with secondary structures to catalyse synthesis of 2′-5′ linked oligoadenylate from ATP. This 2′-5′ oligoadenylate can activate ribonuclease L (RNase L) to degrade viral RNA within infected cells (Kristiansen et al. 2011; Malathi et al. 2005). Gene expression studies on DENV-infected HUVECs also
showed upregulated levels of OAS including OAS2 and OAS3 (Dalrymple and Mackow 2012) (Warke et al. 2003). In addition, an association between clinical outcome in DENV infection and polymorphisms in the OAS1, OAS2 and OAS3 genes has been reported: OAS2-OAS3 and OAS3-OAS2 haplotypes, may be related to increased OAS activity, while OAS2-OAS3 and OAS1-OAS3-OAS2 haplotypes led to reduced OAS activity and increased DENV replication, thereby increasing the risk to develop DHF (Alagarasu et al. 2013). In another study the p42/p46 isoforms of OAS1 and p100 isoform of OAS3 were found to contribute to host defence against DENV infection by blocking DENV replication in an RNase L dependent manner (Lin et al. 2009). A number of studies have been performed over the years to study antiviral effects of the OAS family, with the identification of several different mechanism involved in controlling viral replication in mammalian cells (Kristiansen et al. 2011). Further understanding of the mechanisms behind the association of OAS gene family members with DENV replication may be useful in designing OAS based therapeutics for DENV infection.

MX2 protein is another innate immune effector molecule used by mammalian cells. Higher levels of MX2 gene has been identified in a group of Vietnamese dengue patients with DSS (Simmons et al. 2007). MX2 is reported to be a potent inhibitor of HIV-1 infection as it blocks infection at a post entry point by suppressing viral complementary DNA (Goujon et al. 2013). A study on innate antiviral immunity against DENV infection in a double-deficient IRF-3−/−7−/− mouse model showed that MX2 can be induced via an IRF-3 and IRF-7 independent pathway that does not involve IFN-γ signalling for protection against DENV. As combined absence of IRF-3 and IRF-7 protected from the induction of DENV infection, these results revealed that an IRF-3/IRF-7 independent pathway may be contributing to antiviral immunity against DENV infection (Chen et al.
We found increased MX2 gene expression in DENV-infected LSECs which may be a factor contributing to the low virus replication in LSECs. Thus, further analysis of the role of MX2 in DENV infection will help to elucidate the role of this important host defence pathway against DENV.

In DENV-infected LSEC, XAF1 gene was another prominently expressed interferon inducible gene. DENV infection of HUVECs and Ea.hy926 cells (human umbilical vein cell line) induced XAF1 protein production and XAF1 gene expression in HUVECs, suggesting that XAF1 contributes to DENV induced apoptosis of HUVECs in a XIAP dependent manner (Long et al. 2013). As discussed in chapter 3, DENV infection caused apoptosis of LSECs at 48 hpi and XAF1 may be involved in inducing apoptosis in LSECs. Interestingly, functional analysis of related gene sets showed expression of ‘apoptosis gene set’ was downregulated. This ‘apoptosis gene set – Reactome pathway’ contain 149 genes involved in apoptosis excluding XAF1. Further XAF1 gene is considered under three ‘gene set – Reactome pathways’ – cytokine signalling in immune system, interferon signalling and interferon alpha/beta signalling. Absence of enrichment for apoptosis pathways again emphasise possible mechanism of XAF1 involvement in mediating apoptotic function in LSCEs.

In hCMEC/D3s infected with DENV, differential gene expression analysis by Limma package identified 256 upregulated and 186 downregulated genes compared to mock controls. Within this we identified several interesting differentially expressed genes - miRNA-155, IL-1RN, CXCL5, IFI6, IL21R and IL17RC, of potential importance in DENV infection.

DENV infection significantly enhanced the expression levels of microRNA (miRNAs)-155 in hCMEC/D3s. The miRNAs are a class of small, regulatory non coding RNAs and
that function through binding to the 3’-UTR of a target mRNA to induce degradation and suppress translation (Wu et al. 2013). miRNAs play important roles in modulation of immune responses and regulation of virus-host interactions (Pareek et al. 2014) and can regulate viral replication either positively (proviral) or negatively (antiviral) (Wu et al. 2013). Insertion of target sequences of miRNAs including miR-122, miR-124a, miR-128a, miR-142, miR-218 or let-7c, into the 3-UTR of flavivirus genome has been found to restrict virus replication. Pham et al. showed that insertion of the miR-142 target site into the DENV genome restricted replication of virus in hemotopoietic cells (Pham et al. 2012). The exact biological function of miR-155 during viral infection remains unknown. Upon vesicular stomatitis virus infection of macrophages, markedly elevated miR-155 levels regulate the antiviral innate immune response by promoting type I IFN signalling via SOCS1 (Wang et al. 2010). Similarly, over expression of miR-155 in activated macrophages exerts an anti-HIV-1 effect by targeting HIV-1 dependency factors involved in post-entry, pre-integration levels, leading to a significant suppression of HIV-1 infection (Swaminathan et al. 2012). Infection of human microglial cells by the Flavivirus Japanese encephalitis virus (JEV) led to induction of miR-155 and restriction of JEV replication through negative modulation of innate immune responses (Pareek et al. 2014). Thus, miR-155 exerts different antiviral mechanisms depending on the virus and the environmental stimulation generated by the particular virus in a certain cell type (Pareek et al. 2014). Over expression of miR-155 may be a factor contributing to the limited DENV replication, or a substantial reduction in susceptibility to infection of hCMEC/D3s, and further functional studies will be needed to gain insight into the potential mechanisms of this inhibition. In addition, it will be of interest to investigate miR-155 as a target for the prevention and treatment of DENV infection.
DENV infection of hCMEC/D3 cells induced IL-1 receptor antagonist gene (IL-1RN). IL-1RN encodes for IL-1 receptor antagonist (IL-1Ra) protein, that is produced in two isoforms: secreted IL-1Ra (sIL-1Ra) and intracellular IL-1Ra (icIL-1Ra). sIL-1Ra is actively released from cells while icIL-1Ra retained in the cytoplasm (Dewberry et al. 2000; Redlitz et al. 2004). IL-1Ra is considered as the naturally occurring inhibitor of IL-1 as it binds with both IL-1 receptor type I and type II, hindering signalling. IL-1 is important in initiating inflammatory reactions and stimulation and activation of endothelial cells to induce expression of adhesion molecules, which promote monocyte recruitment. IL-1RN gene was found to be associated with DSS in a Vietnamese patient population (Simmons et al. 2007). Increased circulating IL-1Ra levels in dengue patients were associated with antipyretic actions to counteract increased concentrations of IL-1β (de-Oliveira-Pinto et al. 2012). The ratio of IL-1β to IL-Ra related to the severity of sepsis and septic shock syndrome. In DENV infection IL-1β is considered to be important due to its biological effects on the central nervous system and on endothelial permeability changes. Studies of the contribution of IL-1β and IL-1Ra to DENV disease severity are limited. A study to investigate association between polymorphisms of IL-1β and IL-1Ra with DSS in a Thai population showed, that IL1β −31C , or IL-1Ra 2/4 independently carry risk for DSS (Sa-Ngasang et al. 2014).

DENV infection of hCMEC/D3s downregulated IFI6 gene expression at 48 hpi. IFI6, belonging to the IFN-induced gene family, is involved in immune response. DENV infection induced IFI6 gene expression in HUVECs and Ea.hy926 cells (Huang et al. 2014; Long et al. 2013). It acts as an antiapoptotic factor by delaying type I IFN-induced apoptosis in cells and was shown to inhibit apoptosis in DENV-infected Ea.hy926 cells (Huang et al. 2014). IFI6 also has more targeted antiviral specificity against the flavivirus
YFV (Schoggins et al. 2011), which suggests that IFI6 may also have antiviral activity against DENV. Thus downregulated IFI6 expression in hCMEC/D3s may facilitate viral survival.

DENV infection of hCMEC/D3s led to reduced expression of IL21R gene. IL21R gene encodes a cytokine receptor for IL21. The IL21 receptor was discovered in 2000 and was of great interest as it is made up of a common cytokine receptor γ chain like IL-2, IL-4, IL-7, IL-9 and IL-15 (Spolski and Leonard 2005). A protective role of IL-21 has been demonstrated in chronic viral infections such as hepatitis C virus infection in humans (Feng et al. 2013) and lymphocytic choriomeningitis virus (LCMV) infection in rodents (Elsaesser et al. 2009). The role of IL21 in acute viral infections such as DENV infection is not clearly understood. One study showed that higher levels of IL21 are produced by DENV-infected patients during primary and secondary infections and are associated with production of DENV-specific IgM and IgG antibodies, which may play a protective role during infection (Vivanco-Cid et al. 2014). Although IL21 receptor is expressed on vascular endothelial cells, an effect of IL21 cytokine on endothelial activation or vascular leakage has not been reported.

Functional analysis of LSECs gene clusters revealed that IFN α related genes were upregulated in DENV-infected cultures. In virus infected cells, type I interferon response occurs during viral entry and, viral component synthesis and release. Synthesised IFNs bind with IFNα receptor and activate the JAK/STAT pathway. More than 100 IFN stimulated genes are induced and provide an antiviral role (Green et al. 2014; Navarro-Sánchez et al. 2005). Pre-treatment of human cells with IFNs can protect those cells from DENV infection although post treatment has not been able to inhibit DENV replication. Although multiple IFN inhibition mechanisms have been reported with other virus
including HCV and pestiviruses the mechanisms used by DENV remain unclear (Green et al. 2014; Muñoz-Jordán et al. 2003). Further Dalrymple and Mackow (2012) showed innate IFN and ISG responses are highly induced at 24 to 48 hpi in HUVECs and interestingly demonstrated that DENV-infected HUVECs limit viral spread by secreting IFN-β (Dalrymple and Mackow 2012). Therefore, the IFN α related gene network generated for DENV-infected LSECs provides additional information on IFN system during DENV infection and future studies are required to determine how different IFN responses inhibit DENV spread in endothelial cells.

In LSEC IFN α network, ubiquitin specific peptidase 18 (USP18: also known as UBP43) gene was identified to interact with ISG15 and IRF7, USP18 gene encodes for a protein in the UBP family that cleaves ubiquitin from ubiquitinated protein substrates. Upregulation of USP18 gene in DENV-infected HUVECs, monocytes and B cells has been previously reported (Warke et al. 2008). USP18 deficient mice were shown to be resistant to fatal lymphocytic choriomeningitis virus (LCMV) and vesicular stomatitis virus (VSV) infections. The enhanced IFN-mediated response detected in USP18 knockout mice decreased viral replication and viral antigen expression. Further USP18 has been identified as an ISG15 deconjugating protease (Rosario et al. 2004). Another group showed that USP18 binds the IFN α receptor subunit and blocks JAK1 interaction with the receptor leading to JAK1 activity inhibition (Fuchs et al. 2006). These studies showed that USP18 functions as a negative regulator of IFN mediated responses and, thus, inhibition of USP18 can enhance the protective role of IFN. Therefore, USP18 may be a potential target for antiviral therapies.

In this chapter, the gene expression of LSECs and hCMEC/D3s revealed many differentially expressed genes, gene clusters and host response pathways, that were
upregulated during DENV infection. Specific genes regulated in response to DENV, such as miR-155, USP18 and OAS genes have been implicated in DENV infection of microvascular endothelial cells for the first time. Further functional analysis of these genes with respect to host response pathways will help to understand key pathogenic mechanisms in DENV infection. In addition, the molecular mechanisms surrounding the activation of USP18 in LSECs, and miR155 in hCMEC/D3s, will need to be further examined to understand their role in DENV infection and potential as antiviral targets.
Figure 6.1 Total RNA expression in DENV-infected endothelial cells

Microvascular endothelial cell lines LSECs and hCMEC/D3s were seeded in 24 well plates. After 24 hours cells were infected with DENV at MOI 1 or mock control with culture medium alone. At 48 hpi total RNA was extracted and quality was determined. (A) Agilent Bioanalyzer electropherograms of RNA isolated from LSECs and hCMEC/D3s showing peaks for the 18S and 28S ribosomal RNA. (B) Agarose gel electrophoresis displays the same high-quality total RNA preparation by demonstrating sharp bands for the 28S and 18S ribosomal RNA.
Figure 6.2 Microarray dataset quality assessment

The arrayQualityMetrics package was used to detect outliers in microarray dataset. (A) A heatmap of the distances between arrays was generated to indicate clustering of arrays due to biological or experimental factors. The colour scale is chosen to cover the range of distances encountered in the dataset. (B) Outlier detection was performed by the signal intensity distributions of the arrays. (C) Quantile normalisation was used to normalise LSEC and hCMEC/D3 microarray dataset.
Microvascular endothelial cell line LSECs were infected with DENV at MOI 1 or mock control with culture medium alone. At 48 hpi total RNA was extracted and gene expression was determined by Illumina human HT 12 Expression Beadchip. (A) The volcano plot showing the association between the p-values and the \( \log_2 \) of the fold change. An absolute fold change greater than two and a p-value less than 0.05 in DENV-infected LSECs compared to mock control was considered significant. Colours indicate red for upregulated, green for downregulated and black for no change. (B) Heat map of the microarray gene expression profile of DENV-infected LSECs. Colour intensity is derived from mean relative expression fold changes and colours indicate red for upregulated, green for downregulated and black for no change.
Figure 6.4 Identification of differentially expressed genes in DENV-infected hCMEC/D3s

Microvascular endothelial cell line hCMEC/D3s were infected with DENV at MOI 1 or mock control with culture medium alone. At 48 hpi total RNA was extracted and gene expression was determined by Illumina human HT 12 Expression Beadchip. (A) The volcano plot showing the association between the p-values and the log\(_2\) of the fold change. An absolute fold change greater than two and a p-value less than 0.05 in DENV-infected hCMEC/D3s compared to mock control was considered significant. Colours indicate red for upregulated, green for downregulated and black for no change. (B) Heat map of the microarray gene expression profile of DENV-infected hCMEC/D3s. Colour intensity is derived from mean relative expression fold changes and colours indicate red for upregulated, green for downregulated and black for no change.
Figure 6.5 Differentially expressed genes in DENV-infected LSECs

Microvascular endothelial cell line LSECs were infected with DENV at MOI 1 or mock control with culture medium alone. At 48 hpi total RNA was extracted and gene expression was determined by Illumina human HT 12 Expression Beadchip. Differentially expressed genes in DENV-infected cultures compared to mock control were identified by bioconductor R package “Limma” analysis. Gene expression of (A) IFI44L (B) IL1A (C) IRF7 (D) MX2 (E) OAS1 (F) OAS2 and (G) OAS3 are shown.
(f) OAS1

P < 0.0001
1.7-fold higher

(g) OAS2

P < 0.0001
1.6-fold higher

(h) OAS3

P < 0.0001
1.4-fold higher
Figure 6.6 Differentially expressed genes in DENV-infected hCMEC/D3s

Microvascular endothelial cell line hCMEC/D3 were infected with DENV at MOI 1 or mock control with culture medium alone. At 48 hpi total RNA was extracted and gene expression was determined by Illumina human HT 12 Expression Beadchip. Differentially expressed genes in DENV-infected cultures compared to mock control were identified by bioconductor R package “Limma” analysis. Gene expression of (A) miR-155 (B) IL1RN (C) IFI6 (D) CXCL5 (E) IL17RC and (F) IL21R are shown.
Figure 6.7 Enrichment map for DENV-infected LSECs

Microvascular endothelial cell line LSECs were infected with DENV at MOI 1 or mock control with culture medium alone. At 48 hpi total RNA was extracted and gene expression was determined by microarray analysis. The functional enrichment analysis was performed by ClueGO, Cytoscape plug-in. Each gene set is represented by a node with different size which is proportional to the number of genes. The connecting lines represents the percentage of overlap and its thickness represents the percentage of overlapping. Red nodes represent upregulated gene sets and green nodes represent downregulated gene sets. Labels for the clusters were manually assigned: 1) Interferon response 2) Virus response 3) Endothelial cell migration 4) Biosynthetic process.
Figure 6.8 Enrichment map for DENV-infected hCMEC/D3s

Microvascular hCMEC/D3s were infected with DENV at MOI 1 or mock control with culture medium alone. At 48 hpi total RNA was extracted and gene expression was determined by microarray analysis. The functional enrichment analysis was performed by ClueGO, Cytoscape plug-in. Each gene set is represented by a node with different size which is proportional to the number of genes. The connecting lines represents the percentage of overlap and its thickness represents the percentage of overlapping. Red nodes represent upregulated gene sets and green nodes represent downregulated gene sets.
**Figure 6.9 Functional gene sets in DENV-infected LSECs**

GSEA analysis of DENV-infected LSECs compared to mock control is shown. The enrichment score reflects the degree to which gene set in a certain pathway is overrepresented at the top (upregulated) or bottom (downregulated) of the rank gene list.
Figure 6.10 Functional gene sets in DENV-infected hCMEC/D3s

GSEA analysis of DENV-infected hCMEC/D3s compared to mock control is shown. The enrichment score reflects the degree to which gene set in a certain pathway is overrepresented at the top (upregulated) or bottom (downregulated) of the rank gene list.
Figure 6.11 Functional linkage network of IFN α response to DENV infection
<table>
<thead>
<tr>
<th>Sample</th>
<th>RNA Concentration (ng/µl)</th>
<th>rRNA ratio (28s/18s)</th>
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<tr>
<td>LSEC mock control 1</td>
<td>487</td>
<td>2.5</td>
</tr>
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<td>LSEC mock control 2</td>
<td>389</td>
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<td>LSEC mock control 3</td>
<td>299</td>
<td>2.5</td>
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<tr>
<td>LSEC DEN-infected 1</td>
<td>473</td>
<td>2.5</td>
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<tr>
<td>LSEC DEN-infected 2</td>
<td>452</td>
<td>2.6</td>
</tr>
<tr>
<td>LSEC DEN-infected 3</td>
<td>383</td>
<td>2.5</td>
</tr>
<tr>
<td>hCMEC/D3 mock control 1</td>
<td>257</td>
<td>2.6</td>
</tr>
<tr>
<td>hCMEC/D3 mock control 2</td>
<td>241</td>
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<tr>
<td>hCMEC/D3 mock control 3</td>
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<td>2.8</td>
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<tr>
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<td>2.5</td>
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<td>135</td>
<td>2.6</td>
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<tr>
<td>hCMEC/D3 DENV-infected 3</td>
<td>334</td>
<td>2.6</td>
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**Table 6.2:** Microarray data filter

<table>
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<tr>
<th>Probes</th>
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<th>hCMEC/D3 samples</th>
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<tr>
<td>Discarded</td>
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<td>25,773</td>
</tr>
<tr>
<td>Passed</td>
<td>23,170</td>
<td>21,543</td>
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Chapter VII

General Discussion
7.1 Discussion

DENV has rapidly expanded its range and has become a global economic and health threat. It is endemic or epidemic in almost all tropical countries, with approximately half of the world's population residing in DENV-endemic regions (Guzman et al. 2010). More than 50 million new infections are estimated to occur annually (Simmons et al. 2012). Symptomatic DENV infection can present with a wide range of clinical manifestations, from mild febrile illness to severe life threatening DHF/DSS. Severe DHF/DSS conditions are clinically challenging, as no vaccines or antiviral drug treatments are available. A key observation in the diagnosis of DHF/DSS is sudden and transient vascular leakage, which rapidly worsens the patient’s condition (Whitehead et al. 2007). The vascular endothelium has therefore become the focus of DENV studies, and research into the role of endothelial cells during DENV infection has increased over the years. In spite of a large number of studies, the precise responses of endothelial cells to DENV infection, particularly in the natural target organs of DENV, are not clearly understood.

This thesis will contribute two major findings to DENV research. Firstly, it identifies a microvascular endothelial cell type from an organ naturally targeted by DENV that can be used to characterise the endothelial cell response to DENV infection. Secondly, in-depth studies on the gene expression patterns of the host cell will contribute to the identification of host genetic factors that are potential antiviral targets in cases of DENV infection.

The receptiveness of human endothelial cells to DENV infection and the subsequent effects of infection are subject to debate, mainly because of the variation in endothelial cell types used in research. As described in Chapter One, the use of macrovascular
endothelial cells, as well as microvascular endothelial cells from organs not targeted by DENV, has made it difficult to draw conclusions about DENV infection of endothelial cells. Consequently, there is a need for a more physiologically relevant model to study DENV infection. Accordingly, this study has characterised DENV infection in microvascular endothelial cell types from the human liver and brain, which are target organs of DENV infection. In Chapter Three, LSECs and hCMEC/D3s were shown to be permissive to DENV in varying degrees, which may be due to vascular bed-specific differences in the structure and function of the capillary endothelium in different organs (Peyrefitte et al. 2006).

DENV infectivity levels obtained for LSECs in this study were around 10%, in accordance with an earlier study by Peyrefitte et al. (Peyrefitte et al. 2006). Many studies have reported low levels of DENV infectivity in endothelial cells (Arevalo et al. 2009; Diamond et al. 2000; Warke et al. 2003), although a recent study showed that more than 80% of HUVECs can be infected with DENV when heparin is removed from the culture medium (Dalrymple and Mackow 2012). In Chapter Six, the interferon response pathway was identified as upregulated in DENV-infected LSECs. Upregulation of many interferon-stimulating genes such as OAS1, OAS2, OAS3 and MX2 may have antiviral effects that control DENV replication, and may contribute to limit DENV infection, resulting in the low infectivity levels seen in LSECs. A recent study also showed that DENV2 was able to productively infect a small percentage of primary endothelial cells and to induce IFN-β driven innate immune responses that may have limited the further infection of endothelial cells (Calvert et al. 2015).

In Chapter Three, DENV was shown to induce apoptosis in more than half of the LSEC population. Even though only a low level of LSECs were permissive to DENV infection,
apoptosis caused damage to the LSEC monolayer, with the formation of gaps in intact monolayers leading to increased permeability. DENV-induced apoptosis in different endothelial cells such as HMEC-1, ECV-304, and HUVECs has been previously reported (Avirutnan et al. 1998; Cardier et al. 2005; Lin et al. 2002). Gene expression analysis of LSECs, as discussed in Chapter Six, further revealed that XAF1 gene expression was upregulated in infected cells. It was shown that upregulation of XAF1 expression led to apoptosis of HUVECs in an XIAP dependent manner (Long et al. 2013). An association between DENV-induced apoptosis and vascular leakage has been reported previously. In a dengue haemorrhage mouse model, DENV-induced apoptosis of endothelial cells was responsible for the development of haemorrhage in mice (Lin et al. 2014). Additionally, in DHF patients, hepatic injury by DENV infection was shown to occur primarily through apoptotic destruction of hepatocytes (Pagliari et al. 2014). This study has shown that microvascular endothelial cells from the human liver can be induced by DENV to undergo apoptosis; and that apoptosis may have a role in the pathogenesis of hepatic injury in DHF patients. As DENV may cause mild to severe liver dysfunction leading to hepatic encephalopathy during severe DHF (Lima Mda et al. 2011; Nguyen et al. 1997), further investigation into the association between apoptosis and disease pathology in the liver during DENV infection should be the focus of future research.

In addition to apoptosis, increased levels of vasoactive factors have also been postulated to increase endothelial permeability leading to vascular leakage (Malavige et al. 2012). As shown in Chapter Four, DENV infection induced LSECs to produce higher levels of cytokines, chemokines and vascular growth factors. Therefore, further studies were performed (Chapter Five) using LSEC and hCMEC/D3 cell models to address whether vasoactive factors produced by DENV-infected cells have an effect on endothelial
permeability. DENV infection caused apoptosis of LSECs at 48 hpi, which can damage the intact monolayer and affect permeability. This may result in a misleading positive result, wrongfully interpreting the increase in permeability as an effect of soluble mediators. To determine permeability alterations on LSEC monolayers, UV-inactivated supernatants were used to exclude the effects of apoptosis and direct DENV infection. Overproduction of IL-6, CXCL8, CCL11 and CXCL10 by LSECs is thought to mediate alterations in permeability in LSEC monolayers (Ferreira et al. 2015; Huang et al. 2000; Jamaluddin et al. 2009; Talavera et al. 2004; Vervaeke et al. 2015). Specifically, CXCL8 is responsible for tight junctions and cytoskeletal reorganisation of endothelial cells, leading to vascular leakage (Peyrefitte et al. 2006; Talavera et al. 2004). IL-6 also alters tight junction distribution in endothelial cells and cell morphology to increase endothelial permeability in HUVEC monolayers (Desai et al. 2002). Chapter Five, however, found that supernatants from DENV-infected LSECs did not alter the permeability of LSEC monolayers, regardless of higher IL-6 and CXCL8 levels being present. On the other hand, DENV-infected MDM supernatants caused a transient permeability increase in LSEC monolayers. DENV-infected LSEC supernatants contained higher levels of IL-6, IL-1β, TNF-α, CCL3, CCL4, CCL2, CCL11 and CXCL10, whereas DENV-infected MDM supernatants contained elevated levels of TNF-α, IL-6, CCL2 and IL-1β. When comparing infected supernatants from the two cell types, it is clear that LSEC supernatants also contained the factors that were detected in MDM supernatants. Therefore the transient leakage observed in LSEC monolayers is mediated by unique factors present in DENV-infected MDM supernatants and is not primarily caused by TNF-α, IL-6, IL-1β or CCL2.
Chapter Three showed very low levels of infectivity and replication of DENV in hCMEC/D3s. Permissiveness of DENV to hCMEC/D3 cells has not been previously reported. In Chapter Six, gene expression analysis identified the miR-155 gene as differentially expressed in DENV-infected hCMEC/D3s compared to mock controls. The miR-155 gene has been reported to suppress infection or to limit virus replication in other viral infections such as JEV and HIV-1 (Pareek et al. 2014; Swaminathan et al. 2012). Thus it may also be a factor responsible for the limited DENV replication seen in hCMEC/D3s. The low level of infection in hCMEC/D3s did not cause cells to undergo apoptosis as seen with other endothelial cells.

Interestingly, a low level of DENV infection of hCMEC/D3s induced enhanced production of soluble immunomediators such as IL-6, IL-1β, TNF-α, CXCL8, CCL4, and G-CSF. As many of these factors are reported to induce endothelial permeability, as discussed in Chapter Four, hCMEC/D3 supernatants were assessed for their ability to induce permeability changes in hCMEC/D3 monolayers. Neither DENV-infected hCMEC/D3 nor MDM supernatants altered monolayer permeability of the hCMEC/D3 monolayer.

Brain endothelium displays highly restricted permeability largely attributed to the tight junctions. The hCMEC/D3 cell line displays a stable and physiologically typical endothelial phenotype, and maintains most of the unique structural and biochemical properties of brain endothelium in vivo (Weksler et al. 2005). The inability of MDM supernatants to induce a transient monolayer dysfunction or alterations in permeability, as determined in Chapter Five, may be due to the tight junction organisation maintained by hCMEC/D3 monolayers (Weksler et al. 2005).
The increased endothelial permeability induced by MDM cell supernatants that was observed with LSEC monolayers, but not with hCMEC/D3 monolayers, is consistent with comparisons of liver and brain endothelial cells reported in the literature. Compared to liver endothelial cells, far higher expression levels of tight junction proteins are found in brain endothelial cells, whereas liver sinusoidal capillaries are among the leakiest vessels in the body (Aird 2007; Daneman et al. 2010).

The findings of Chapter Five show for the first time that DENV-infected MDM supernatants can induce a transient permeability increase in microvascular endothelial cells derived from human liver-LSECs. Additionally, the findings of Chapter Five on endothelial monolayer permeability suggest that different microvascular endothelial cells respond differently to DENV infection. The same treatment of DENV-infected MDM culture supernatants caused a transient permeability increase in the LSEC monolayer with no changes to the hCMEC/D3 monolayer. This shows the importance of the use of microvascular endothelial cells from naturally targeted organs of DENV, as the phenotypic heterogeneity of the endothelium will have different responses to DENV infection. It is therefore important to use an endothelial cell type that closely resembles natural DENV infection to study the underlying mechanisms of DENV infection.

The major findings in Chapter Six suggest that host cell gene expression may play a major role in the pathogenesis in DENV infection. In DENV-infected LSECs, the differentially expressed genes identified may be important in therapeutics for DENV infection. Although an association of OAS family members and DENV infection has been shown in previous studies (Alagarasu et al. 2013; Lin et al. 2009), their exact role during DENV infection and their capacity to control DENV replication remain to be understood. MX2 was another gene identified as differentially expressed in DENV-infected LSECs that
may contribute to the low virus replication in LSECs. A possible role for MX2 in antiviral immunity to DENV infection has proposed by Chen et al. (Chen et al. 2013), although this has not yet been tested. Furthermore, the USP18 gene was identified as a potential antiviral target in the IFN-α gene response network analysis. USP18 functions as a negative regulator of IFN-mediated responses (Fuchs et al. 2006; Rosario et al. 2004) and thus inhibition of USP18 may have a protective role against DENV infection. More importantly, the contribution of type I interferon to virus-induced vascular damage was reported in a recent study using variants of lymphocytic choriomeningitis virus in mice (Baccala et al. 2014). Even though IFN-I has been thought to have antiviral effects that protect the host against viral infections, recent studies have shown that these factors contribute to viral disease pathology (Teijaro et al. 2013; Trinchieri 2010). More importantly, a recent study on primary endothelial cells infected with DENV serotype 2 showed that IFN-β driven innate immune responses induced by infection can limit infection and further alter endothelial cell functions (Calvert et al. 2015). In DENV-infected endothelial cells, Calvert et al. report an induction of IFN-β, OAS1, IFIT-1/ISG56, viperin mRNA, total STAT1 protein and STAT1-S727 and Y701 phosphorylation. Antibody blocking of IFN-β reduced OAS1, IFIT-1/ISG56, and viperin mRNA, and led to an increase in DENV RNA production and the number of ds-RNA-containing cells. Our data also shows a strong induction of innate immune response in DENV-infected LSECs; further, it provides information on IFN-α related genes that have been upregulated during DENV infection. Therefore, understanding the molecular mechanisms is critical to identifying its role during DENV infection.

Finally, identification of the upregulated miR-155 gene in DENV-infected hCMEC/D3s is important, as it exerts antiviral mechanisms in different viral infections, including JEV
infection, HIV-1 infection and vesicular stomatitis infection (Pareek et al. 2014; Swaminathan et al. 2012; Wang et al. 2010). In other flaviviruses like JEV, miR-155 negatively regulates innate immune responses to restrict JEV replication (Pareek et al. 2014). Higher levels of miR-155 determined in hCMEC/D3s may have contributed to limited DENV replication in hCMEC/D3s; further investigation into the potential mechanisms of this inhibition will be interesting as a target for the prevention and treatment of DENV infection.

Finally, this study has identified physiologically relevant in vitro microvascular endothelial cell models for the study of DENV infection. LSECs provide a model close to natural DENV infection, in which apoptosis and transient monolayer damage are responsible for permeability alterations, making it a suitable model to study DENV-induced permeability alterations in future. Concurrent use of the hCMEC/D3 model supports the characterisation of specific responses of the microvascular endothelial cell type to DENV infection. Further, analysis of gene expression of endothelial cells in this study has highlighted the relevance of host genetic regulation of DENV infection.
7.2 Conclusion and Future Directions

This is the first study to show that human microvascular endothelial cells from the liver alter their permeability in response to local DENV infection. DENV-induced apoptosis is one factor that is responsible for permeability alterations. Additionally, enhanced production of soluble mediators by DENV-infected endothelial cells causes activation of endothelial cells, as shown by increased expression of CAMs, although it does not alter permeability in endothelial monolayers. On the other hand, MDM supernatants contain vasoactive factors that cause transient permeability changes in LSEC monolayers. Therefore, this study suggests that both apoptosis of endothelial cells and vasoactive factors produced by cells of monocytic lineage are responsible for transient vascular leakage during DENV infection. However as LSEC supernatants also contained the factors that were detected in MDM supernatants, transient leakage observed in LSEC monolayers may be mediated by unique factors present in DENV-infected MDM supernatants. To confirm that elevated levels of TNF-α, IL-6, CCL2 and IL-1β present in MDM supernatants were not involved in altered permeability in LSEC monolayers, future continuation of this work will involve the examination of MDM supernatants, including use of neutralise antibodies against these factors to confirm inhibition of induction of LSEC permeability by MDM supernatants.

Analysis of host cell gene expression found new antiviral targets such as OAS family members, USP18 and miR-155 genes. Future extension of this work involves examination of how these differentially expressed genes are involved in DENV infection and their potential use in drug development. Emerging evidence has suggested a role for vascular endothelial participation in the innate immune response to viral infections, which
could be a potential therapeutic target for vascular leakage (Baccala et al. 2014; Calvert et al. 2015). Our findings also demonstrated an involvement of type I interferon signalling pathways during DENV infection of endothelial cells which may have important implications for the development of DENV infection therapeutics. Therefore, future functional analysis studies are needed on antiviral target genes this study has identified in the type I interferon pathway and which may contribute to finding therapeutics for vascular leakage.
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Shresta, S., Kyle, J. L., Snider, H. M., Basavapatna, M., Beatty, P. R. & Harris, E. 2004. Interferon-dependent immunity is essential for resistance to primary dengue virus infection in mice, whereas T- and B-cell-dependent immunity are less critical. Journal of virology, 78, 2701-2710.


APPENDIX
**Table: Preparation of tissue culture media**

<table>
<thead>
<tr>
<th>Tissue culture media</th>
<th>Preparation</th>
<th>Supplier</th>
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<tbody>
<tr>
<td>OptiMEM growth media with 10% FCS</td>
<td>Add 50 mL of heat-inactivated FCS (HIFCS) to 450 mL of OptiMEM</td>
<td>Life Technologies, AU</td>
</tr>
<tr>
<td>DMEM growth media with 10% FCS</td>
<td>Add 50 mL of HIFCS to 450 mL of DMEM</td>
<td>Life Technologies, AU</td>
</tr>
<tr>
<td>DMEM maintenance media with 2% FCS</td>
<td>Add 10 mL of HIFCS to 490 mL of DMEM medium</td>
<td>Life Technologies, AU</td>
</tr>
<tr>
<td>EBM-2 growth medium with 5% FCS</td>
<td>Add 25 mL of HIFCS, 5 ml of penicillin-streptomycin (Penicillin,10000 units-Streptomycin, 10000μg.ml⁻¹, Gibco/Invitrogen, 15140122), 1.4 mM hydrocortisone (Sigma-Aldrich, cat no: H0135), 5 μg.mL⁻¹ acid ascorbic acid (Sigma-Aldrich, A4544), 1% chemically defined lipid concentrate (Gibco/Invitrogen, 11905031), 10 mM of N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic acid (HEPES) and 1 ng.mL⁻¹ of fibroblast growth factor-basic human (bFGF) (Sigma-Aldrich, F0291) to a final volume of 500 mL of EBM-2</td>
<td>Lonza, AU</td>
</tr>
<tr>
<td>EBM-2 maintenance medium with 2.5% FCS</td>
<td>As described for EBM-2 growth medium except addition of 12.5 mL of HIFCS instead of 25 mL of HIFCS to a final volume of 500 mL of EBM-2</td>
<td>Lonza, AU</td>
</tr>
<tr>
<td>Leibovitz's L-15 growth medium with 10% FCS</td>
<td>Add 50 mL of HIFCS to 450 mL of Leibovitz's L-15 medium.</td>
<td>Life Technologies, AU</td>
</tr>
<tr>
<td>Leibovitz's L-15 maintenance medium with 5% FCS</td>
<td>Add 25 mL of HIFCS to 475 mL of Leibovitz's L-15 medium</td>
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### Table: Instruments used in this study

<table>
<thead>
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<th>Instrument</th>
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<td>Allegra™ X-22R Benchtop Centrifuge</td>
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<td>Allegra® X-15R Benchtop Centrifuge</td>
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<td>Analytical Balances</td>
<td>METTLER TOLEDO</td>
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<td>Benchtop 5075 EL Sterilizer</td>
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<td>CFX96™ Real-Time PCR Detection System</td>
<td>Bio-Rad</td>
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<td>CFX96 Touch™ Real-Time PCR Detection System</td>
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<tr>
<td>System</td>
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<td>Compact Digimage System</td>
<td>Major Science</td>
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<td>Liquid tank LS6000</td>
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<td>Magnetic-Stirrer- RCT basic</td>
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<td>Microfuge 22R centrifuge</td>
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<td>NanoDrop 2000c UV-Vis Spectrophotometer</td>
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<td>pH meter - Seven Easy S20</td>
<td>METTLER TOLEDO</td>
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<td>Rotofix 32a benchtop centrifuge</td>
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<td>Self-contained ice flakers ice machine AF200</td>
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<td>Sensor Microwave Oven</td>
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<td>Spectrafuge™ Mini Laboratory Centrifuge</td>
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<tr>
<td>WiseBath® WB Digital Precise Water Bath</td>
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<tr>
<td>xMark Microplate Spectrophotometer</td>
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<tr>
<td>X-Ray Viewing box</td>
<td>Dalcross</td>
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<td>Primer</td>
<td>Sequence</td>
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<td>ICAM-1</td>
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<td></td>
<td><strong>Reverse:</strong> AACCCCATTCAGCGTCA</td>
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<tr>
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<td><strong>Forward:</strong> CCGGATTGCTGCTCAGATGGGA</td>
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<td></td>
<td><strong>Reverse:</strong> AGCGTGGGATTGGTTCCCTCA</td>
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<td></td>
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<td></td>
<td><strong>Reverse:</strong> CTCTGACACGACCTCCATCC</td>
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<td></td>
<td><strong>Forward:</strong> GACTGGAGCCCTTTTCAGAG</td>
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</table>
Figure: Representative flow diagrams of DENV-infected LSECs

Mock control vs DENV-infected at different time points:
- **0 hrs**
- **12 Hrs**
- **24 hrs**
- **36 hrs**
- **48 hrs**

**7-AAD** (7-Aminoactinomycin D) and **PE Annexin V** (Phycoerythrin Annexin V) staining.
**Table:** Mean fluorescence intensity values from flow cytometry analysis of DENV induced LSEC apoptosis

<table>
<thead>
<tr>
<th>Time (hpi)</th>
<th>MFI of mock control samples</th>
<th>MFI of DENV infected samples</th>
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<tr>
<td>0</td>
<td>2.62</td>
<td>3.03</td>
</tr>
<tr>
<td></td>
<td>3.14</td>
<td>5.1</td>
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<td>12</td>
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<td>24</td>
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<tr>
<td></td>
<td>2.48</td>
<td>14.84</td>
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</table>

**Table:** Mean fluorescence intensity values from flow cytometry analysis of DENV induced hCMEC/D3 apoptosis

<table>
<thead>
<tr>
<th>Time (hpi)</th>
<th>MFI of mock control samples</th>
<th>MFI of DENV infected samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.56</td>
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<tr>
<td></td>
<td>1.36</td>
<td>2.14</td>
</tr>
<tr>
<td>12</td>
<td>0.72</td>
<td>0.88</td>
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<tr>
<td></td>
<td>0.71</td>
<td>0.93</td>
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<tr>
<td>24</td>
<td>0.72</td>
<td>0.91</td>
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<tr>
<td></td>
<td>0.93</td>
<td>1.05</td>
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<tr>
<td>36</td>
<td>0.75</td>
<td>0.65</td>
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<tr>
<td></td>
<td>0.65</td>
<td>0.95</td>
</tr>
<tr>
<td>48</td>
<td>0.9</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>0.88</td>
</tr>
</tbody>
</table>
Reagents

Constituents of media used in experiments
10× Phosphate buffered saline (PBS)

To make 1L:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount to add (for 10× stock)</th>
<th>Final concentration (10×)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>80 g</td>
<td>1.37 M</td>
</tr>
<tr>
<td>KCl</td>
<td>2 g</td>
<td>27 mM</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>14.4 g</td>
<td>100 mM</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2.4 g</td>
<td>18 mM</td>
</tr>
</tbody>
</table>

Dissolve the reagents listed above in 800 mL of distilled H₂O
Adjust the pH to 7.4 with HCl and then add H₂O to 1L
Dispense the solution into aliquots and sterilize them by autoclaving for 20 min at 121 °C
Store PBS at room temperature

2.5% Trypsin

To make 100 mL:

Dissolve 2.5 g trypsin (Sigma-Aldrich) in 100 mL of 1× PBS
Filter the solution using 0.22 µm syringe filter and dispense into 2 ml aliquots
Store at – 20 °C
**1% EDTA**

To make 100 mL:

Dissolve 1 g EDTA (Sigma-Aldrich) in 100 mL of 1× PBS

Sterilize solution by autoclaving for 20 min at 121 °C

Dispense into 2 ml aliquots and store at – 20 °C

**Trypsin/EDTA working solution**

Thaw 2 mL trypsin and 2 mL EDTA stocks and mix with 96 mL sterile 1× PBS

Store reconstituted solution at 4 °C

**0.4% Crystal violet stock solution**

Dissolve 2 g of crystal violet in 20 mL ethanol and filter to remove precipitate

Add 450 mg of NaCl and then add distilled H2O to 500 mL

**Crystal violet/ 3.7% Formaldehyde solution**

To make 200 mL:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount to add</th>
</tr>
</thead>
<tbody>
<tr>
<td>37% Formaldehyde</td>
<td>20 mL</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.18 g</td>
</tr>
<tr>
<td>0.4% Crystal violet stock solution</td>
<td>50 mL</td>
</tr>
</tbody>
</table>

Dissolve the reagents listed above in 200 mL of distilled H2O
1% Methylcellulose

To make 100 mL:

Heat 98 mL of 1X Opti-MEM at 65 °C for 5 minutes

Then add heated Opti-MEM to 1g of methylcellulose (Sigma-Aldrich)

Place the solution on rotator at room temperature for 30 minutes

Just before adding overlay to cells add 2ml of FCS