PYROPTOSIS: AN ALTERNATIVE PROGRAMMED CELL DEATH IN DENGUE VIRUS (DENV) INFECTION

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Pyroptosis: An Alternative Programmed Cell Death in Dengue Virus (DENV) Infection

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Certificate of Originality

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Cheung Ka Tik

Abstract

Dengue fever (DF) is a mosquito-borne disease caused by dengue virus (DENV), which is a member of the family Flaviviridae. DENV is categorised into five serotypes (DENV-1, -2, -3, -4 and -5). DF is a self-limiting illness; however, it can also progress to more severe forms, including Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS). DHF and DSS are severe febrile illnesses that can progress to hypovolemic shock with characteristic hemostatic abnormalities and increased capillary leakage. The outcome of DENV infection depends on the interactions between host cells and DENVs. The host cells are thought to respond to viral infection by initiation of cell death or secretion to the host of stimulating factors, like cytokines.

It has been reported that host cells undergo cell death in response to DENV infection. Apoptosis is perhaps the most widely recognised process of programmed cell death in DENV infection. However, other types of cell death exist, including autophagy, oncosis and caspase-1-dependent programmed cell death (also known as pyroptosis). These may also contribute to the mechanism of cell death in DENV infection. Given that pyroptosis can promote pathogen clearance by a more aggressive approach than silencing cell death (e.g., apoptosis and autophagy) in response to DENV, which in turn leads to inflammation, the mechanism by which this occurs in DENV is still not completely clear. In addition, one of the major explanations of the vascular leakage that occurs in DHF or DSS is endothelial cell (EC) death induced by DENV. Pyroptosis in DENV-infected ECs could offer an alternative explanation.

In this study, macrophages and ECs were used as *in-vitro* cell models to demonstrate that pyroptosis could be an alternative process of programmed cell death in DENV infection. We hypothesised that both macrophages and ECs would be able to undergo pyroptosis and provide the sources of IL-1β production during DENV-2 infection with the activation of caspase-1. We further hypothesised that pyroptosis in ECs could be induced by DENV-2 infection and increase membrane permeability.

Our results found an increase in expression of NALP3, ASC and caspase-1. It is suggested that DENV triggers activation of caspase-1 through the sensor NALP3 and induces the production of the pro-inflammatory cytokines IL-1β in macrophages and ECs. Finally, elevated LDH activity showed evidence of cell lysis and demonstrated that cell death occurred after DENV infection. Therefore, we can conclude that the activation of pyroptosis in macrophages and ECs could be triggered by DENV infection *in-vitro*. Further, plasma leakage is the hallmark of DHF, which is associated with a sudden increase in systemic vascular permeability. In the current study, our results indicated that the pyroptosis in ECs induced by DENV-2 infection increased membrane permeability. This is the first evidence that pyroptosis in ECs contributes to the mechanism of vascular leakage during DENV-2 infection.

This study also demonstrated that the production of IL-1β and activation of pyroptosis in macrophages and HUVECs during DENV-2 infection were regulated not only by caspase-1 but also by caspase-4 and that caspase-4 was upstream of caspase-1 in the activation pathway. Caspase-4 thus appears to be a key mediator of inflammation and pyroptosis in macrophages during DENV-2 infection. Additional studies are required to elucidate further the molecular mechanisms involved. Further, this study

showed that while apoptosis may not be a major type of cell death induced by DENV infection in macrophages and ECs at higher multiplicities of infection, it will be the major type of programmed cell death in both macrophage and ECs when DENV occurs at a lower infectious dose. The fate and amount of DENV-infected macrophages may affect the level of DENV and pro-inflammatory cytokines in patients, in turn affecting severity.

From the results of the present study, it can be concluded that both macrophages and ECs are able to undergo pyroptosis and provide sources of IL-1β production during DENV-2 infection with the activation of caspase-1. Caspase-4 was also found to be involved in the activation of caspase-1. In another finding, pyroptosis in ECs can be induced by DENV-2 infection, resulting in increased membrane permeability. These results provide an in-depth understanding of pyroptosis as an alternative process of programmed cell death and of caspase-1 and -4 activations during DENV infection. Our results shed light on the pathogenesis of DENV infection and may also provide new knowledge for the future development of therapeutic targets that could help to develop better management of severe dengue disease.

Publications

Conference paper:

1. Cheung, KT, Sze, DMT, Chan, KH & Leung, PHM. 2013. Involvement of caspase-4 in the production of IL-1 beta and pyroptosis in human macrophages during dengue virus infection. In 3rd Journal Conference on Dengue and Dengue Hemorrhagic Fever. Bangkok, Thailand, 21–23 Oct 2013.

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List of Abbreviations

AIM2	Absent in melanoma 2
PEC	Antibody plaque-forming cells
ADE	Antibody-dependent enhancement
ASC	Apoptosis-associated speck-like protein
BMDM	Bone marrow-derived macrophages
CAD	Caspase-activated DNase
CARD	caspase initiation and recruitment domain
cIAP	Cellular inhibitor of apoptosis protein
CSF-1	Colony stimulating factor-1
CLEC5a	C-type lectin domain family 5, member a
CPE	Cytopathic effect
DAMPs	Danger-associated molecular patterns
DCs	Dendritic cells
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DHF	Dengue haemorrhagic fever
DNA	Deoxyribonucleic acid
DSS	Dengue Shock Syndrome
DENV	Dengue virus
ER	Endoplasmic reticulum
ECs	Endothelial cells
ELISA	Enzyme-linked immunosorbent assay
ECM	Extracellular matrix
GAPDH	Glyceraldehyde-3-phsophate dehydrogenase

GEMM-CGU	Granulocyte-erythrocyte-megakaryocyte-macrophage colony forming unit
GM-CFU	Granulocyte-macrophage colony forming unit
HSP	Heat shock protein
HCV	Hepatitis C virus
HMEC-1	Human dermal microvascular EC line
PBMCs	Human peripheral blood mononuclear cells
HUVEC	Human Umbilical Vein Endothelial cell
HIV	Immunodeficiency Virus
INCL	Infantile neuronal ceroid lipofuscinosis
ICAD	Inhibitor of caspase-activated DNase
IFN	Interferon
IPC	Interferon-producing cell
IFI200	Interferon-inducible family member
IL	Interleukin
LCs	Langerhans cells
LPS	Lipopolysaccharide
LSGS	Low serum growth supplement
M-CSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex
MMP	Metalloproteinase
MEM	Minimal essential medium
M-CFU	Monocyte precursors
MoDC	Monocyte-derived DC
MOI	Multiplicity of infection
mDC	Myeloid DC

NAAT	Nucleic acid amplification test
NK	Natural killer cells
NLRC4	NLR family card domain-containing protein 4
NLR	Nod-like receptor
NOD-like	Nucleotide oligomerization domain receptors
OD	Optical density
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern recognition receptors
PBS	Phosphate buffered saline
PRNT	Plaque reduction neutralization test
pDC	Plasmacytoid DC
PARP	Poly(ADP-ribose) polymerase
PAGE	Polyacrylamide gel electrophoresis
PYD	PYIN effector domain
RIPA	Radioimmunoprecipitation assay buffer (RIPA)
ROS	Reactive oxygen species
RES	Reticuloendothelial system
RT-PCR	Reverse transcription polymerase chain reaction
ORF	Single open reading frame
SDS	Sodium dodecyl sulfate
SF	Suppressor cytokine
TS	Suppressor T- cells
$TCID_{50}$	Tissue culture 50% infectious dose
TLR	Toll-like receptor
TBST	Tris-buffered saline containing 0.5% Tween-20
TNF-α	Tumour necrosis factor-α

WHO	World Health Organization

Chapter 1: Introduction

1.1 Dengue virus

Dengue is an endemic viral disease with a worldwide distribution that is present in most urban centres of the tropics and subtropics. The World Health Organization (WHO) estimated that over the last 50 years, cases of the disease have increased approximately 30-fold, with the geographic area covered by the disease continuing to expand in Asia and South America (Figure 1-1). Urbanisation has led to a dramatic increase in the incidence of the disease and created the ideal conditions for increased transmissions of the mosquito-borne disease. Today, there are an estimated 100 million cases of dengue, of which over 500,000 are severe forms of the disease (i.e., dengue haemorrhagic fever [DHF] or dengue shock syndrome [DSS]). Further, each year 250,000 fatal cases occur, mainly in children under the age of 15.

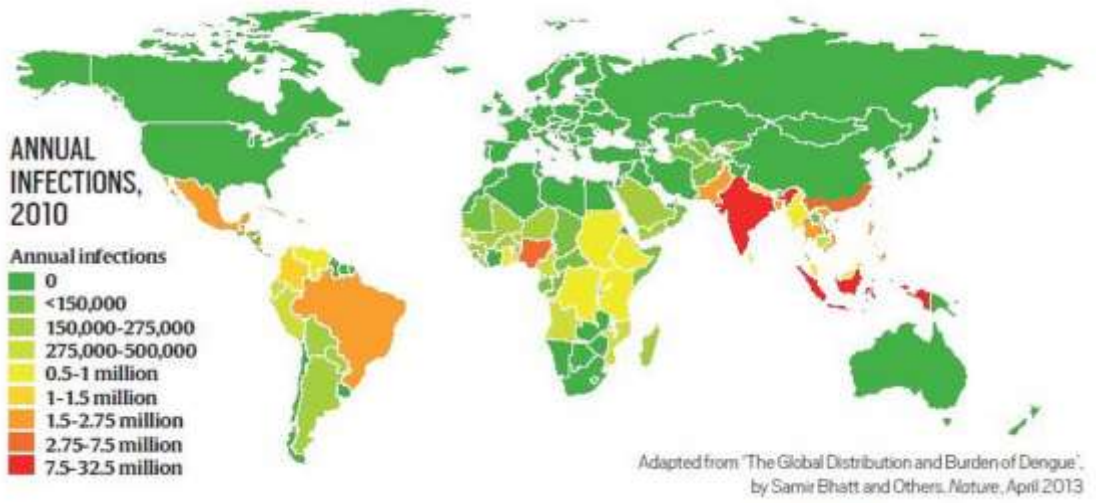


Figure 1-1: The distribution of dengue across the world 2010. Source: Adapted from 'The Global Distribution and Burden of Dengue' by Samir Bhatt and Others. Nature April 2013.

1.1.1 Structure of DENV

Dengue virus (DENV) belongs to the Flavivirus (of the Flaviviridae) family, characterised as an enveloped, single-stranded and positive-sense RNA virus. The virus genome is estimated to be 11,000 nucleotide bases in length. It encodes three structural proteins: the membrane (M), capsid (C) and envelope (E) and seven non-structural proteins (i.e., NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). They are glycoproteins that can be exclusively present in infected host cells and are a prerequisite for the replication of the virus (Figure 1-2) (Avirutnan et al., 2007; Beatty et al., 2015; Libraty et al., 2002). The dengue virion comprises a spherical particle with a diameter of 40–50nm. It is packed with virus capsid (C) protein and surrounded by an envelope (Figure 1-3).

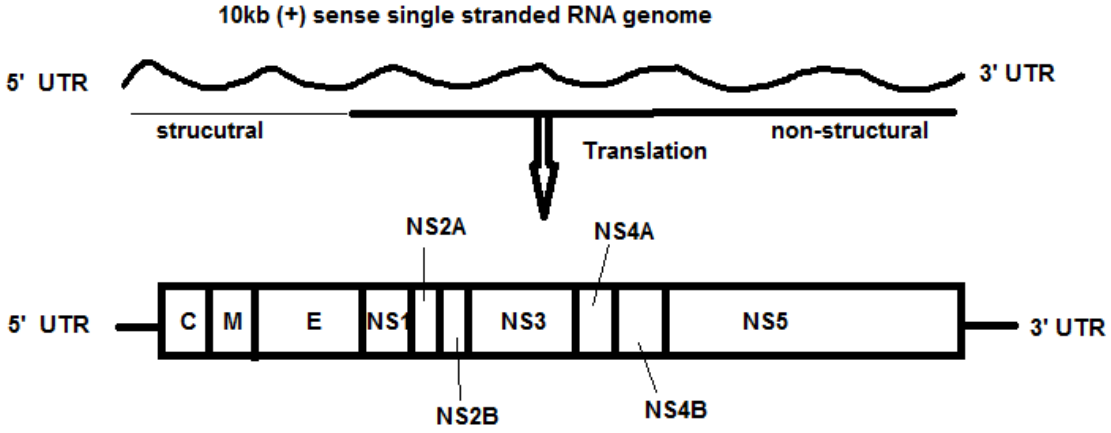


Figure 1-2: The genome and poly-protein organisation of DENV

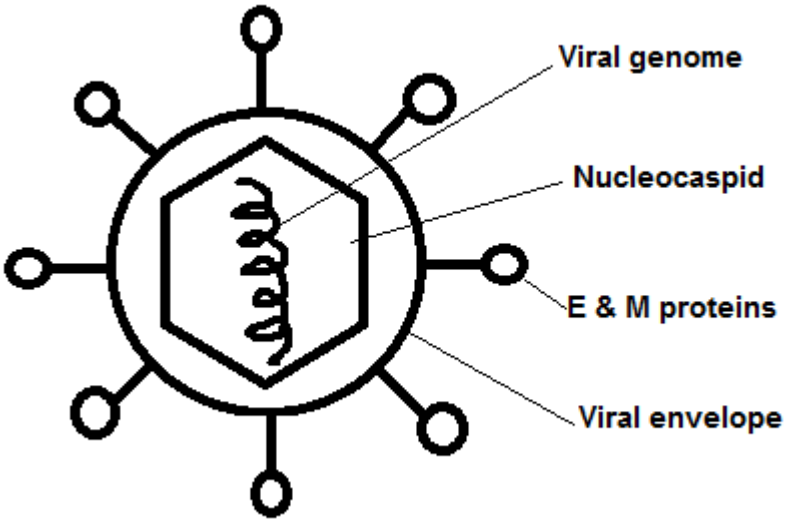


Figure 1-3: Structure of the mature dengue virion arrangement

There are five antigenically different serotypes (i.e., DENV-1, DENV-2, DENV-3, DENV-4 and DENV-5). DENV-5 was only found in October 2013 (M. S. Mustafa, Rasotgi, Jain, & Gupta, 2015) and there is still limited information available for the new DENV serotype. Further genetic and epidemiology studies are needed to identify new dengue strains.

Among the four well-known DENV serotypes, each share around 65% of the genome, a degree of genetic relatedness similar to that between West Nile virus and Japanese encephalitis virus (Rodenhuis-Zybert, Wilschut, & Smit, 2010). The four known DENV serotypes have the capacity to inflict the full range of the disease, can cause similar symptoms in infected humans and have the same ecological niche circulation (Rodriguez-Roche et al., 2005). The envelope (E) protein consists of 180 monomers. It contains

significant information that assists in the classification of B epitopes. It is the main surface element of the dengue virion. Multiple epitopes exist in three respective domains that are bound by antibodies that resemble the domains in the crystal structure. Infected cells produce a glycoprotein called the NS1 protein; however, this protein is not incorporated into the virus (Rodenhuis-Zybert et al., 2010). The NS1 protein forms a multimeric structure or a protein with two or more polypeptide chains that can be seen on the surface of infected cells and is sometimes released as a soluble molecule in *in-vitro* cell culture and within the living organism (Libraty et al., 2002). Early on in the assembly of the virion, DENV pre-M and NS1 proteins may form a heterodimer with the E protein that is then cleaved by the host cell-expressed furin during the final stage of virion maturation before release (Stadler, Allison, Schalich, & Heinz, 1997). The NS2A protein plays a role in virus assembly (Leung et al., 2008) while the NS3 protein contains serine protease, which is the cofactor of NS2B in viral replication (Arias, Preugschat, & Strauss, 1993). The N-terminal of NS5 contains methyltransferase activity, which is responsible for viral RNA cap formation and internal RNA methylation. NS5 also plays a role in the evasion of innate immune responses (Zhao et al., 2015). There is limited information on NS4A and NS4B, but they have been shown to play a role in viral replication and interferon (IFN) α/β response (Zou et al., 2015).

1.1.2 The life cycle of DENV

The female *Aedes* mosquito, specifically the *A. aegyptus*, is mainly responsible for the spread of DENV (Bandyopadhyay, Lum, & Kroeger, 2006). Other *Aedes* species that carry and spread the disease include *A. polynesiensis*, *A. scutellaris* and *A. albopictus* (Bandyopadhyay et al., 2006). These species are distinguishable from *A. aegyptus*, as they normally bite during the day, mostly in the early morning, or the early evening (Watts, Burke, Harrison, Whitmire, & Nisalak, 1987). DENV is mainly hosted by humans, but can also be spread though non-human primates. Once bitten, humans have a high tendency to be infected, typically after a period of five days of the viremia. The virus usually comes from the intestinal tracts of the mosquito and is passed through the salivary glands after the extrinsic incubation period, which can be about 10 days but is usually faster in environments with high ambient temperatures (Watts et al., 1987). As mosquitoes feed on blood, the virus is transmitted through the mosquitos' saliva and enters the skin of the host human. As it bypasses the skin, DENV binds to Langerhans cells (LCs) in the skin tissues. Binding between viral proteins and LC membrane proteins allows the virus to enter the LCs and specific C-type lectins (i.e., dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), mannose receptor and C-type lectin domain family 5, member a (CLEC5A)) (Rodenhuis-Zybert et al., 2010). Reports have shown that DC-SIGN is the major passage or entry point of DENV (Tassaneetrithep et al., 2003). It may be that the DC-SIGN, a nonspecific receptor for foreign material on dendritic cells (DCs), contributes to DCs as professional antigen-presenting cells. (Guzman et al., 2010). As the DCs become infected with the virus, they will mature and migrate to a local or regional

lymph node, where they then present viral antigens to T cells, triggering cellular and humoral immune responses (e.g., antibodies and IFN production). These responses are exhibited in an individual's body as symptoms (e.g., fever, flu-like symptoms and severe joint pains). In cases of severe infection, the virus is produced within the body at critically high rates and may cause other types of cells and organs to become infected (e.g., the liver parenchymal cells, macrophages in lymph nodes and spleens and peripheral blood monocytes) (Jessie, Fong, Devi, Lam, & Wong, 2004). Patients will become febrile and infective after 7–10 days of the viraemic phase. Thereafter, patients may either recover by themselves or progress to leakage phase, leading to severe forms of dengue disease (e.g., DHF or DSS). In a single gonotrophic cycle, *A. Aegypti* is known to feed on multiple human hosts for a blood meal. When a mosquito bites a person who has DENV in his or her blood, the mosquito becomes infected with DENV. An infected mosquito can later transmit that virus to healthy people by biting them. In this way, DENV is spread from humans to mosquitos and then to humans or primates in a cycle of transmission (Figure 1-4).

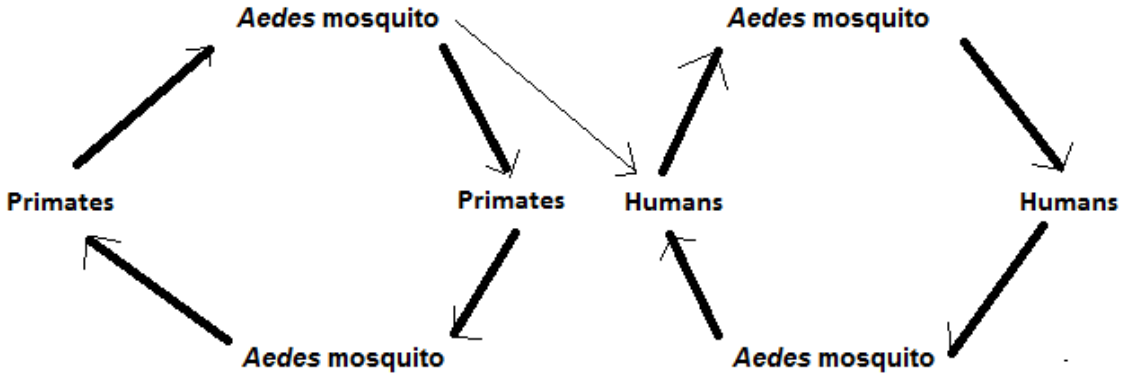


Figure 1-4: The sylvatic and urban dengue transmission cycles, from human/primate to mosquito to human/primate

1.1.3 Clinical manifestations of DENV infection

Dengue infections may cause symptoms or be asymptomatic but give rise to an undifferentiated fever, dengue fever (DF), DHF(Grade I-IV) or DSS (Figure 1-5) (Guzman & Kouri, 2004). Commonly, an undifferentiated fever manifests from a primary infection (i.e., in a patient not previously exposed to DENV), but it may also manifest in a secondary infection (i.e., in a patient who has previously been exposed to DENV). Other types of viral infection may not be clinically distinguishable from a DENV infection. Both primary and secondary infections can manifest in DF; however, DF is more likely to be caused by a primary infection than a secondary infection. Secondary infection by a different DENV serotype has been confirmed as an important risk factor for the development of DHF or DSS (Kalayanarooj, 2011), and secondary infection commonly results in one of these severe forms of dengue disease. A new classification of DF, developed in 2009, is divided into dengue without warning signs, dengue with warning signs and severe dengue (Kalayanarooj, 2011).

1.1.3.1 Dengue without warning signs

The presenting symptoms of dengue without signs may include sudden high fever (usually lasting 2–7 days), severe headaches, arthralgia, myalgia, anorexia, abdominal discomfort and a maculopapular rash. Younger children tend to have symptoms such as diarrhoea, coryza, rash and seizures and sometimes develop headaches, abdominal pain and vomiting (Figure 1-5) (Pancharoen, Mekmullica, & Thisyakorn, 2001). Haemorrhagic

symptoms are not commonly observed, but petechiae, gastrointestinal bleeding epitasis and gingival bleeding have been observed in a few infected individuals (Pancharoen et al., 2001). The pre-infection condition of the infected individual's body strongly influences recovery time, which can be uneventful, but delayed.

1.1.3.2 Dengue with warning signs

Dengue with warning signs (formerly DHF) is typically observed from secondary dengue infection, but is sometimes also observable in primary infections, especially if the patient is a child. High fever, haemorrhagic phenomena and features of circulatory disturbances are characteristics of this class (Guzman & Kouri, 2004). Around the time of fever defervescence, some patients develop signs of thrombocytopenia, enhanced vascular permeability with leakage of intravascular fluid (Figure 1-5).

1.1.3.3 Severe dengue

Severe dengue (formerly DSS) is a fatal dengue disease, distinguishable from dengue with warning signs (DHF) by the occurrence of cold blotchy skin, circular cyanosis and circulatory failure (Pancharoen et al., 2001) (Figure 1-5). The fluid leakage leads to shock when a critical volume of plasma is lost into the extravascular space and the cardiac output becomes insufficient to maintain the individual's blood pressure. Without proper treatment, shock may cause death within 12–36 hours after onset (Figure 1-5). Therefore, it

is important to distinguish as early as possible patients with severe rather than non-severe forms of the disease, as intravenous hydration can reduce the fatality rate of the disease.

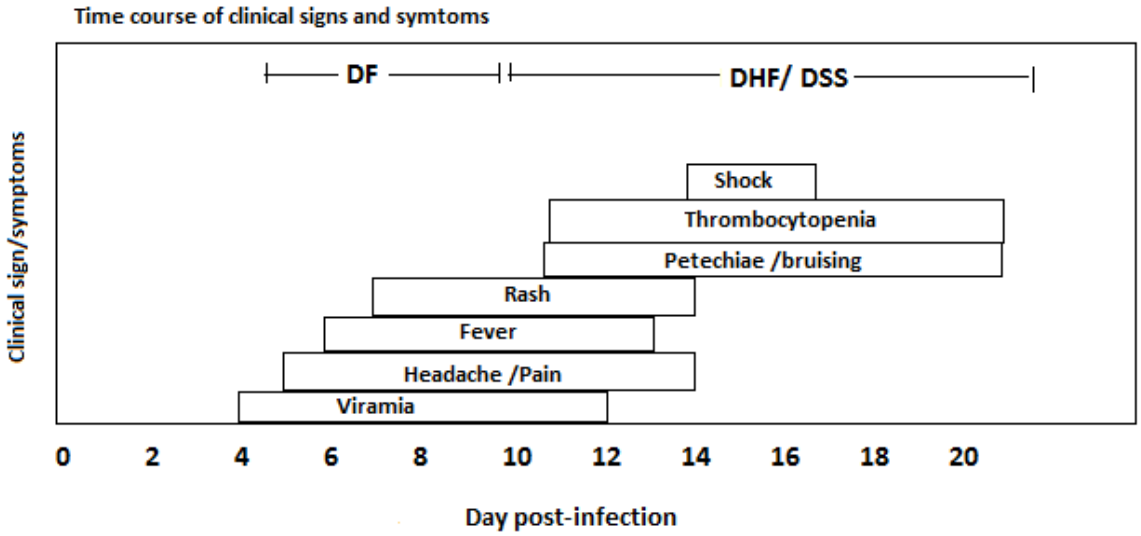


Figure 1-5: The time course of clinical signs and symptoms of DF and DHF

1.1.4 Immune responses to DENV infection

Once bitten by a dengue-carrying mosquito, DENV enters the body and replicates within the mononuclear phagocytic cells (e.g., the monocytes, macrophages, DCs and B cells). In some cases, mast cells, liver cells and endothelial cells (ECs) also become infected, as the virus also replicates inside these cells (Ho et al., 2001; Huang et al., 2000; King, Marshall, Alshurafa, & Anderson, 2000). Once inside the body, DENV incubates within 7–10 days. The patient goes through a febrile and infective state, followed by a viraemic phase. Subsequently, the patient may either recover spontaneously or progress to a leakage phase that can lead to dengue with warning signs or severe dengue (Kalayanarooj, 2011). The severity of the disease is correlated with the peak viremia (Libraty et al., 2002).

Clinical and experimental investigations suggest that immune responses to DENV can be both protective and harmful. The antibody, cytokines and cellular responses have been observed to differ among individuals with DF or DHF/DSS.

1.1.4.1 Antibody responses to DENV

1.1.4.1.1 Antibody responses associated with protection

Once the primary infection takes place, western blotting has clearly demonstrated that antibodies form against both structural and non-structural viral proteins such as prM, E and NS-1. Studies on mice have shown that the adaptive transfers of immune serum or monoclonal antibodies (specific to pre-M, E or NS1 viral proteins) are saved from the fatality of DENV infection (Sukupolvi-Petty et al., 2010). There have also been cases in which antibody responses, particularly NS3 and NS5, have been found to counter other non-structural proteins, like NS1 and NS4. However, the responses were noted to be particularly weak in primary infections (Valdes et al., 2000).

Following natural DENV infections in humans, the serological responses to the E protein are highly cross-reactive serotype. Most E protein-specific human monoclonal antibodies bind to multiple DENV serotypes, which is in line with findings on patient serums (Schieffelin et al., 2010). In addition, antibodies specific to NS1 and pre-M protein are highly cross-reactive serotype. This cross-reactivity of the specific antibodies makes the

assessment of the protective effects of DENV-specific antibodies when exposed to DENV challenging.

In secondary infection, the titers, or the concentration of the specific antibodies necessary to neutralise the virus, have been shown to be proportional to the severity of the disease (Endy et al., 2004); however, it is noted that other studies have reported conflicting results (Endy et al., 2004). DENV-specific IgG antibodies of the particular subclasses IgG1, IgG3 or IgG4 can also bind to complement proteins to initiate their activation; for example, NS1-specific antibodies initiate the complement-dependent lysis of infected cells. This does not fully explain the protective effects *in vivo*; however, the fixation of complement antibodies (specific to the pre-M and/or E proteins) has been shown to slow down, decrease or even stop viral infections (Mehlhop et al., 2007). The specific subclasses of antibody production and their levels during the initial stage of dengue infection have been shown to have a significant protective effect (Vaughn et al., 1997). The DENV-specific antibodies neutralise the infection by inhibiting the virus binding to cell-surface receptors or by acting directly on E proteins (Schieffelin et al., 2010).

1.1.4.1.2 Antibody responses associated with dengue disease

The pathogenesis of severe dengue infections is thought to have a major role in antibody-dependent enhancement (ADE). ADE is only observable in secondary dengue infection (K. D. Yang, Yeh, Yang, Chen, & Shaio, 2001), where the sub-neutralising dengue antibodies already developed and present in the body of the patient form complexes with DENV; for instance, the Fc portion of these antibodies binds to FcγRI and FcγRII (i.e.,

the adjacent cells that increase the number of cells infected with DENV) (Boonnak, Slike, Donofrio, & Marovich, 2013; Chotiwan, Roehrig, Schlesinger, Blair, & Huang, 2014). As an example of ADE in the presence of sub-neutralising dengue antibodies, DENV-1 enhances DENV-2 infection of mononuclear leucocytes, which in turn increases lymphocyte proliferation and decreases IFN-γ production (Lin et al., 2002).

Compared to patients with DF, patients with DHF and DSS have higher levels of DENV-specific IgG_1 and IgG_4 and lower levels of IgG_2 (Koraka et al., 2001). The activation of the complement system augments the permeability of the vessels causing coagulation irregularities. Thus, the prevailing IgG subclass (specific to DENV) may play a significant role in the development of severe dengue diseases (Koraka et al., 2001).

The exact function of neutralising antibodies is unknown; however, dependent upon caspase, anti-NS1 antibodies have been reported to stimulate EC death (i.e., apoptosis) (Lin et al., 2002). Following antigen binding, IgG subclasses differ in their ability to prompt the typical complement pathway; for example, IgG_1 is observed to be more effective than IgG_2. Elevated concentrations of IgG_1 and IgG_4 and low concentrations of IgG_2 have been reported in patients with DHF and DSS (Koraka et al., 2003). Further, total IgE antibody levels are statistically higher in patients with a history of DENV infections (Koraka et al., 2003), and one study found that patients with DHF or DSS had higher overall antibody levels and more IgE antibodies specific to DENV than did those patients with DF (Guzman et al., 2010). In addition, DENV has the ability to induce IL-6 with the involvement of IgE production. The increased level of IgE during acute DENV infection possibly reflect

immune memory (Vercelli, Jabara, & Geha, 1989). Thus, the role of the IgE antibodies in the development of the disease is raised concerns.

Thrombocytopenias are common in patients with DHF. The contributing mechanisms may include: a) specific antibodies for dengue, b) IgM types of anti-platelet antibodies, c) a hypo-cellularity of bone marrow that may lead to increases in the number of abnormal megakaryocytes and d) destroyed platelets in the spleen or liver (Chuang et al., 2011). Anti-platelet antibodies may result in the lysis of platelets when the complement is present. Similarly, patients with either DHF or DSS have higher levels of anti-platelet antibodies than do those with DF. This may result in a higher degree of thrombocytopenia in patients with DHF (Lin et al., 2001). When the antibodies specific to a virus are present, the DENV-2 strain or serotype attaches to the platelets and thus performs an immune-mediated clearance function in the platelets.

Further, the anti-B cell antibodies present in patients with DHF may contribute to humoral immune responses during infection. Reports suggest that the number of B cells increases while the number of T cells decreases in patients with DHF (J. Fink, Gu, & Vasudevan, 2006). This decrease may be due to the T cell antibodies found in the serum (Chaturvedi et al., 1999). Such changes are more noticeable during DHF or DSS. DENV can induce different subclasses of antibodies and auto-antibodies that contribute to the severity of the disease (Lin et al., 2001).

1.1.4.2 Cytokine responses in dengue infections

1.1.4.2.1 Cytokine responses associated with protection

The immune cells (e.g., the monocytes, B-lymphocytes and mast cells) infected with DENV can secrete cytokines. Recently, researchers have disagreed on what cytokines are predominantly secreted by these cells during DF and DHF (Tang et al., 2010). In patients with DHF, the levels of tumour necrosis factor-α (TNF-α), interleukin (IL)-2, IL-6 and IFN-γ in the serum during the first few days of infection indicate these as the cytokines most secreted by the cells during this period, followed by IL-10, IL-5 and IL-4 secretion (Chaturvedi et al., 1999; Nguyen et al., 2004; Tang et al., 2010). Lymphocytes infected by DENV secrete IFN-α at higher levels than IFN-γ (Kurane, Meager, & Ennis, 1986). IFN-γ is secreted at the onset of the infection and peaks during the time of defervescence or at the same time that viraemia disappears (Pandey et al., 2015). The amount of IFN-α is higher in patients with DHF than in those with DF; however, there seems to be no difference between the levels of IFN-α in patients with different DHF grades (Kurane et al., 1993). Scientists suggest that IFN-α slows the monocyte infection caused by DENV and thus plays a significant role in controlling primary infections of DENV (Diamond et al., 2000). In the case of IFN-γ levels, these are similar in patients with DHF and DF. It has also been suggested that the levels of different types of cytokines secreted during DENV infection are correlated with primary or secondary dengue infection (Pandey et al., 2015).

IL-12 may also play a certain function in the protection of the disease as it is only detected in high levels in patients with DF and has not been detected in patients with grades III or IIV DHF (Rathakrishnan et al., 2012). However, more research in this area is needed. IL-2 and IFN-γ are T-helper 1 (Th-1)-type responses observed during the onset of the disease; IL-5 and IL-4 type responses follow thereafter. The T-helper 2 (Th-2) responses appear to predominate in DHF and DSS sera while the Th-1 responses appear to offer protection against severe infections. Researchers disagree as to which of these two responses predominate; for example, studies have found that Th-1 responses may be suppressed in severe dengue infection, another study also report that Th-2 responses outweigh Th-1 responses. A shift in Th-1 to Th-2 responses was observable in patients with severe dengue infection (Chaturvedi, 2009).

1.1.4.2.2 Cytokine responses associated with dengue diseases

Patients with DHF secrete more TNF-α, IL-6, IL-13, IL-18 and cytotoxic factors than patients with DF. Cytokines have been reported to cause increases in the permeability of vessels and the development of shock (A. S. Mustafa, Elbishbishi, Agarwal, & Chaturvedi, 2001; Vitarana, de Silva, Withana, & Gunasekera, 1991). The level of IL-1 beta, IFN-gamma, IL-4, IL-6, IL-13, IL-17 and GM-CSF were significantly increased in patients with severe severe dengue when compared to mild dengue diseases (Bozza FA et al., 2008) The concentrations of IL-8 have been found to be higher in patients suffering from severe dengue infections and appear to be highest in deceased patients (Pandey et al., 2015). Elastases are secreted by activated neutrophils and may contribute to endothelial

injury while simultaneously activating the complement, coagulation and fibrinolytic systems. The high concentrations of both IL-8 and elastases in the serum are associated with severe cases of infections; thus, these concentrations appear to play an important role in the pathogenesis of dengue infections (Bosch et al., 2002; Pandey et al., 2015). IL-8 is also secreted by the ECs and could potentially cause inflammation and chemo-attraction.

IL-13 and IL-18 have been observed at increased levels in patients with severe dengue infections. The highest levels of IL-13 and IL-18 have been reported in patients with grade IV DHF. Conversely, higher levels of the transforming growth factor-β (which inhibits Th-1 responses and enhances Th-2 responses) are correlated with the severity of the disease and inversely correlated with the levels of IL-12 (A. S. Mustafa et al., 2001). IL-12 is produced at low concentrations in DCs after DENV infection. Low concentrations of IL-12 in DHF probably occur when its induction by IFN-γ has failed during DENV infection (Rathakrishnan et al., 2012). According to Ho et al. (2001), IFN-γ up-regulates the monocyte Fc gamma receptors and thus enhances the immunity of DENV infection. Conversely, TNF-α can delay the survival of DCs through the up-regulation of factors for anti-apoptosis inside the cells. The delay in the survival of DENV in DCs could possibly lead to severe dengue infections (Ho et al., 2001).

The cytotoxic factor secreted by $CD4^+$ T cells stimulates the production of the pro-inflammatory cytokines IL-1α, TNF-α and IL-8 by the macrophages. The levels of cytotoxic factors are linked to the severity of the disease (with DHF/DSS patients having the highest levels). Additionally, high levels of antibodies for the cytotoxic factors have

been shown to defend against severe dengue disease, as seen in patients with mild forms of dengue disease (Chaturvedi, Elbishbishi, Agarwal, & Mustafa, 2001).

IL-6 levels are higher in both DHF and DSS patients (Fredeking et al., 2015). The mast cells and ECs mainly secrete IL-6 cytokines. IL-6 is an endogenous pyrogen that can cause inflammation or increase body temperature and enhances the ECs' permeability. It has been found that the levels of TNF-α, IFN-γ, IL-10 and the soluble TNF receptor in the infected cells are considerably higher in DENV-infected patients than in patients with no infection (Pandey et al., 2015). Haemorrhagic manifestations have also been observed in patients with high levels of TNF-α; however, platelet destruction is present in those with high levels of IL-10. The up-regulation of IL-10 cytokine production in genetically susceptible individuals is a well-known response to cell infection; virus-antibody complexes are a consequence of cellular responses to infection. It has been suggested that IL-10 could down-regulate platelet function and thus contribute to the destruction of platelets during dengue infections (Azeredo et al., 2001). In general, the cytokines TNF-α, IL-6, IL-10, IL-13 and IL-18 are the major contributors of pathogenicity in dengue infection.

1.1.4.4 Cellular responses to dengue infection

1.1.4.4.1 Cellular responses to dengue infection associated with protection

Cellular responses involve the activation of DCs, macrophages, natural killer (NK) cells and antigen-specific T-lymphocytes. These cells are essential in clearing infections in host cells and also provide immunity from future infections. DCs are antigen-presenting cells that react to the class I and II major histocompatibility complex (MHC) molecules of T cells following the stimulation of immunogenic peptides and then migrate to the paracortex of lymph nodes (Liu, Kanzler, Soumelis, & Gilliet, 2001). They influence the process of innate and adaptive immunity by serving as messengers. The premature $CD34^{+}$ transforms into $CD11c^{+}$, $CD1a^{-}$ immature and $CD11c^{-}$, $CD1a^{-}$ premature DCs. The $CD11c^{+}$, $CD1a^{-}$ immature DCs move into the skin's epidermis and eventually become LCs. The $CD11c^{+}$, $CD1a^{-}$ premature DCs move into the skin's dermis and other tissues and transform into interstitial premature DCs (Ito et al., 1999). The interstitial DCs become the myeloid and plasmacytoid DCs (pDCs). Myeloid DCs (mDCs) can secrete IL-10 while the body fights off DENV infection. They then gain the ability to take up sufficiently high doses of INF-α, a form of IFN-producing cell. Monocyte-derived DCs (MoDCs) are also naturally immune cells and are differentiated by monocytes under inflammatory situations. MoDCs are known to be 10-times more permissive to DENV infection than are monocytes or macrophages (S. J. Wu et al., 2000). When DCs are activated by DENV, they secrete TNF-α and IFN-α. Studies have found that the number and frequency of circulatory pre-MoDCs decreases when there is severity during an early acute viral stage (Rothman, Medin, Friberg, & Currier, 2014). The number and frequency of pDCs are noted to be high and the circulatory pDC is sufficiently stable with DF; however, an initial decrease in pDCs levels,

specifically in children with late–early DHF, has also been observed (Pichyangkul et al., 2003).

Other than MHCs, the toll-like receptor (TLR) is another major receptor that recognises pathogens. The human body has approximately 11 different TLRs. The nuclear transcriptase factor NF_kB is downstream of the response of TLR with pathogen ligand and also prompts the expression of different genes of co-stimulatory molecules or cytokines. Different TLRs are expressed by different DCs. The mDCs express TLR2 and TLR4 and the pDCs express TLR7 and TLR9. Patients with early DF infection have been shown to have highly expressed TLR3 and TLR9 receptors on DCs (Torres et al., 2013). TLR3 targets dsDNA to trigger the production of INF-β, IL-1 and IL-6; while TLR2 ligands the lipoprotein of bacteria, TLR-5 targets the flagellin of some bacteria and TLR-7 only targets RNA virus (Torres et al., 2013).

The NK cells eliminate virus-infected cells using cytotoxic granules or through recognising and prompting lyses of antibody-coated target cells via the antibody-binding receptors CD16 and CD56. They are then quickly recruited into infected organs or tissues by chemoattractant factor (a product of infected cells). Studies suggest that early activation of NK cells could be important in the process of fighting primary dengue infection (Shresta, Kyle, Robert Beatty, & Harris, 2004). The number of NK cells was noticeably higher in patients with DF compared to patients with DHF (Beltran & Lopez-Verges, 2014). The severity of DENV infection was related to the activity of NK cells (Beltran & Lopez-Verges, 2014). There is insufficient evidence to draw conclusions about how NK cells

affect the outcome of DENV infection; however, one experimental study showed that the early activation of NK cells with B-lymphocytes is effective in fighting off primary dengue infection. This may be because DENV causes the MHC class I of immune cells to be up-regulated (Yossef, Rosental, Appel, Hershkovitz, & Porgador, 2012). By being in close physical contact, DCs and NK cells establish a private cross-reaction, whereby the DCs induce NK cell propagation and the NK cells mediate the IMDCs and NK-dependent DC maturation (Della Chiesa, Sivori, Castriconi, Marcenaro, & Moretta, 2005). In general, an increase in the number of NK cells suggests a good prognosis in DENV infection.

An increase in the number of atypical lymphocytes (antigen-stimulated lymphocytes) is very common during DENV infections (Chaturvedi et al., 1999). From the variety classifications of T cells, $CD8^+$ T cells have been observed to be effective in the control of early DENV infection; however, the strong propagation of $CD8^+$ T cells could also be connected to the disease (Yauch et al., 2009). Conversely, in $CD4^+$ T cells, peak IFN-γ secretion has been observed during cell exposure to a homologous antigen (Mangada & Rothman, 2005). A study done on mice showed that dengue-specific $CD4^+$ T cells were at low levels during primary infection, but were more responsive during secondary infection (Beaumier & Rothman, 2009).

Monocytes and macrophages are immune cells that serve different roles in protecting the host from DENV replication. This view is supported by the fact that monocytes and macrophages undergo apoptosis when they come into contact with DENV and they are capable of phagocytosis. Specifically, they phagocytose the infected apoptotic

cells or apoptotic bodies and up-regulate the immune response through secretion of cytokines (Espina, Valero, Hernandez, & Mosquera, 2003; Honda et al., 2009). Besides apoptosis, no study mentions other forms of cell death (e.g., pyroptosis) playing a protective role in DENV infection.

1.1.4.4.2 Cellular responses to dengue infection associated with dengue diseases

Different receptors (such as C-type lectin, TLRs and MHC) can be used to identify pathogens. The DC-SIGN, a type II membrane protein with C-type lectin ectoderms, has the important role of connecting the DCs and T cells (Geijtenbeek, Engering, & Van Kooyk, 2002). DENV infections are intensely inhibited in the knock-down of the DC-SIGN receptor of DCs (Tassaneetrithep et al., 2003). Evidence suggests that the number of DC-SIGN receptors on host cells is closely related to the severity of the disease (Tassaneetrithep et al., 2003; F. Zhang, Ren, & Zuo, 2014). Low production of TLR3 and TLR9 has been observed in the DCs of patients with more severe DENV infections (Torres et al., 2013). Further, observable increases in the expression of TLR2 in patients with DHF have been observed (Torres et al., 2013).

Unlike DCs, T cells cannot independently identify the intact virus; rather, T cells require the existence and detection of viral peptides on the surface before they can transform into dengue-specific T cells. T cells have been shown to increase immunity to DENV through the production of IFN-γ by this dengue-specific T cell; however, IFN-γ appears unnecessary for protection (Lazo et al., 2010). The specific role of DENV-specific

T cells in humans continues to be challenging to define; however, evidence shows that there is little to no difference between the T cell propagation response and the cytokine secretion levels of DENV-infected patients hospitalised for the treatment of subsequent infections as compared to patients with their first DENV infection (Mangada et al., 2002). Thus, it appears that T cell propagation and cytokine responses are not influential in protecting humans from DENV infections.

In relation to the subsequent rise of the cytokine levels of patients with DHF, this may be because the cytokines produced by the T cells have a pleiotropic effect that includes the induction or improvement of inflammatory responses and modification of vascular permeability (Rathakrishnan et al., 2012). However, there is insufficient evidence on the level of T cell activation associated with severity of the disease. It might be distinguished by the factors existing prior to the infection (e.g., memory T cells, immunisation of the host, and the amount of virus injected or the replication kinetics of the infective virus strain); however, the research is inconclusive. One study observed a specific response pattern of memory T cells represent a high risk for the severity of dengue disease (Mangada et al., 2002); however, no exclusive factor can explain or distinguish between the severities of dengue cases (Mangada et al., 2002).

Monocytes and macrophages are two of the cell types that most noticeably make DENV susceptible to phagocytic action and, under the theory of ADE in heterologous infection, could cause increased virus production. Despite the role of monocytes and

macrophages in DENV infection having been studied for decades, the role of the pathogenesis of DHF and DSS remains poorly defined (K. D. Yang et al., 2001).

1.1.5 Laboratory diagnosis of DENV infection

Over the many decades since the first clinical presentation of DENV, laboratory analyses of the disease have proven essential through the development of different procedures to diagnose infection, including serology, isolating the virus, the molecular technique, and viral genome or viral antigen detection. Of these techniques, virus isolation has emerged as the standard, as it offers the most valuable and specific results. However, facilities that support viral culture are not usually available.

1.1.5.1 Virus isolation

Virus isolation is the gold standard technique used by laboratories to confirm DENV infection. In practice, DENV is usually separated by the plasma or serum from the leukocytes of an infected patient during the febrile phase (i.e., the first 3–5 days). In post-mortem specimens, DENV can be isolated from the lungs, liver, lymph nodes, spleen, cerebrospinal fluid, thymus and ascitic or pleural fluid (Guzman & Kouri, 2004). Conventionally, DENV isolation has been conducted in animal cell cultures (i.e., Vero cell line or baby hamster kidney cell lines) or new-born mice, before replacing the cells of the mosquito. This is because these are cheap and easy to maintain at room temperature.

Although effective in determining DENV infection in humans, this technique is expensive and time-consuming. Thus, it is currently only used for research purposes (Kao et al., 2001).

1.1.5.2 Serological diagnosis

Serological diagnosis is another important method commonly used to diagnose DENV infection in humans. Procedures include enzyme-linked immunosorbent assay (ELISA), neutralisation tests, haemagglutination inhibition tests and complement fixation tests. Unlike the virus isolation procedure, many laboratory scientists currently use the serological procedure, as it is simple and cost effective. Upon second exposure to DENV, certain secondary immune responses generally initiate immediately, leading to the production of high levels of IgG due to memory B cell stimulation (Teixeira & Barreto, 2009). This stimulation is caused by the previous DENV infection. There is also increased production of IgM in response to the current infection (Figure 1-6) (Guzman & Kouri, 2002). Usually, IgG and IgM compete for antigen binding and thus are significant in identifying primary and secondary DENV infections (Table 1-1) (Guzman & Kouri, 2004; Teixeira & Barreto, 2009).

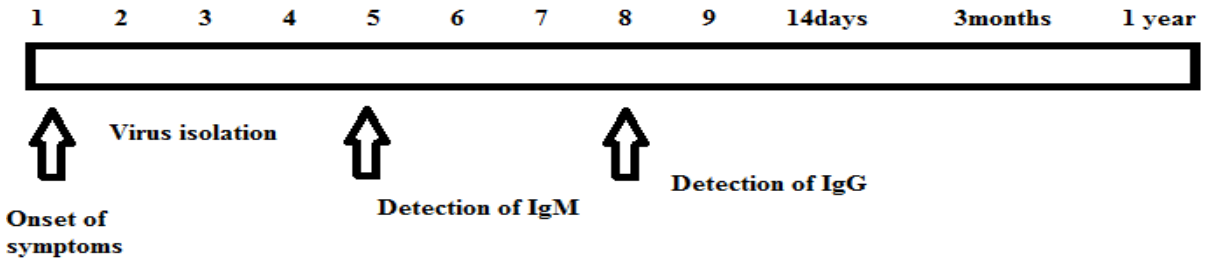


Figure 1-6: General timeline of primary DENV infection from identification and isolation of the virus to detection of IgM and IgG

Table 1-1: The laboratory criteria for confirmed and probable dengue infection

Laboratory criteria for confirmed and probable dengue infection	**Laboratory criteria for probable dengue infection**
Virus isolation by tissue culture	Serum IgM positive
Genome detection in blood or tissue samples	Elevated IgG titer in blood samples
Antigen detection on leucocytes and tissue	
IgM or IgG seroconversion	

1.1.5.2.1 IgM ELISA

To detect the presence of dengue-specific IgM in a serum test, the first step is usually to capture all the IgM in the serum on solid phase media. When compared to different techniques used to determine DENV infection, the MAC-ELISA is especially effective, as the specificity and sensitivity of any sample test collected after approximately five days of DF are 98% and 90%, respectively (de la C Herrera, Cabrera, Garcia, & Gilart, 2006). According to de la C Herrera et al. (2006), dengue-specific IgM can be collected from whole blood (using a filter paper) and saliva. Both whole blood and saliva give a sensitivity and specificity of approximately 98.1% and 98.5% and 90.3% and 92.0%, respectively (Balmaseda et al., 2003; de la C Herrera et al., 2006). A limitation of using the MAC-ELISA to determine dengue-specific IgM in patients is the risk of false-positive results due to the cross-reactivity of dengue-specific IgM and other flaviviruses, especially in places where multiple flaviviruses co-circulate. Another limitation is that some tests may show nonspecific sera reactivity in individuals with leptospirosis and malaria (Hunsperger et al., 2009).

1.1.5.2.2 IgG ELISA

ELISA is an important technique used to confirm the presence of DENV infection in patients. It is especially important in detecting dengue-specific IgG. IgG ELISA is used to classify both primary and secondary DENV infections. Serum dilution is used in titering dengue-specific IgG (Vazquez, Bravo, Perez, & Guzman, 1997). One disadvantage of the IgG ELISA is that it lacks specificity within flavivirus serocomplex groups. However, research has shown that, due to a lack of cross-reactivity in sera, the response of IgG to prM membrane glycoprotein is usually specific to an individual flavivirus (e.g., there is no cross-reactivity in a patient infected with DENV or Japanese encephalitis virus) (Cardosa, Wang, Sum, & Tio, 2002). In addition, it has been noted that the IgG specific for NS5 protein has the potential to discriminate between DENV infections, including those caused by the St Louis encephalitis, dengue and West Nile viruses (Wong et al., 2003). In general, IgG assays are very useful for seroepidemiological studies to identify DNEV infection.

1.1.5.2.3 IgM:IgG ratio

The ratio of IgM to IgG, especially on the M and E protein-specific DENV, can be helpful in distinguishing DENV infection in the primary and secondary stages of infection. For this, the IgG and IgM capture ELISAs are the best and most commonly used assays. Under this method, a ratio of more than 1:2 (IgM:IgG) describes dengue infections in the primary stage. Conversely, if the ratio is less than 1:2 or 1:4, the infection is described as secondary (Shu et al., 2003). Research has shown that these ratios vary based on whether a

patient has a classical DENV infection or a serologically non-classical infection (Falconar, de Plata, & Romero-Vivas, 2006). Thus, the cut-off ratio of IgM:IgG is not well established (Falconar, de Plata, & Romero-Vivas, 2006).

1.1.5.3 Nucleic acid amplification tests

In the last few decades, numerous DENV infection reverse transcriptase assays have been developed. These techniques have mainly been used for different amplification techniques and to target different genes. According to Rosario Dominguez, Suarez Moran, Rodriguez Roche, Soler Nodarse and Guzman Tirado (1996), the most frequently used nucleic acid amplification tests (NAATs) include the nested real-time reverse transcription polymerase chain reaction (RT-PCR) assay, single real-time RT-PCR assay and one-step multiplex real-time RT-PCR assay. For the nested polymerase chain reaction (PCR), the reaction usually consists of a primary reverse transcription, a first amplification step and a subsequent amplification step in the converse region, which is serotype-specific. Once these steps have been completed, electrophoresis is used to differentiate the results based on size. Compared to virus isolation cell cultures, the sensitivity of real-time RT-PCR assays usually varies between 25 and 80% (Klungthong et al., 2015; Raengsakulrach et al., 2002).

The real-time RT-PCR assay is helpful because it permits the quantification of virus titers in a single step. Detection of the amplified targets can be easily assessed using fluorescent probes rather than post-amplification electrophoresis. There are two types of

real-time RT-PCR; that is, singleplex (for detecting one single serotype per reaction) and multiplex (for detecting all four known serotypes in every single run) (Raengsakulrach et al., 2002). An important advantage of the real-time RT-PCR is that it can determine viral titer in the early stage of infection. Thus, it can be used to predict the severity of the infection (Vaughn et al., 2000).

1.1.5.4 Antigen detection

Dengue antigens can be detected in many human body tissues, including the spleen, liver and lymph nodes. Enzymes and colorimetric substrates are used with antibodies targeting dengue-specific antigens to help to detect these antigens (Vaughn et al., 2000). NS1 antigen detection as become a routine rapid diagnostic test for DENV infection because it can be able to detect DENV infection from Days 1 to 8 (Shu et al., 2003).

1.1.5.5 Neutralization test

The plaque reduction neutralization test (PRNT) is considered as t*he* 'gold standard' to quantify and characterize the circulating levels of anti-DENV *neutralizing* antibody. It has a higher sensitivity than other tests like hemagglutination and enzyme immunoassay. However, false negative results may be seen if wrong cell line *was* used for the assay. The test is *now* being used by dengue vaccine developments, academic researches and public health setting but not for routine patient diagnosis (Thomas et al., 2009).

1.1.6 Treating the DENV infection

Treating the majority of dengue infections is challenging, especially as there is a lack of specific medications to treat these infections. Many specialists recommend infected patients manage the disease to obtain symptomatic relief. However, in addition to managing the symptoms, it is important to balance and maintain fluids for quick recovery (Kalayanarooj, 2011) and seek early admission to hospital before shock begins.

Those suffering from the febrile phase and DF usually engage in similar management practices based on the symptoms presenting. Research has shown that sufficient bed rest, replacing fluids, analgesics and supportive care are significant in the early treatment of DENV infections. It is recommended that infected patients use paracetamols as an antipyretic because other non-steroid anti-inflammatory medication (e.g., dicolfenac sodium and aspirin) can cause stomach irritation and gastrointestinal bleeding. In using paracetamols, patients should not take more than 60mg/kg per day, as this drug has the potential to cause liver damage (Alam et al., 2004). On occasion, patients may suffer from high fevers even after taking paracetamols. If this occurs, tepid sponging is recommended. In addition to paracetamols, patients can take acetaminophen to treat and relieve DF. If the severity of the dengue infection worsens, proper management is required. In managing fluids and when taking aggressive medications, particular attention must be paid to signs of hemorrhaging. The WHO has published guidelines to help in the treatment and management of all dengue infections, especially in small hospitals (Guzman et al., 2010).

1.1.7 Dengue control and prevention strategies

Over the last 10 years, many specialists have adopted a similar global strategy to control and prevent dengue infections. This strategy focuses on three key aspects: planning and response surveillance, lowering the burden of the dengue infection and improving control of the vector by changing behaviours. Recently, enhanced and validated strategies and tools for preventing and controlling the infection have been established. These strategies and tools are accessible to all clinicians and other practitioners in the public health sector and include the development of a rapid commercial test for diagnosis, especially in countries with high endemic rates; the development of insecticide products to deal with mosquito bleeding; and the setting up of universal tactical frameworks for assimilated vector management.

1.1.7.1 Vector control

Proper prevention alternatives are required to address the high endemic rates of DENV transmission. A number of vector control components have been established and integrated into a number of control and prevention programmes; however, the delivery of these strategies and programmes is insufficient, especially among public health practitioners. To eliminate the principal DENV vectors (e.g., *A. aegypti*), consideration must be given to the general behaviours of the vector in relation to humans and preventive and control methods established (Rodriguez, Bisset, & Fernandez, 2007). This will assist in combining various methods of vector control, especially in relation to environmental and

chemical vectors such as the application of adulticides and larvicides. In relation to chemical vector control methods, specialists recommend that various control chemicals be stored in domestic water (e.g., cooking and drinking water). This method is effective in certain dosages and has been used to control *Aedes spp.* (Heintze, Velasco Garrido, & Kroeger, 2007). Other strategies can also be used to reduce the vector source, including clean-up campaigns; cleaning and emptying containers (both in households and public settings such as schools, green areas and cemeteries); installing water supply systems; managing and controlling solid wastes; and proper urban planning. In addition to these interventions, infrastructure should be heavily invested in to improve and enhance easily accessible, reliable and safe solid waste disposal and water supply systems.

The WHO guidelines state that it is important to enhance social mobilisation planning and communication in relation to the dengue disease, especially in respect of proper control and preventive measures. Based on Alam et al.'s (2004) suggestions, properly organised control services need to be developed that use partnership strategies and new tools (based on the principle of 'integrated vector management systems') to significantly reduce the transmission of DENV among humans.

1.1.7.2 Developing a DENV vaccine

Numerous challenges exist in relation to the prevention and control of DENV in communities. Scientists have shown a great interest in this issue. One satisfactory method

being developed by scientists is the effective use of vaccines (Hombach, 2007). A number of issues have slowed this initiative, including the complexity of DENV infection pathology, the lack of funds being invested to develop the vaccine, and the need to control concurrently all four of the main virus serotypes (the relationship between DHF/DSS and DENV and secondary DENV infection has imposed significant challenges on the development of a vaccine) (Hombach, 2007). Any vaccine needs to induce a strong immune response against all four DENV serotypes as well as in formerly immune persons. There is currently a lack of awareness of the methods that can be used to induce protective immunity against DENV infections. Specialists provide protective immunity by neutralising antibodies; however, more research is needed to find a method that helps to improve cellular immune responses. Any DENV vaccine must be reactogenicity free, able to induce life-long protection against any infection of DENV, and affordable.

Some of the approaches currently being used to develop DENV vaccines include the use of live attenuated viruses, inactivated viruses, deoxyribonucleic acid (DNA) vaccines, subunit vaccines, chimeric viruses, cloned engineered viruses and, finally, DENVs attenuated as backbones (Durbin et al., 2005; Raviprakash et al., 2006; Whitehead et al., 2003). Significant steps have been made in the development of vaccines against DENVs; For example, a dengue vaccine has been licensed in 2015, Dengvaxia® (CYD-TDV). It is a tetravalent dengue vaccine which is developed by Sanofi Pasteur. Approximately five additional dengue vaccine candidates are in clinical development, with two candidates developed by Butantan and Takeda and be expected to begin Phase III trials in early 2016.

Researchers have stated that fewer more fully licensed dengue vaccines should be readily available in 3 or less years (George, 2014).

1.2 Macrophages

1.2.1 The background to macrophages

Macrophages were originally identified by Metchnikoff (Van Furth et al., 1972). They are the main differentiated cells of a phylogenetically primitive system of cells known as the mononuclear phagocyte system (MPS). These cells have an important function in both innate and adaptive immunity and were initially assigned to the reticuloendothelial system (RES), which comprises the ECs, reticular cells, fibroblasts, monocytes and histiocytes. These cells were later placed in the MPS, which comprises the promonocytes, monoblasts, blood macrophages and monocytes (Van Furth et al., 1972).

1.2.2 Macrophage origin, development and distribution

Ontogenetically, the origin of macrophages is the ectoderm of the yolk sac (Moore & Metcalf, 1970). When the yolk sac reaches maturity, the bone marrow-derived stem cells yield the MPS (Metcalf & Moore, 1970). In responding to particular growth factors and cytokines, stem cells go through the two chronological differentiating processes of ‘maturation’ and ‘commitment’ (Ogawa, 1993). Within the bone marrow, IL-1, IL-3 and/or IL-6 are involved in activating resting stem cells to enter the cell cycle. The division of

stem cells yields pluripotent myeloid cells known as the granulocyte-erythrocyte-megakaryocyte-macrophage colony forming unit (GEMM-CFU). This precursor becomes a progenitor of macrophages and granulocytes, called the granulocyte-macrophage colony forming unit (GM-CFU), in the presence of IL-1 and IL-3. IL-3 and granulocyte-macrophage colony stimulating factor (GM-CSF) provoke the proliferation of these myeloid precursors (i.e., the GM-CFU). The macrophage colony-stimulating factor (M-CSF) (also referred to as the colony stimulating factor-1 [CSF-1]) induces differentiation to monocytic precursors (i.e., M-CFU) apart from the proliferation. These precursors (i.e., the M-CFU) subsequently differentiate further into monoblasts in the presence of IL-3, GM-CSF and M-CSF (Valledor, Borras, Cullell-Young, & Celada, 1998) (Figure 1-7).

The least mature cell of the MPS is the monoblast, which divides into promonocytes (the direct precursors of the blood monocytes that eventually move through the blood vessel walls into various organs and tissue systems and become a resident there) (van Furth et al., 1972). The maturation and differentiation of cells from the monocyte precursors (i.e., M-CFU) to tissue macrophages occur in the presence of growth factors M-CSF, IL-3 and GMCSF (Valledor et al., 1998). Throughout myelopoiesis, external differentiating signals in the form of growth factors or cytokines control the expression of a set of transcription factors that together determine the expression of myeloid-specific genes and the generation of monocytes and macrophages. The transcription factor PU.2 has an important function in this process, as it enhances the expression of the M-CSF receptor (Valledor et al., 1998).

Macrophages are derived from monocytes that grow in the bone marrow. They enter the bloodstream, circulate all over the body and squeeze through the endothelium into tissues. Once in the tissues, they are called macrophages or tissue macrophages. Macrophages are widely spread throughout the body and take specific names according to the organ in which they are prominent; for example, in the lymph nodes, lungs (alveolar macrophage), connective tissues (histocytes), spleen, liver (Kupffer cells), gastrointestinal tract, skin (histocytes), bone (osteoclasts), central nervous system (microglia), kidney (mesangial cells), serous cavities (pleural and peritoneal macrophages) and synovium (type A cells) (Figure 1-7). These cells exhibit great structural and functional heterogeneity in diverse organs (Adams & Hamilton, 1984). A cardinal feature of the MPS is that macrophages can alter once within a specific tissue. Tissue macrophages can complete proliferative capabilities and are not dependent upon bone marrow stem cells to maintain their population, despite being derived from circulating monocytes (Hamilton, 1993). Inflammatory peritoneal or murine resident macrophages and bone marrow-derived macrophages (BMDM) have been applied in the dissection of the complex processes of macrophage biology *in-vitro*. The cells come from the peritoneal cavity; whether inflammatory or resident, these cells are heterogeneous populations and show a spectrum of functional and morphological phenotypes (Cohn, 1978). A population with more homogenous macrophages can be acquired by culturing bone marrow progenitors in liquid cultures with the presence of M-CSF or other CSFs, like GM-CSF (Warren, Mao, Rodriguez, Miao, & Aderem, 2008).

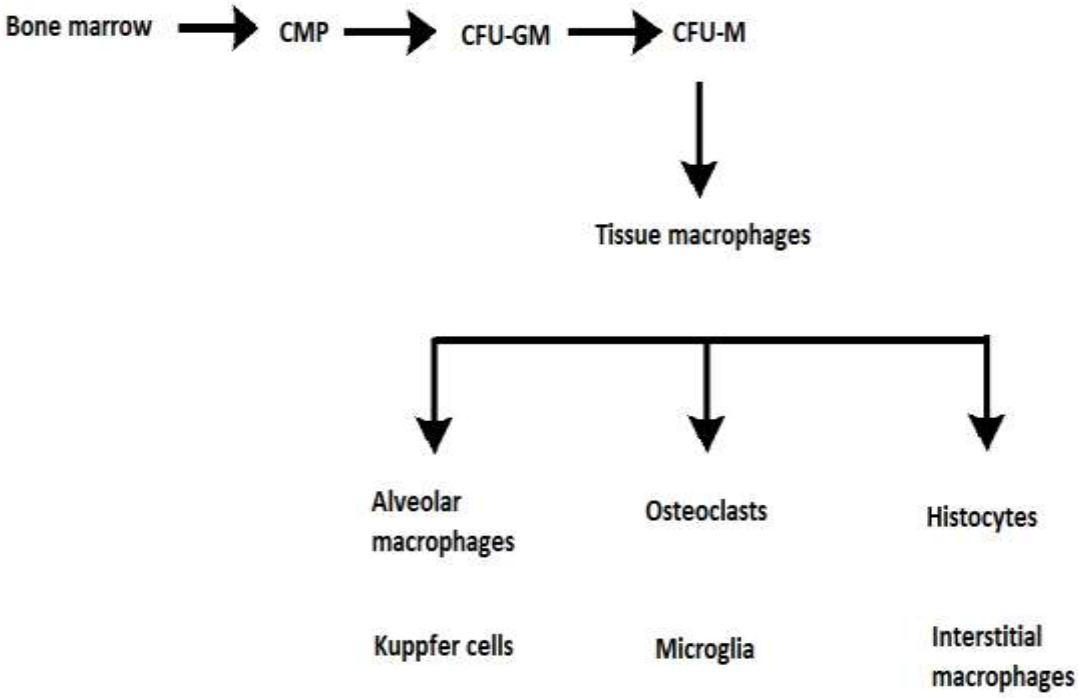


Figure 1-7: Macrophage differentiation map

1.2.3 Macrophage activation and functions

George Mackaness was the first to put forward the concept of 'macrophage activation' (Mackaness, 1962). Mackaness theorised that individuals who recover from infections caused by one microorganism might gain the capacity to defend against infections from the same pathogens and, temporarily, against a wide spectrum of unrelated microorganisms. This theory was directly related to macrophage-mediated killing. The antimicrobial activity creates the concept of defining the term 'macrophage activation' (Mackaness, 1962). The concept of macrophage activation was later broadened to incorporate the destruction of tumours following the discovery that the stimulation of such a cytolytic function was due to a multistep cascade of events (Adams & Hamilton, 1984).

The word ‘activation’ is relatively controversial, as macrophages have pleiotropic properties that cannot be provoked in a concerted manner. A variety of definitions has been proposed for the activation of macrophages. Macrophage activation has also been the subject of many reviews (Adams & Hamilton, 1984; Galli, Borregaard, & Wynn, 2011; Italiani & Boraschi, 2015; Valledor et al., 1998; Van Furth et al., 1972). Nathan (1986) defined macrophage activation as the enhancement (through immunologically specific reactions) of the immunologically nonspecific capability of the macrophage to destroy microbial pathogens. Activation changes the morphology and functional activity of macrophages, with phagocytic macrophage activation being initiated by cytokines, including macrophage activation factor (MAF) and macrophage migration-inhibitory factor (MMIF), immune complexes C3b, and various peptides, polysaccharides and immunologic adjuvants. M-CSF causes the cell line to proliferate and mature into macrophages. Macrophages originate from the circulating monocytes that differentiate into macrophages during inflammation, cellular immunity and the production of pro-inflammatory cytokines (Wu et al., 2013). Their activation generally refers to the activation and suppression of various genes that encode the proteins important to the role being activated (Adams & Hamilton, 1984; Hamilton, 1993).

The lack of permanency of macrophage activation is important. This activation is controlled by suppressive and inductive signals. Once activated, the cessation of inductive signals and the application of suppressive signals return the cells to their basal state (Adams & Hamilton, 1984; Hamilton, 1993). Numerous signals control the activation of macrophages, including various cytokines, some surface molecules of T cells, colony

stimulating factors, growth factors and small inflammatory proteins (Adams & Hamilton, 1984; Hamilton, 1993; Krishnan, Robertson, & Thwaites, 2013; Stout, 1993).

Macrophages are part of the family of mononuclear leukocytes and act as the key regulators of the innate immune response. They are also regarded as the first line of host defence in fighting harmful microorganisms. Macrophages ingest pathogens, digest them and present their antigens with MHC class II molecules on their cell membranes to B lymphocytes and T cells to generate antigen-specific immune responses (adaptive immunity). Macrophages also help in the opsonisation of the virus or the cells with antibodies attached to control and eliminate the virus (Kyle, Beatty, & Harris, 2007). Differentiation occurs in response to inflammatory or immunologic stimuli being rendered beside the activity of foreign elements (e.g., the phagocytosis of pathogens, dead cells and cellular debris, or inflammatory and immunological processes) (Italiani & Boraschi, 2015). IFN-γ is then released by NK cells after the initiation of the host innate immunity response. This stimulates macrophages to trigger microbial activity and produces highly reactive chemical species (e.g., reactive oxygen species [ROS]) to destroy microbes (Gordon, Pluddemann, & Martinez Estrada, 2014).

The activated macrophages then move rapidly through the blood vessel walls to the inflamed tissue. At this stage, the macrophages are highly phagocytic and thus can engulf unwanted cells. After the stimulation, they secrete a variety of products, including enzymes (e.g., lysozyme, chemokines and cytokines [i.e., IL-1, IL-6, IL-12, IL-18, TNF-α, and IL-

10]) that activate tissue repair, other immune cells, inflammation and host defences (Italiani & Boraschi, 2015).

1.2.4 The classification of macrophages

Macrophages are versatile cells that play different roles in immunity, there are two main groups classified into M1 and M2. M1 macrophages are macrophages that stimulate inflammation, whereas M2 macrophages have the ability to decrease inflammation and promote tissue repair (Mills, 2012). M1 macrophages are able to metabolize arginine to the "killer" molecule nitric oxide, whereas M2 macrophages have the ability to metabolize arginine to the "repair" molecule ornithine. LPS and IFN-gamma can activate M1 macrophages and produce high levels of IL-12 and low levels of IL-10. However, the M2 macrophages are broadly referred to macrophages that function in building processes like tissue repair and wound healing, and those that produce anti-inflammatory cytokines like IL-10 to turn off damaging immune system (Mosser & Edwards, 2008). M2 is the resident tissue macrophages, it can be further elevated by IL-4 and secrete high levels of IL-10, TGF-beta and low levels of IL-12. Tumor-associated macrophages that actively promote tumor growth are beyond to the M2 phenotype.

1.2.6 The role of macrophages in dengue infection

Macrophages play a key role in homeostatic, inflammatory and immunological processes and are also considered the major target cells in DENV infection, as they create room for intracellular viral replication (Halstead, 1989; Honda et al., 2009). In the process of DENV infection, the virus reproduces within the macrophage (Chaturvedi, Nagar, & Shrivastava, 2006). Consequently, the infected macrophages are stimulated to produce various innate cytokines (e.g., TNF-α, IL-6, IL-10 and IL-18) (Srikiatkhachorn & Green, 2010). The making of cytokines has been associated with dengue haemorrhage development and increased the severity of DF (Chaturvedi et al., 2006; Wu et al., 2013). It has been suggested that DENV can infect macrophages through different attachment receptors dependent on the cell type, such as the C-type lectins, DC- and L-SIGN or through a trypsin-resistant Fc receptor. Reyes-Del Valle et al., (2005) have shown that heat shock protein (HSP) 90 and HSP70 act as receptor complexes in human cell lines and in macrophages. When a virus enters the target cell, generally beginning with the reaction of viral envelope (E) glycoproteins with specific entry receptors and co-receptors, sometimes need to trigger the virus-cell membrane fusion.

The efficiency of DENV replication by macrophages is higher than that of peripheral lymphocytes, however, it is lower than that of human lymphoblastoid cell lines (Theofilopoulos et al., 1976). The clinical outcomes may be determined by the degree of DENV replication in the early phase of infection, which range from asymptomatic infection and febrile illness (i.e., DF) to life-threatening hemorrhagic disease (i.e., DHF and DSS).

Interstitial DCs and LCs (the cells of macrophage lineage) provide the first line of the innate defence against the invading DENV in the skin, where it replicates after the initial bite by an infected mosquito (S. J. Wu et al., 2000). Early activation of NK cells and type-I IFN-dependent immunity may decrease viral replication during the initial stages of DENV infection (Beltran & Lopez-Verges, 2014; S. J. Wu et al., 2000).

Patient's previous exposure to DENV is one of the major risk factors for DHF and DSS. Primary DENV infections usually result in non-complicated DF and the development of both humoral and cellular immunity; both of which are long term and protect the host from reinfection by the same serotype. However, this anti-dengue immune response is cross-reactive in nature, it does not have long-term cross-protection to other serotypes. Instead, an association of DHF with secondary infections has been observed (Chotiwan et al., 2014), providing the dengue pathogenesis. ADE can enhance the infection of macrophages and monocytes, both *in-vitro* and *in-vivo* (Kliks, Nimmanitya, Nisalak, & Burke, 1988; Schieffelin et al., 2010). ADE start from the infection of mononuclear phagocytes through their Fc receptors by immune complexes formed by DENVs and non-neutralising antibodies. These non-neutralising antibodies due to previous heterotypic dengue infection or from low concentrations of dengue antibodies of maternal origin in infant sera (Kliks et al., 1988). One working hypothesis of dengue pathogenesis is that severe from of disease in infants with primary infection and in older people with secondary infection is the consequcne of ADE during the infection of phagocytes. This hypothesis is consistent with the available evidence (Ubol et al., 2007).

DENV-infected macrophages present DENV antigen to B cells both *in-vitro* and *in-vivo*, generate their clonal expansion, as shown by increasing the virus-specific IgM antibody plaque-forming cells. The consequcne of these cells intend to the number of DENV-infected macrophages. During DENV infection, different cytokines are produced that are specific to dengue and have not been reported in any other type of virus infection. Most of the cytokines are secreted by macrophages in patients with DENV disease (Table 1-2). The functions and effects of these cytokines on immunity and pathogenesis have been discussed in section 1.1.4.2.

Table 1-2: Cytokines secreted by macrophages in patients with DENV infection

Cytokines	**DF**	**DHF**	**Reference**
IL-1β	↑ ↑ ↑	↑	(Kuno & Bailey, 1994; Rathakrishnan et al., 2012)
IL-6	↑	↑ ↑ ↑	(Chaturvedi, Agarwal, Elbishbishi, & Mustafa, 2000)
IL-8	↓	↑ ↑ ↑	(Chaturvedi et al., 2000; Pandey et al., 2015)
IL-10	↓	↑ ↑ ↑	(Chaturvedi et al., 2000; Pandey et al., 2015)
IL-12	↑ ↑ ↑	↑ ↑ ↑	(Chaturvedi et al., 2000; Pacsa et al., 2000)
IL-18	↑	↑ ↑ ↑	(Chaturvedi et al., 2000)
TNF-α	↑ ↑ ↑	↑ ↑ ↑	(Chaturvedi et al., 2000)
TGF-β	↓	↑ ↑ ↑	(Laur et al., 1998; Pandey et al., 2015)

Note: increased (↑), markedly increased (↑ ↑ ↑), decreased (↓)

DENV-infected macrophages can send a signal to recruit suppressor T-1 cells (TS1), which secrete a suppressor cytokine (SF1). These cytokines then recruit second subpopulations of suppressor T-2 cells (TS2) and produce another soluble suppressor

cytokine (SF2). The SF2s induce the third subpopulation of suppressor T-3 cells (TS3), which suppress the antigen-specific humoral response via acting on B cells and T helper cells (Shukla & Chaturvedi, 1981). This suppression can limit the effect of ADE-mediated DENV replication; however, it also delays the elimination of DENV by suppressing neutralising bodies produced by the host. It is reported that nitrates and calcium ions serve as intracellular signals of macrophages in the transmission of suppressor signals to T cells (Khare & Chaturvedi, 1995). The transmission of the suppressor signal from TS1 to TS2 is controlled by the calcium channel block treatment of macrophages by blocking the influx of calcium in a dose-dependent manner (Khare & Chaturvedi, 1995). DENV is capable of inducing increased levels of nitrates when co-cultured with human Kupffer and spleen cells. Increased levels of nitrates are found in DF patients, but not in DHF or DSS patients (Valero, Espina, Anez, Torres, & Mosquera, 2002). In addition to the basic functions of macrophages, like ingestion, digestion and elimination of DENV by phagocytosis, macrophages can serve as host cells for the replication of DENV, complicating the immune response by ADE or immune suppression.

1.4 Vascular endothelium

DHF and DSS are characterised by microvascular plasma leakage. Presently, the precise molecular mechanisms causing microvascular leakage are unknown, but the disruption of the EC barrier may be a critical factor. Thus, it is important to understand the basic structure of the vascular endothelium.

1.4.1 The basic structure of the vascular endothelium

ECs adhere to each other at the following main junctions: (A) tight junctions, (B) adherens junctions and (C) gap junctions (see Figure 1-8). Integrins on the cell surface fix the cells to the extracellular matrix (ECM). The relationship between the ECM and integrins produces signals that hinder EC migration and proliferation, but also stimulates cell–cell and cell–ECM adhesion (Mehta & Malik, 2006). Together, these intercellular and ECM interactions form the endothelial barrier (Bazzoni & Dejana, 2004; Mehta & Malik, 2006). In models that study vascular endothelium permeability, disturbing the function of integrins results in the permeation of slightly impermeable or impermeable molecules (Bazzoni & Dejana, 2004; Mehta & Malik, 2006). Consequently, the proteins involved in the adherens junctions (e.g., the vascular EC-specific transmembrane protein, VE-cadherin) are examined regularly. Junctional adhesion proteins are also responsible for connecting the cytoskeletal and signalling proteins through their cytoplasmic tails, thus providing anchoring room to acting microfilaments and intracellular signal transduction (Dejana,

2004). This association allows for the regulation of functional permeation and stabilises the adherens junctions (Mehta & Malik, 2006).

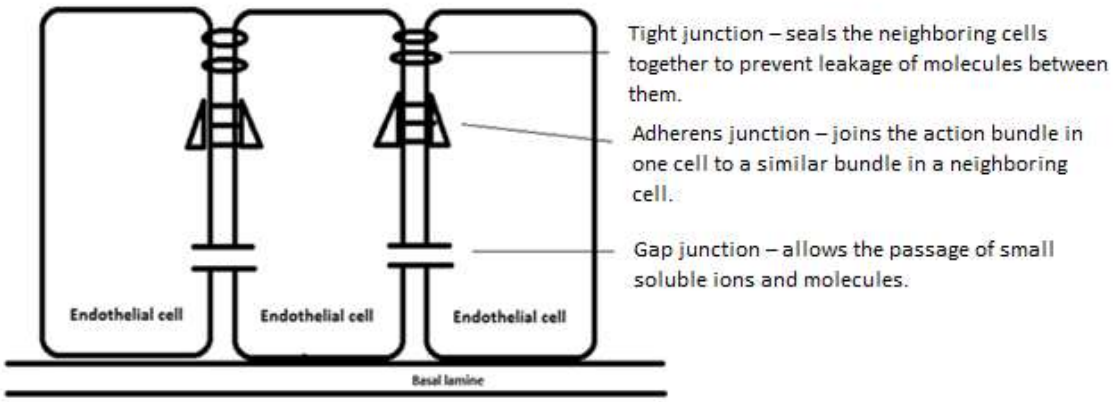


Figure 1-8: The structure of healthy endothelium and the functions of the junctions

1.4.2 The role of ECs in DENV infection

1.4.2.1 Endothelium damage caused directly by DENV

The apoptosis of DENV-infected ECs has been reported as the mechanism by which vascular endothelial barrier integrity is lost causing vascular leakage syndrome (as observed in patients with DHF and DSS) (Avirutnan, Malasit, Seliger, Bhakdi, & Husmann, 1998; H. C. Chen, Hofman, Kung, Lin, & Wu-Hsieh, 2007; Liew & Chow, 2004). Evidence suggests that DHF patients have decreased ECs by means of apoptosis; however, it is not clear if these cells underwent apoptosis as a direct or indirect consequence of DENV infection (Limonta, Capo, Torres, Perez, & Guzman, 2007). Previous studies reporting a significant apoptosis of DENV-infected ECs *in-vitro* were conducted in the

ECV304 cell line (Avirutnan et al., 1998; Liew & Chow, 2004), derived from a bladder carcinoma rather than of endothelial origin (Kiessling, Kartenbeck, & Haller, 1999). Therefore, this theory requires re-examination.

The release of inflammatory cytokines by DENV-infected ECs is also thought to trigger vascular leakage *in-vitro*. Infected HUVECs and human dermal microvascular EC line (HMEC-1) monolayers generate inflammatory cytokines, IL-8 and IL-6, that are capable of inducing permeability of vessels (Huang et al., 2000; Talavera, Castillo, Dominguez, Gutierrez, & Meza, 2004). Further, IL-8 is involved in recruiting lymphocytes, neutrophils and eosinophils to the production site. Thus, it is possible that DENV-infected ECs may inititate effector cells to the infection by generating chemokines, thereby aggravating local inflammation and, consequently, inducing a sequence of events leading to vascular leakage. DENV infection was also shown to induce 269 genes and suppress 126 genes in HUVEC (Warke et al., 2003). Genes that were up-regulated included those with functional roles in defence, immune responses, stress, cell adhesion and wounding, and inflammatory and anti-viral responses. Genes that were down-regulated included cytoskeletal genes and membrane proteins (Warke et al., 2003).

Thus, there is evidence that DENV infects EC *in-vitro*; however, the issue of whether DENV infects ECs *in-vivo* remains controversial. One study examined tissue specimens obtained from biopsies and autopsies of patients with DHF, DSS or DF and found the DENV antigen in sinusoidal ECs of the liver and vascular ECs of the lung (Jessie et al., 2004). However, other autopsy studies of dengue patients have not found any proof

of pathological harm to the endothelium or the presence of DENV antigens in ECs (Halstead, 1989). This failure to detect the DENV antigen in ECs may be explained by the rapid occurrence of DHF or DSS after defervescence and the clearance of viraemia (Vaughn et al., 2000). New mice models of DENV-ADE show a significant infection of the liver sinusoidal ECs *in-vivo* (Zellweger, Prestwood, & Shresta, 2010). Thus, conclusions on cellular tropism drawn from autopsies may not be representative of the actual target cells of DENV during acute infection.

1.4.2.2 Endothelium damage caused by indirect mechanisms

Vascular leakage may occur independently of direct DENV infection of ECs. A key observation in DHF and DSS patients is that symptoms of severe illness, including haemorrhagic manifestations and vascular leakage, appear as the patient's condition suddenly deteriorates around the time of defervescence and the clearance of viraemia (George, 2014). Thus, a strong inflammatory response may spoil the EC barrier function.

It appears that activated immune cells participate in the vascular leakage observed in DHF and DSS patients through the secretion of inflammatory cytokines. Elevated levels of TNF-β, IL-6, IL-lβ, IL-8, IFN-γ and MCP-1 have been documented in the serum of patients with DHF and DSS (Y. R. Lee et al., 2006; Pang, Cardosa, & Guzman, 2007). The probable contributors of these cytokines include T cells, DENV-infected or activated monocytes and macrophages, B cells and mast cells. Monocytes and macrophages infected

in-vitro with DENV or under ADE conditions have been shown to release factors into a culture medium that activate and increase the permeability of the HUVEC barrier (Carr et al., 2003; E. Lee, Pavy, Young, Freeman, & Lobigs, 2006). These factors include IL-1 (Anderson, Zafar, Nizam, & Berry, 1997), IL-6 (Huang et al., 2000), IL-8 (Huang et al., 2000) and MCP-1 (E. Lee et al., 2006). Dengue-specific T cells may also activate the generation of cytokine and vascular leakage. Studies show that patients with secondary heterotypic dengue infections have many dengue-specific CD8+ T cells that produce high levels of cytokines, IFN-γ and TNF-α (Mongkolsapaya et al., 2003). Additionally, isolated human primary B cells can be infected with DENV directly or under ADE conditions *in-vitro,* with these infected B cells releasing IL-6 and TNF-α (Y. W. Lin et al., 2002).

It should be noted that in a peripheral blood mononuclear cell (PBMC) culture, only monocytes can be infected with DENV or DENV-antibody immune complexes (not T or B cells) (Kou et al., 2008). Immature DCs can also be infected with DENV overproduce matrix metalloproteinase (MMP)-9 and, to a lesser extent, MMP-2. MMP-9 and MMP-2 activate the vascular permeability connected with a loss of VE-cadherin and F-actin reorganisation (Luplertlop et al., 2006). Further, in addition to blood mononuclear cells, mast cells infected with DENV under ADE conditions exude factors that activate ECs and up-regulate EC adhesion molecules. However, this activation relies on TNF-β and does not stimulate EC permeability. The schematic diagram in Figure 1-9 summarises the possible interactions between DENV, DENV-antibody immune complexes and PBMCs that result in the generation of inflammatory cytokines and most probably cause vascular leakage.

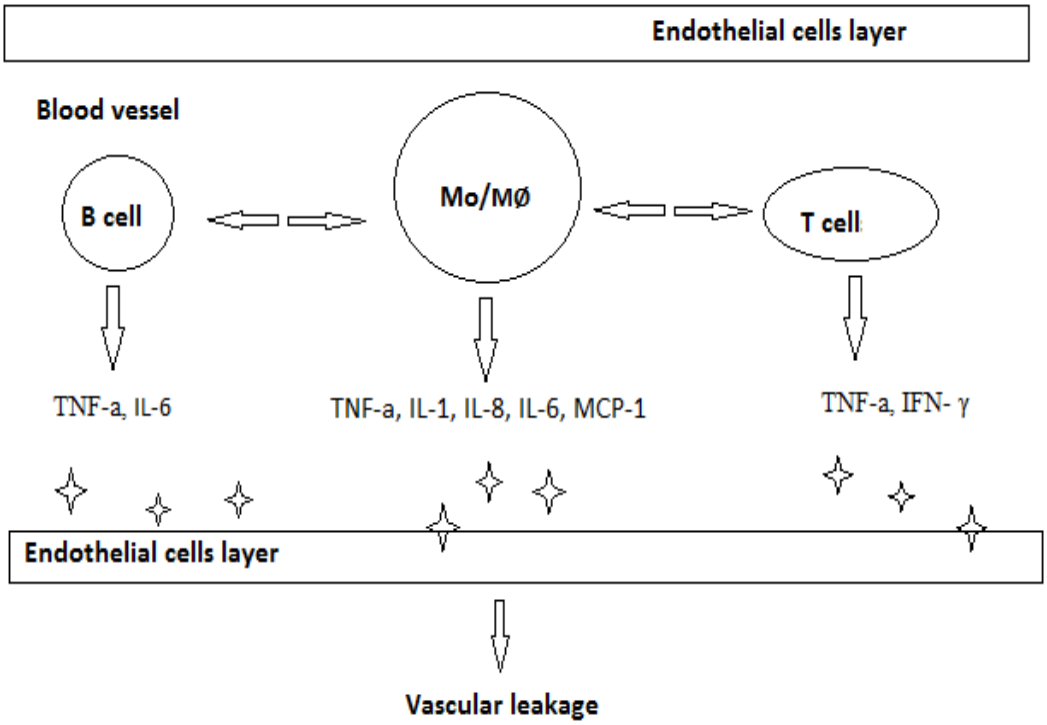


Figure 1-9: The role of immune cells in vascular leakage during DHF and DSS

1.5 Pyroptosis

Eukaryotic cells have the ability to initiate various programmes of cell death with diverse morphological and physiological results. Apoptosis was initially a well-recognised programmed cell death. Programmed cell death is widely functional through several genetically definite pathways, as the cell plays an active role in damaging itself; for example, by autophagy, caspase-1-dependent cell death and oncosis (also known as pyroptosis) (Bailie, Stowe, & Schmitt, 1978). Pyroptosis has recently been recognised as the pathway of host cell death induced by a variety of microbial agents (e.g., Francisella, Legionella and Salmonella,), non-infectious stimuli and host factors created during myocardial infarction (Y. Yang, Jiang, Zhang, & Fan, 2015).

Caspase-1 is primarily known as an IL-1β-converting enzyme and is the main known protease used to process the inactive precursors of IL-1β and IL-18 into active inflammatory cytokines (Goldstein, 1998). However, caspase-1 activation results not only in the making of activated inflammatory cytokines, but also in a quick cell death categorised by plasma-membrane breakage and the release of pro-inflammatory intracellular matters (Rollof, Nordin-Fredriksson, & Holst, 1989). Caspase-1-dependent cell death, or pyroptosis, is a unique form of cellular self-destruction facilitated by caspases. While not initially differentiated from apoptosis (Bergsbaken, Fink, & Cookson, 2009), the mechanism features and results of pyroptosis are quite different from those in apoptosis (Bergsbaken et al., 2009; Rollof et al., 1989). The term pyroptosis, from the Greek '*pyro*' (meaning fire or fever) and '*ptosis*' (meaning a deterioration), refers to the integrally inflammatory procedure of caspase-1-dependent programmed cell death (Yu & Finlay, 2008).

1.5.1 Mechanism and characteristics of pyroptosis

Pyroptosis is a method of programmed cell death known for its exceptional molecular mechanism and morphological features (Duprez, Wirawan, Vanden Berghe, & Vandenabeele, 2009). This is a vigorous method of cell loss in which the host eliminates unused cells, and it is strongly controlled and measured by a genetical pathway (Galluzzi et al., 2012). The initialisation of pyroptosis can be activated by a diversity of microbial agents or non-infectious stimulants (Broz, von Moltke, Jones, Vance, & Monack, 2010; Keller, Ruegg, Werner, & Beer, 2008). It is viewed as an innate immune response that

eradicates invading intracellular pathogens and is described by monocytes, macrophages and DCs (Duprez et al., 2009). Microbial pathogens that trigger the procedure include *Salmonella enterica, Shigella flexneri, Listeria monocytogenes, Francisella spp.* and *Legionella spp.* (Bergsbaken et al., 2009; Duprez et al., 2009; Sauer et al., 2010; Yeretssian, Labbe, & Saleh, 2008). This cell death pathway is exclusively facilitated by the proteolytic enzyme caspase-1 (Bergsbaken et al., 2009). The precise pathway of caspase-1 differentiates it from apoptosis, autophagy, oncosis and necrosis (Broz et al., 2010).

Pyroptosis apperants rapid plasma membrane damgae and the release of pro-inflammatory intracellular contents, which contrasts with the packaging of cellular contents and the non-inflammatory phagocytic uptake of membrane-bound apoptotic bodies that characterises apoptosis. The rupture of cell membranes during pyroptosis results from caspase-1-dependent processes and the formation of plasma membrane pores (Bergsbaken et al., 2009; S. L. Fink, Bergsbaken, & Cookson, 2008) (Figure 1-10). Caspase-1-dependent plasma membrane pores dissipate cellular ionic gradients, producing a net increased osmotic pressure. This leads to water influx, cell swelling (S. L. Fink & Cookson, 2007) and eventual osmotic lysis, releasing inflammatory intracellular contents (Figure 1-10). It is reported that *Salmonella* infection or treatment with lethal toxins from *Bacillus anthracis* can result in the formation of plasma membrane pores with a functional diameter of 1.1–2.4 nm (S. L. Fink et al., 2008), and that the formation of these pores is dependent on caspase-1 activity (Bergsbaken et al., 2009).

While the fatal cleavage of chromosomal DNA is a feature of apoptotic cell death, DNA damage also occurs during pyroptosis (Hilbi, Chen, Thirumalai, & Zychlinsky, 1997). During apoptosis, the caspase-mediated proteolysis of inhibitor of caspase-activated DNase (ICAD) releases caspase-activated DNase (CAD). CAD cleaves DNA between nucleosomes to produce oligonucleosomal DNA fragments of approximately 180 bp (S. L. Fink & Cookson, 2006). Even purified caspase-1 can able to cleave ICAD *in-vitro*, ICAD degradation does not occur in pyroptosis (S. L. Fink & Cookson, 2006). DNA cleavage in pyroptosis results from the action of an unidentified caspase-1-activated nuclease and that does not achieve oligonucleosomal DNA fragmentation pattern characteristic of apoptosis (Rollof et al., 1989). DNA cleavage is accompanied by marked nuclear condensation, but unlike in apoptosis, nuclear integrity is maintained (Bergsbaken & Cookson, 2007). The break down of the actin cytoskeleton was found in cells undergoing pyroptosis, but the importance and mechanism of this destruction remain unclear (Bergsbaken & Cookson, 2007). Caspase-1-dependent degradation of cellular inhibitor of apoptosis protein (CIAP) also occurs in parallel with pyroptosis but the mechanism is also unknown (Wickliffe, Leppla, & Moayeri, 2008). Caspase-1 cleaves and inactivates metabolic enzymes during pyroptosis. Furthermore, the identification of new proteolytic targets of caspase-1 could provide an insight into the mechanism and novel features of pyroptosis.

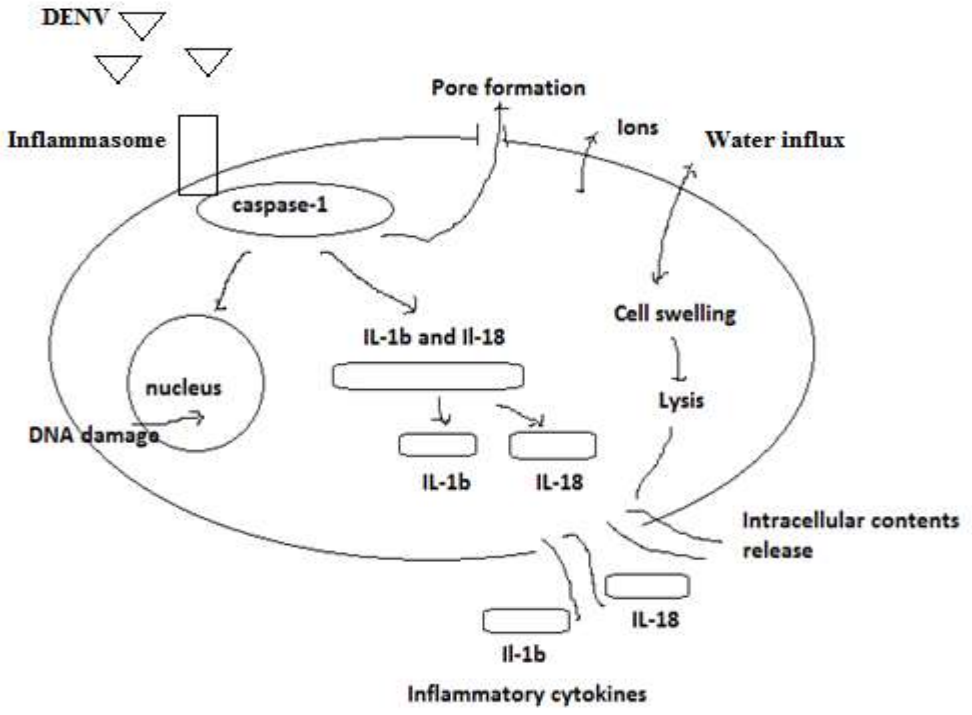


Figure 1-10: The mechanism of pyroptosis

1.5.2 Caspase-1

Caspase-1 is a distinctive chracterirstic of pyroptosis, and it is the enzyme that control the process of cell death (Figure 1-11) but not in apoptosis. There was no defects in apoptosis on Caspase-1-deficient mice and it can develop normally (Zengaffinen & Lange, 1997). The apoptotic caspases, such as caspase-3, caspase-6 and caspase-8, are not involved in pyroptosis (Talan, Citron, Abrahamian, Moran, & Goldstein, 1999) and substrates of apoptotic caspases, including poly (ADP-ribose) polymerase and ICAD, do not proceed proteolysis during pyroptosis (J. Fink et al., 2006). Additionally, release of cytochrome c and the loss of mitochondrial integrity, which is believed to induce apoptotic caspases, do not occur during pyroptosis (Cervantes, Nagata, Uchijima, Shibata, & Koide, 2008).

Caspase-1, formerly known as the IL-1β-converting enzyme (ICE), is an affiliate of the caspase family of cysteine proteases and acts as an essential effector protein in the course of pyroptosis. Caspase-1 contains two heterodimers that link to form a tetramer (Winkler & Rosen-Wolff, 2015). Caspase-1 can cleave to cellular substrates that would lead to irritation or cell loss (Martinon, Burns, & Tschopp, 2002; Thornberry & Lazebnik, 1998). Initially, caspase-1 is synthesised as an inactive protein domain of 45 kDa, consisting of two subunits (i.e., p20 and p10) (Figure 1-11) (Martinon et al., 2002). The mature caspase-1 is formed by two pro-caspase-1 subunits (i.e., p20 and p10) joined together as a dimer and then further combined together as a heterodimer (Figure 1–11). The caspase-1 precursor can be changed enzymatically into a biologically active form by the autoproteolytic splitting of the two subunits if the active location is situated at the p20 unit (Broz et al., 2010). The two pro-caspase-1 subunits are then detached (Chowdhury, Tharakan, & Bhat, 2008).

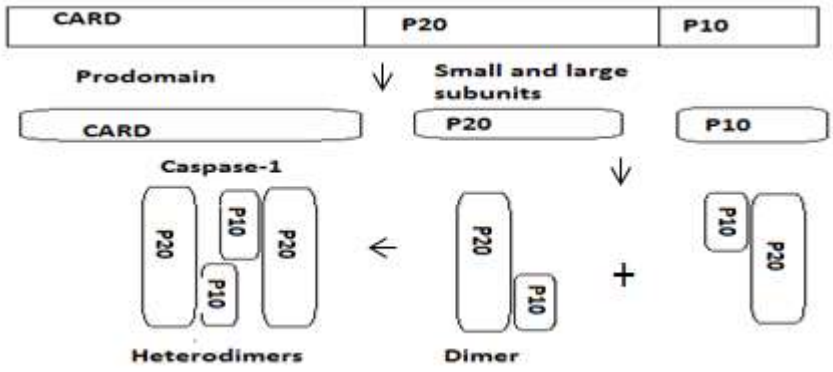


Figure 1-11: Structure of caspase-1

In general, caspases are classified into three main groups: inflammatory caspases, initiator caspases and effector caspases. Inflammatory caspases are involved in the

activation of cytokine. A range of signals activates initiator caspases and, consequently, triggers effector caspases. Effector caspases are also responsible for degrading cellular proteins (Hebert, Valdes, & Bentley, 2009). Activated caspase-1 is an inflammatory caspase responsible for triggering the production of pro-inflammatory cytokines such as IL-1β and IL-18. Caspase-1 assists in repairing other machinery (e.g., lipid membrane biogenesis) and is used for cell endurance after harm, for example, from bacterial pore-forming toxins (Lamkanfi, 2011).

It is recognised that the initiation of caspase-1 is activated within a large cytosolic multiprotein complex called inflammasome. Upon caspase-1 activation, the inflammasome is gathered and used to trigger undeveloped pro-caspase-1 to create active caspase-1 (Duprez et al., 2009; Yeretssian et al., 2008). Originally, the pro-inflammatory cytokines IL-1β and IL-18 are manufactured as precursor inactive particles and are sedentary in nature (Davis, Wen, & Ting, 2011). Caspase-1 has an enzymatic activity for the activating and maturing of IL-1β and IL-18 in the cytoplasm (Keller et al., 2008) whereby the N-terminal amino acids of the pro-inflammatory cytokines is removed by caspase-1 resulting in a ‘mature’ bioactive form.

1.5.4 Nod-like receptors

In nod-like receptors (NLRs), nowadays known as nucleotide oligomerisation domain receptors, cytoplasmic proteins identify endogenous or microbial molecules and the molecules formed in reaction to stress that may have a diversity of functions in variable inflammatory and planned cell death (Inohara & Nunez, 2003). NLRs are encoded by genes from a large family that found in many animal species (there are more than 20 NLR genes in humans). NLRs can be separated into two main subfamilies: the NLRs (formerly called the NODs) and the NLRPs (formerly called the NALPs) (Inohara & Nunez, 2003; Strober, Murray, Kitani, & Watanabe, 2006). A number of NLRs are thought to be pattern recognition receptors (PRRs), able to sense the microbial yield in the cytoplasm of cells, although some members have diverse functions. Thus, NLRs have a key role in nonspecific immune reactions (Strober et al., 2006). NLRs can also induce the activation of the cysteine protease caspase-1 (Martinon, Mayor & Tschopp, 2009), which results to caspase-1-dependent pyroptosis and the release of the inflammatory cytokines IL-18 and IL-1β (Figure 1-10) in response to the risk signals from bacterial, viral and host molecules and poisonous foreign products. Potassium efflux is a common reaction to many of these stimuli, and blockage of caspase-1 activation can prevent potassium efflux (Kahlenberg & Dubyak, 2004). However, potassium efflux alone will not activate caspase-1 (Kahlenberg & Dubyak, 2004). Further, preventing potassium effluxes will block the caspase-1 activation mediated by another NLR, NLRP1b (also known as NALP1b). Thus, the activation signal of NLRP3-dependent caspase-1 may not be directly controlled by potassium efflux ; however, it can create an circumstances that is encouraging for detection and caspase-1 activation (Pelegrin & Surprenant, 2007).

The NLR protein NLRC4 (the NLR family card domain-containing protein 4, also referred to as IPAF) mediates the preception of various bacterial pathogens that exist extracellularly (e.g., *Pseudomonas*) or intracellular orgnaims (e.g., *Shigella*, *Salmonella*, *Listeria* and *Legionella*,) during infection, and allocates the necessities for the caspase-1 activation. The pathogens stimulate virulence determinants to host cells by translocation systems (Warren et al., 2008). The appearance of flagella in the cytoplasm of macrophage cytosol stimulates NLRC4-dependent pyroptosis, showing that NLRC4 directly recognises flagella (Lightfield et al., 2008); however, this contact has not been confirmed. Remarkably, NLRC4-dependent caspase-1 activation has been described during the infection of *Listeria Shigella* and *pseudomonas* mutants, which have no flagella-generating ability (Lightfield et al., 2008). The studies show that NLRC4, like NLRP3, can react with additional bacterial components that remain unclear.

NLRP1b recognises the cytosolic toxin released by *B. Anthracis*, a metalloprotease that can cleave to host mitogen-activated protein kinases (MAPKs). The proteolytic action of the toxin is essential for caspase-1 activation, but MAPK cleavage alone is inadequate. This suggests that an unidentified fatal toxin is involved (S. L. Fink et al., 2008). Proteasome action is also a requisite for the activation of caspase-1 in responding to lethal stimuli (Wickliffe et al., 2008). Numerous NLR proteins in addition to those mentioned above have been involved in caspase-1 activation (Martinon et al., 2009). The NLR neuronal apoptosis inhibitory protein 5 (NAIP5) has the ability to activate caspase-1 in *Legionella* infections; however, it does not appear to be essential to all bacteria that stimulate caspase-1 via NLRC4 (Lightfield et al., 2008). The exact role of NAIP5 in pyroptosis remains unknown.

NLRP3 (NACHT, LRR and PYD domains comprising protein 3, also known as NALP3), one of the NLR proteins, reacts to numerous stimuli, including pore-forming toxins (Mariathasan et al., 2006), extracellular ATP released by pathogens (Mariathasan et al., 2006), uric acid crystals (Shek et al., 2009), virus DNA (Pontillo et al., 2012), viral RNA (Kanneganti et al., 2006), asbestos (Dostert et al., 2008) and ultraviolet B irradiation (Feldmeyer et al., 2007). However, the mechanism of NLRP3 senses this different category of signals is unclear. It is clear that host cells react to most of these stimuli via the generation of signals that react with NLRP3; however, additional studies are necessary to define how NLRP3 directly distinguishes or involve in the reaction to such a wide series of molecules.

1.5.5 Pyroptosis signalling

Pyroptosis is a part of the innate response against 'danger stimuli' and limits the spread of infection by inducing host cell death (Duprez et al., 2009; Lara-Tejero et al., 2006). The process of cell death starts by a selection of complex molecular pathways. To date, the exact molecular mechanism of pyroptosis is unknown; however, the beginning of pyroptosis is activated by the recognition of dangerous signals, usually known as pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), by a limited number of germline-encoded receptors, or PRRs (Medzhitov & Janeway, 2002). The role of PRRs is to perceive the occurrence of the dangerous signals (Figure 1-12).

The molecules of PAMPs are not present in the host and are derived from diverse classes of pathogens with different chemical structures. These molecules are released into the host's extracellular or intracellular space during pathogen invasion. Lipopolysaccharide (LPS) of gram-negative bacteria is the best known of the PAMPs (Suzuki et al., 2007). Viral PAMPs largely consist of nucleic acids, such as double-stranded RNA, uncapped single-stranded RNA and cytosolic DNA. In contrast, DAMPs are the non-microbial endogenous particles released from the host under cellular stress, tissue damage or cell lysis. The recognition of DAMPs by PRRs can induce downstream signalling pathways of pyroptosis (G. Y. Chen & Nunez, 2010). The PRRs only exist in the cytoplasmic or endosomal membrane, the extracellular space, the intracellular vesicle and the cytoplasm of immune cells (X. Zhang & Mosser, 2008). Cells such as macrophages, neutrophils, monocytes and ECs articulate the PRRs and are able to sense the microbial motifs of a number of hazard signals (Hansen, Vojtech, & Laing, 2011). Once detected, these signals activate and trigger the transcription of the genes related to immune response. The PRRs then allow the immune cells to sense attacking pathogens at the site of infection.

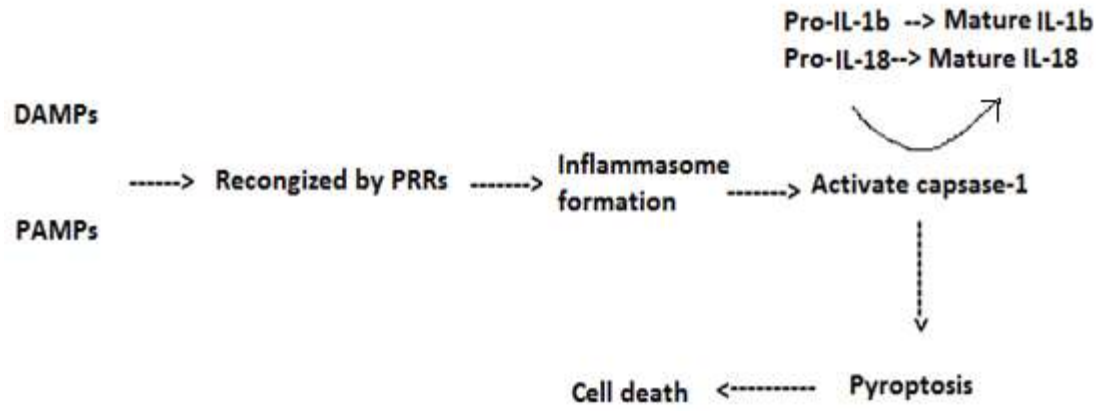


Figure 1-12: Schema for caspase-1 activation leading to cell death by DAMPs and PAMPs

Presently, the mammalian PRR family is divided into numerous separate subfamilies on the basis of structure and function, including the membrane-bound toll-like receptors (TLRs), the new interferon-inducible family member (IFI200) (which is absent in melanoma 2 [AIM2]), the retinoic acid-inducible gene-I RIF-I similar receptors (RLRs) and the nucleotide-connecting oligomerisation spheres (NLRs) (Yeretssian et al., 2008). The NLRs are an intracellular PRR located in the cytoplasm, and in humans comprise 16 family members (Alnemri, 2010). Based on their phylogenetic relationships, the NLR family can be divided into NODs and NLRPs (Martinon et al. 2009). Many NLR family members are similar in domain structure and contain a multidomain protein. They are categorised by a triplicate arrangement of a carboxy-terminal ligand detecting leucine-rich repeat (LRR) area, a central nucleotide-binding oligomerization (i.e., NOD or NACHT [NAIP, CIITA, HET-E and TP1]) domain and an amino-terminal protein–protein interaction domain, which is an amino-terminal caspase initiation and recruitment domain (CARD) in NOD proteins or an amino-terminal PYIN effector domain (PYD) (Figure 1-13) (Duprez et al., 2009; Schroder & Tschopp, 2010). The exception is NLRP10, which only has a NACHT and whose PYD domain does not have an LRR domain (Schroder & Tschopp, 2010).

In NLR, the LRR domain plays the role of the sensor of the inflammasome. LRR identifies and interacts with cytosolic ligands, like the PAMPs and DAMPs. Consequently, the LRRs of NLRP have been considered the innate signalling receptors of inflammasome activation. LRR proteins are short motifs, normally 20–29 residues in length, with a typical leucine-rich pattern (Kobe & Kajava, 2001). It may be that they are involved in the modulation and auto-regulation of NLR actions (Martinon et al., 2009). However, the mechanism for LRR detecting ligands remains unknown. Another NLRP component, the

NACHT domain, offers an alternative significant organisation of the NLRP and is common to all NLR family members. It acts as an oligomerization domain that facilitates the formation of oligomers subsequent to NLRP activation (Agostini et al., 2004; Martinon et al., 2009). The protein–protein interaction domains of the NLR proteins are carboxy-terminal CARD and amino-terminal PYD (part of the death fold superfamily). The death fold domains comprise protein–protein interaction motifs that act as bridge receptors to bind members of the same family to induce cell death; for example, a CARD14 domain interacts with a CARD domain and a PYD domain with a PYD domain (Franchi, Eigenbrod, Munoz-Planillo, & Nunez, 2009; Martinon et al., 2009) (Figure 1-13). As soon as NLRs sense danger signals or microbial motifs by LRRs, the NLRP undergoes conformational reorganisation. The NACHT domain of the NLRP enables nucleotide binding and the oligomerisation of NLRP, which in turn activates the recruitment of downstream components, usually through an adaptor protein, apoptosis-associated speck-like protein (ASC) (Figure 1-13).

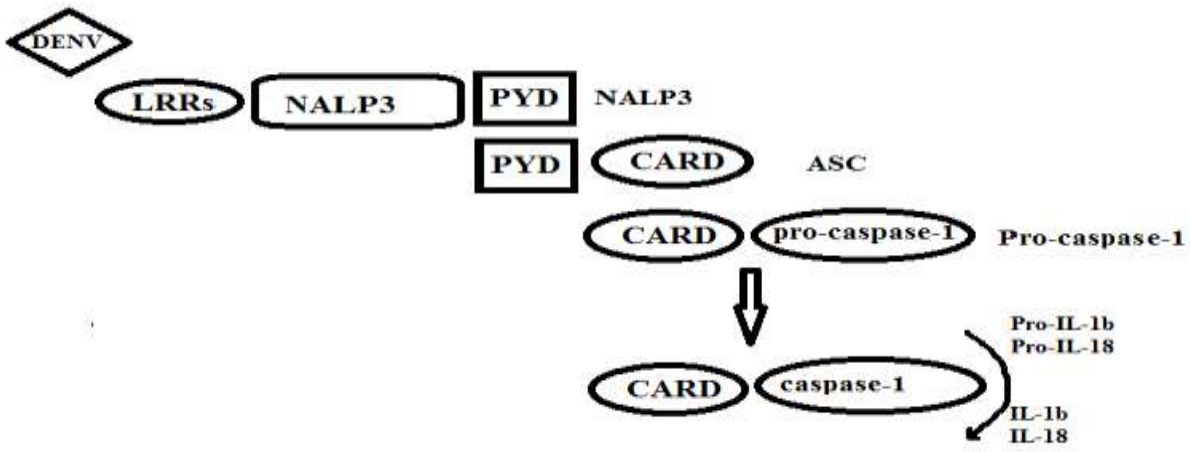


Figure 1-13: Inflammasome active caspase-1

ASC is a bipartite 22-kda protein comprising two areas: an amino-terminal PYD and a carboxy-terminal CARD (Masumoto et al., 1999) (Figure 1-13). The ASC protein is significant in the activation of NLR inflammasome-dependent pyroptosis due to acting as a connector between NALP inflammasome and pro-caspase-1. The assembly of NALP inflammasome is connected via a PYD–PYD interaction between the ASC and NLR (Figure 1-13). The pro-caspase-1 is then recruited and connects to NLR inflammasome via a CARD–CARD protein interaction (McIntire, Yeretssian, & Saleh, 2009; Schroder & Tschopp, 2010). Finally, NLR inflammasome contacts the PYD-based or CARD-based molecules in relation to different signals and activates downstream caspase-1 pathways (Masumoto, Taniguchi, & Sagara, 2001). The ASC pyroptosome creates a stage for caspase-1 and activates the production of inflammatory cytokines. Wu et al. (Kumar et al., 2013) confirmed that the ASC pyroptosome might also be induced by the anti-viral molecules, R837 or lipopeptides, LPS, SAT and MSU. The primary signal of stimulus and regulation in this situation is different from the inflammasome. Thus, it differs from inflammasome-dependent pyroptosis, which does not have a nod-like scaffolding protein.

1.5.6 Inflammasome and caspase-1 establishment

The signalling pathway for activating caspase-1 and pyroptosis is complicated. According to Yu and Finlay (2008), NALP1 and NALP3 inflammasome are necessary to activate the caspase and produce inflammatory cytokines (Yu & Finlay, 2008). NALP3 inflammasome is involved in anti-viral immunity in all members of the NLR inflammasome complexes. Viral RNA can be recognised by NALP3 inflammasome,

triggering caspase-1 activation and the production of IL-1β and IL-18. The NLR structure of the NALP3 protein, a subfamily of NLR, consists of NACHT, PYD domains and LRR. The function of the NLR protein is to work as a core scaffold for the activation of caspase-1. Figure 1-14 depicts the recruitment of pro-caspase-1, its connection to the NLR protein and the creation of the doughnut-shaped inflammasomes (Martinon et al., 2009; Schroder & Tschopp, 2010). The activation of caspase-1 leads to pore formation on the cell membrane, leading to increased osmotic pressure within cells and cell swelling. Due to water influx and cellular ionic loss, the cell eventually lyses (S. L. Fink & Cookson, 2006). Through this process, the cell releases IL-1β and IL-18 via the small pores in the cell membrane or through cell lysis.

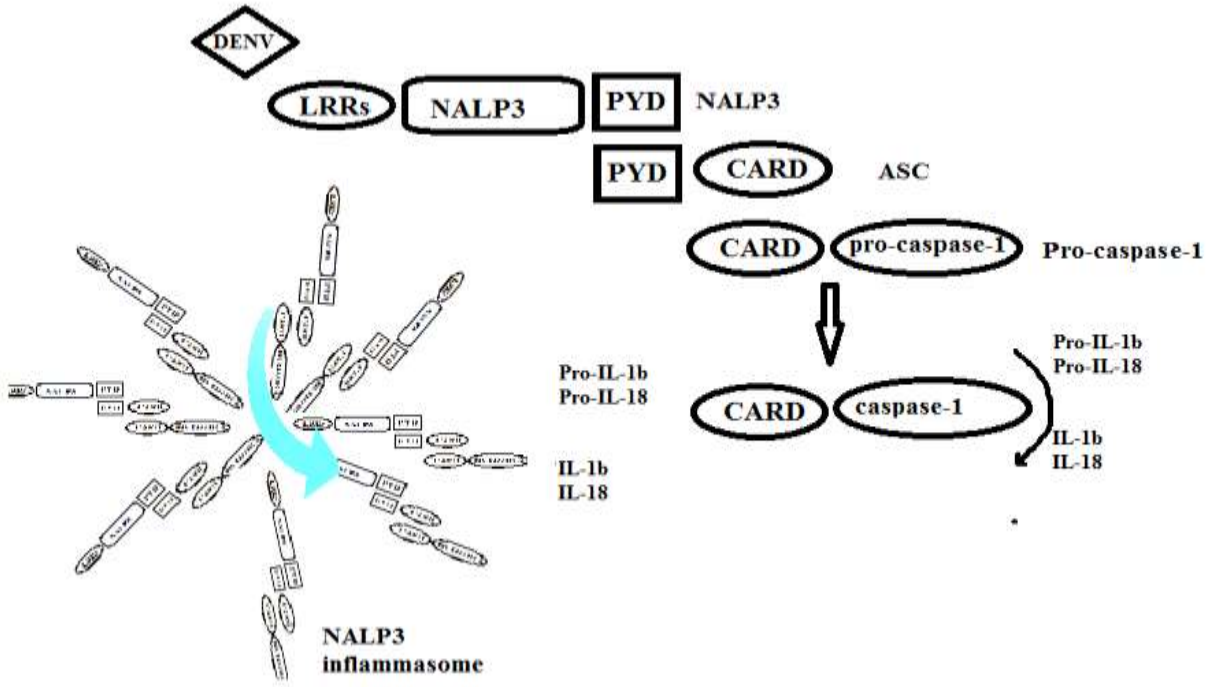


Figure 1-14: Assembly of NALP3 inflammasome

1.5.7 Pro-inflammatory cytokines IL-1β and IL-18 production

IL-1β and IL-18 are the inflammatory cytokines that can undergo caspase-1-dependent activation and production in the process of pyroptosis. It is a potent endogenous pyrogen that induces fever, leukocyte tissue migration and the expression of diverse cytokines and chemokines (Kiessling et al., 1999). IL-18 stimulates IFN-γ production and is crucial for the T cells activation, and also for macrophages and other cell types (Dinarello, 1999). Both IL-1β and IL-18 play important parts in the pathogenesis of inflammatory and many other autoimmune diseases (Kiessling et al., 1999). The process of cell death is not required for any cytokine, however, their production stimulate to the inflammatory response and undergoing pyroptosis. The inflammatory cytokines IL-1β and IL-18 do not have secretion signals and the releasing mechanism has not been clearly determined. The caspase-1-dependent pores formation in the plasma membrane is reported as temporally corrlation with cytokine production in macrophages (Noisakran & Perng, 2008; Oishi, Saito, Mapua, & Natividad, 2007), may suggest that cytokine secretion occurs together with these pores (Figure 1-10). It is noted that the release of activated IL-1β and IL-18 is not require cell lysis. It is because the caspase-1-dependent pore formation or cytokine secretion was not prevented by pharmacological inhibition (Oishi et al., 2007). Thus, the secretion of cytokine and lysis are separate downstream events of caspase-1-dependent pore formation (Figure 1-10).

1.5.8 Caspase 4

Caspase-4 is believed to be an inflammatory caspase because its encoding gene is located in the same chromosomal locus as the caspase-1 gene (Sollberger, Strittmatter, Kistowska, French, & Beer, 2012). It is reported that caspase-4 respond to several bacterial infections, including *Shigella flexneri*, *S. typhimurium* and enteropathogenic *E. coli*, in human intestinal epithelial cells (Knodler et al., 2014; Kobayashi et al., 2013). Three groups of genes were involved in the cloning of caspase-4 including Ice homology protein 2 (Ich-2), TX and ICERELII (Faucheu et al., 1995; Kamens et al., 1995; Munday et al., 1995). These groups were cloned from human monocytic cells with a 52% homology to caspase-1. The characteristic dynamic site of the pentapeptide QACRG appears to have been produced as a huge pro-domain with a long CAR domain, and can cause apoptosis in cases of overexpression. The caspase can cleave and activate itself as well as pro-caspase-1 proteins. However, unlike caspase-1, caspase-4 does not hold IL-1β-converting enzyme activity.

Caspase-4 is found on human chromosome 11q22.2–q22.3. The exons and introns of the gene extend over an area of approximately 25,700 bp at this locus. Caspase-4 is programmed by nine exons interspersed by 10 intron areas. The caspase-4 gene's alternative cleaving causes three transcript deviations to programme different isoforms; that is, the gamma, alpha and delta. The longest isoform is programmed by the alpha. Isoform gamma consists of an exclusive 5' end fragment; the decoding start codon is not used by the isoform alpha. Translation commences at a downstream start codon and results in an N-

terminally truncated protein. Isoform delta comprises an exclusive internal fragment that is not present in alpha and that leads to a translation frame change. This isoform can generate two polypeptides. The first matches the N-terminal section of isoform alpha and has a different C-terminus while the second is similar to the N-terminal truncated isoform alpha.

For a number of years, little information was available in relation to caspase-4, as it does not have a correct mouse homologue. Caspase-12 and -11 showed 48% and 59% homology with caspase-4, respectively. According to Hitomi et al. (2004), caspase-4 has a function in the endoplasmic reticulum (ER) stress-mediated apoptosis. These authors showed that caspase-4 is confined to the ER and contributes to ER stress-induced apoptosis and amyloid beta (aβ)-induced apoptosis (Hitomi et al., 2004). Some studies, including using a putative caspase fluorogenic substrate (i.e., LEVD-AFC), have disagreed with this finding (Obeng & Boise, 2005). It is likely that the function of caspase-4 in ER stress-induced apoptosis is cell-line specific.

A number of studies have been recorded of caspase-4 induced ER stress-mediated apoptosis, including the induction of mutant alpha-anti-trypsin in the ER and ensuing apoptosis (Hidvegi, Schmidt, Hale, & Perlmutter, 2005). For example, in apoptosis in response to bortezomib, a stress inducer was used to elevate the induction of misfolded proteins (Nawrocki et al., 2005); in a celecoxib analogue-induced tumour, cell loss was mediated by ER stress (Pyrko et al., 2007); in ER stress induced by infantile neuronal ceroid lipofuscinosis (INCL), a neurodegenerative disorder was induced (Kim et al. 2006); in a plasma cell, apoptosis was mediated by ER stress (Pelletier et al., 2006); in a

proteasome inhibitor, NPI-0052 was used to induce apoptosis (i.e., to treat chronic lymphocytic leukaemia) (Ruiz et al., 2006); and in cephalostatin, ER stress-mediated apoptosis was induced (Pyrko et al., 2007). Based on the studies, caspase-4 has been shown to have the ability to control cell death.

Little is known about the relationship between caspase-1 and -4 in the induction of IL-1β production and pyroptosis. One study shows that caspase-4 is required for the activation of the inflammasome, with the authors describing the physical interaction of caspase-4 with the central molecule of caspase-1 (Sollberger et al., 2012). Another study shows that capsase-4 provides a dual function in inflammation and ER stress-induced apoptosis (Bian, Elner, & Elner, 2009). A recent study showed that caspase-4 mediates the secretion of both IL-1α and IL1β (Vigano et al., 2015). However, the mechanism by which caspase-4 controls or activates caspase-1 is unknown.

1.5.9 Pyroptosis in host response and disease pathology

The activation of caspase-1 and pyroptosis clearly contributes to a reduction in the growth of intracellular pathogens. Although pyroptosis induces local inflammation that could enhance tissue disruption and pathogen dissemination, it also plays an important role in limiting pathogen replication and enhancing innate and adaptive immune responses. For example, caspase-1 activation can help to clear pathogens like *Shigella* (Chung et al., 2009), *Salmonella* (Chaturvedi et al., 2000), *Legionella* (Ankarcrona, Dypbukt, Brune, & Nicotera, 1994), *Listeria* (Sauer et al., 2010) and *Francisella* (X. Wang et al., 2007). It has been

reported that a lack of caspase-1 activation in macrophages infected with *Legionella* leads to the bacteria replicating within an ER-derived compartment that has a similar function to an immature autophagosome (A. O. Amer & Swanson, 2005). In fact, higher levels of caspase-1 activation in infected macrophages can stimulate caspase-1-dependent delivery of *Legionella* to lysosomes and the degradation of the bacteria (A. Amer et al., 2006). The killing efficiency of mycobacteria by stimulating trafficking of the bacteria to lysosomal compartments was also enhanced by caspse-1 activation (Master et al., 2008). The activation of macrophages suppresses *Yersinia*-mediated inhibition of pyroptosis and enhances *Francisella*-induced pyroptosis (Bergsbaken & Cookson, 2007; Henry, Brotcke, Weiss, Thompson, & Monack, 2007). However, not all bacteria were able to be removed by caspase-1 activity (A. O. Amer & Swanson, 2005), and bacteria or other pathogenic organisms may produce different virulence factors that control the interaction of intracellular portion. How caspase-1 allows leucocyte to overcome the bacterial factors and results to the control of pathogen replication *in-vivo* require further study.

Caspase-1 activation also affects the development of adaptive immune responses. In connection to IL-12, IL-18 plays an important role in inducing the Th-1-type $CD4^+$ T cells differentiation and enhancing the IFN-γ production (Basu & Chaturvedi, 2008). The caspase-1 activation to magnify the adaptive immune responses development and is supported by the study that the non-microbial activators of caspase-1 can provide support in pathogen clearance (Franchi, Eigenbrod, Munoz-Planillo, & Nunez, 2009; Martinon et al., 2009).

Numerous studies have shown that virus infection control is dependent on NALP3 inflammasome and caspase-1. The list of viral pathogens that activate NALP3 continues to grow, with seven new viruses reported to activate NALP3 inflammasome in the last two years, including DENV (Hottz ED et al., 2013). Recently, hepatitis C virus (HCV) was reported to active NALP3 in a reactive oxygen species-dependent manner, but the detailed mechanism is unknown (Burdette et al., 2012). In HCV, the activation of NALP3 inflammasome was found to help to produce IL-18 from monocytes *in-vitro* and activate the NK cells that facilitate the control of HCV infection (Serti et al., 2014). However, these responses are short-term, and inflammasome has been suggested to contribute to the pathogenesis of HCV infection (Negash et al., 2013). Human Immunodeficiency Virus (HIV) has also been found to initiate NALP3 inflammasome and induce pyroptosis on monocytes (Hernandez, Latz, & Urcuqui-Inchima, 2014), and it appears to be important for neuronal damage of infected microglial cells (Walsh et al., 2014). In addition, pyroptosis plays a role in the depletion of $CD4^+$ T cells in HIV patients (Doitsh et al., 2014). NALP3 inflammasome activation in neuron cells has been found to be essential for the control of West Nile virus replication and the prevention of central nervous system damage (Ramos et al., 2012). Further, other viruses can be recognised by NALP3, inducing pyroptotic cell death to clear virus infection; these include Rift Valley Fever virus (Ermler et al., 2014) and Chikungunya virus (Ng et al., 2009). In general, NALP3 inflammasome activation induces capsase-1 activity, which provides significant protection against highly pathogenic viruses but also contributes to the immunopathology of some diseases.

Pyroptosis protects against infection and induces pathological inflammation. Caspase-1 activity and pyroptosis can play a role in protective host response to infectious

diseases but systemic caspase-1 activation and pyroptosis can be destructive. Mutations in NLR proteins can lead to inappropriate caspase-1 activation, which is associated with hereditary auto-inflammatory syndromes (Cardier et al., 2006). Further, caspase-1 is involved in the pathogenesis of several diseases, including myocardial infarction (Lamkanfi & Dixit, 2010), cerebral ischemia (Simon & van der Meer, 2007), inflammatory bowel disease (Costa, Fagundes, Souza, & Teixeira, 2013) and neurodegenerative diseases (Espada-Murao & Morita, 2011). All are characterised by cell death and inflammation cell death. Deficiency in Caspase-1 deficiency or pharmacological inhibition may provide protection against the cell death, inflammation and organ dysfunction that are associated with the diseases that make caspase-1 as an attractive therapeutic target.

1.6 Research gaps and the objectives of this study

DF is a significant public health concern, especially in the tropics and subtropics. This disease is caused by DENV, a mosquito-borne virus of the Flaviviridae family. DENV causes DF, which is a self-limiting illness; however, DF can also progress to more severe forms, including DHF and DSS. These complications are more frequent during secondary infection with a different DENV serotype. DHF and DSS are severe febrile illnesses that can progress to hypovolemic shock with characteristic hemostatic abnormalities and increased capillary leakage (Neeraja, Lakshmi, Dash, Parida, & Rao, 2013).

Numerous studies have shown that both primary and transformed cells undergo cell death in response to DENV infection. Apoptosis is perhaps the most widely recognised

programmed cell death in DENV infection. Apoptotic cell death occurs through two major pathways: an intrinsic pathway which controlled by events occurring inside the cell and an extrinsic pathway (where the death signal comes from outside the cell). The two pathways have been involved in mediating cell death in DENV-infected cells (Torrentes-Carvalho et al., 2009). However, other types of cell death exist, including autophagy, oncosis and caspase-1-dependent programmed cell death (also known as pyroptosis). These may also contribute to the mechanism of cell death in DENV infection.

Recently, pyroptosis has been observed in DENV-infected cells (Wu et al., 2013), and has also been identified as a pathway of host cell death stimulated by a numberous of microbial infections (e.g., *Legionella* and *Salmonella*, *Francisella*) and non-infectious stimuli, including host factors produced during myocardial infarction (Bergsbaken et al., 2009). Pyroptosis is an important innate immune response to viral infection and has mainly been described in cells of the mononuclear phagocyte lineage. IL-1β is secreted in the process of pyroptosis with the activation of caspase-1. The activation of Caspase-1 can result in the stimulation of activated inflammatory cytokines and rapid cell death characterised by rupture of plasma membrane and the release of pro-inflammatory intracellular contents (Schroder & Tschopp, 2010). It has been reported that serum levels of IL-1β correlate with disease severity in dengue patients (Srikiatkhachorn & Green, 2010). Macrophages are an important target for DENV replication and are also the major source of pro-inflammatory cytokines (S. J. Wu et al., 2000). These cells generate inflammatory mediators and promote the dissemination of the virus during the initial phase of the dengue disease (Y. C. Chen, Wang, & King, 1999). Much effort has been expended on trying to elucidate the pathogenesis of this disease, including the role of pro-inflammatory cytokine

production and direct cellular damage leading to plasma leakage during DENV infection (Chaturvedi et al., 2000; Espada-Murao & Morita, 2011). Given that pyroptosis can promote pathogen clearance by acting as an alarm signal that recruits immune cells to the site of infection, a more aggressive approach than silencing cell death (e.g., apoptosis and autophagy) is required. Pyroptosis protects against infection and stimulate pathological inflammation. Thus, pyroptosis could play a role as a protective host response to infection. The mechanism by which this occurs in DENV infection is still not completely clear.

Another study shows evidence of EC damage in DHF patients, but the mechanism is unclear (Beatty et al., 2015; Cardier et al., 2006). One of the major explanations of the vascular leakage that occurs in DHF or DSS is EC death induced by DENV. Apoptosis of dengue-infected ECs has been reported *in-vitro* (Lin et al., 2002). Moreover. the release of inflammatory cytokines by DENV-infected ECs has been reported and is thought to trigger vascular leakage *in-vitro* (Beatty et al., 2015). However, the mechanisms involved in the cell death causing plasma leakage and the production of these cytokines remain unclear. As mentioned, DENV could induce other forms of cell death, like pyroptosis. Hence, we hypothesise that pyroptosis in DENV-infected ECs could be an alternative explanation of the vascular leakage syndrome observed in DENV-infected patients.

In this study, macrophages and ECs will be used as *in-vitro* cell models to demonstrate that pyroptosis could be an alternative programmed cell death in DENV infection. We hypothesise that both macrophages and ECs are able to undergo pyroptosis and provide the sources of IL-1β production during DENV-2 infection. We further

hypothesise that pyroptosis in ECs could be induced by DENV-2 infection and increase membrane permeability.

Pyroptosis and mature IL-1β production are both caspase-1-dependent processes. However, one study suggested that the inflammasome can also be activated by other caspases, such as caspase-4 (Sollberger et al., 2012). Currently, human caspase-4 and -5 are poorly characterised. These caspases are most homologous to caspase-11 in mice, but their functions are not well known. Caspase-4 is believed to be an inflammatory caspase because its encoding gene is located in the same chromosomal locus as the caspase-1 gene (Sollberger et al., 2012). It is reported that caspase-4 responds to several bacterial infections, including *Shigella flexneri, S. typhimurium* and enteropathogenic *E. coli*, in human intestinal epithelial cells (Knodler et al., 2014; Kobayashi et al., 2013). However, few data are available concerning the relationship between these two caspases in the induction of IL-1β production and pyroptosis during DENV infection. In this study, we seek to demonstrate that caspase-4 could be involved in the process of IL-1β production during DENV-2 infection and that it may be upstream in the signalling pathway that controls caspase-1 activation during DENV infection.

Programmed cell death is an efficient effector mechanism that restricts dengue viral growth and dissemination while also enhancing innate and adaptive immune responses. However, the virus can use virulence factors to limit either the action of caspase-1 or caspase-4 activation by performing another form of cell death, such as apoptosis or autophagy. Competition exists between the host and the virus to regulate the activation of

these two caspases, with the outcome dictating the fate—life or death—of the host. It remains unknown how the cells determine their fate after they are infected with DENV. In the present study, we aim to demonstrate the effect of DENV infectious doses on controlling cell death. The fates of DENV-infected cells undergoing pyroptosis or apoptosis in different initial infectious doses are of interest to this study. We hypothesise that DENV-2 infection induces apoptosis in macrophages and ECs at lower infectious doses and that apoptosis is suppressed with higher infectious doses.

A better understanding of programmed pyroptosis will deliver information on the pathogenesis of DENV, also important insights into the role of cell death during DENV infection. Pyroptosis could be a key explanation for both immune responses to, and the pathogenicity of, DENV infection. Another possible outcome of the study is some insight into the future development of therapeutics targets. The overall objective of this study is to investigate how DENV induces pyroptosis in macrophages and ECs. Further, this study seeks to demonstrate the involvement of caspase-4 in the process of pyroptosis and IL-1β production during DENV-2 infection.

Specific objectives

The specific aims of this research are as follows:

1. To investigate pyroptosis and IL-1β production in macrophages during DENV-2 infection.
2. To investigate pyroptosis and IL-1β production in ECs during DENV-2 infection.
3. To investigate the involvement of caspase-4 in pyroptosis and IL-1β production in macrophages and ECs during DENV-2.
4. To investigate the effect of viral dose in apoptotic and pyroptotic cell death.

Chapter 2: Materials and Methods

2.1 Cell culture and differentiation

2.1.1 Primary macrophage preparation

2.1.1.1 Primary macrophage differentiation

The primary macrophages were differentiated by monocytes isolated from human peripheral blood mononuclear cells (PBMC). The source of PBMCs was the Hong Kong Red Cross Transfusion Service, which provided them without disclosing the donor's identities after receiving permission from the Human Subject Ethics Sub-committee of the Hong Kong Polytechnic University. The cells were derived from the white-blood-cell-rich buffy coat fraction of the centrifuged anticoagulated blood. The separation of mononuclear cells was performed by a density gradient created by using a Ficoll-Paque Plus (GE Healthcare UK Ltd., Buckinghamshire, UK). The cells were centrifuged continuously for 30 minutes at 400×g. After centrifugation, ~ 8 x 10^6 cells were dispensed into a T-75 tissue culture flask (Thermo Scientific, USA). The monocytes were separated from all other cell types by incubating overnight at 37 ℃ in the presence of 5% carbon dioxide (CO_2), to allow the PBMCs to adhere to the bottom of the T-75 tissue culture flask. After incubation, the flasks were rinsed three times with phosphate-buffered saline (PBS) (Sigma-Aldrich, UK) to remove the unattached cells. The adherent cells were recovered with a cell scraper and the collected cells were incubated on a new 6-well plate (Thermo Scientific) at a concentration of ~ 3 x 10^5 per well with 10 ng/ml GM-CSF (Sigma) in completed Roswell Park Memorial Institue medium (RPMI) 1640 (Gibco, Thermo Fisher Scientific, UK). This medium included 10% heat-inactivated fetal bovine serum (FBS) (Gibco), streptomycin at a

concentration of 100 µg/ml, penicillin at a concentration of 100 U/ml at 37 ℃ and in the presence of 5% CO_2 for 7 days.

2.1.1.2 Purity and viability of Monocyte-derived Primary Macrophages

Flow cytometer was used to examine the phenotype of the cultured cells. In accordance with a published paper (Buckner, Calderon, Willams, Belbin, & Berman, 2011), two separate antibodies were chosen for checking the purity of the primary macrophages and the cells were stained with each antibody (Table 2-1). For each staining reaction, ~ 3 x 10^5 cells and diluted antibodies were mixed together. Cells were kept on ice for 30 minutes and then were washed with cool PBS (4℃) and fixed with 2% (w/v) paraformaldehyde for 10 minutes at room temperature. Unstained cells were used as negative controls in the flow cytometry studies.

Cell counting was used to determine the number and viability of the cells that grew in the culture. One hundred microliters of Trypan blue dye (Sigma-Aldrich, UK) was mixed with 100 µl of cell suspension, and 20 µl of this mixture was then placed on a hemocytometer and counted under a phase-contrast microscope (Nikon, Japan). Counting was repeated four times.

Table 2-1: Surface markers for confirming the growth situation of monocyte-derived macrophages

Surface marker	**Fluorescence dye conjugated**	**Function**	**Expression in macrophages after differentiation**	**Dilution /Volume of antibody used**	**Company**
CD14	PerCP	Pattern recognition receptor Differentiation marker	Down-regulated	10 µl	(Abcam, Cambridge, UK)
CD68	FITC	Antigen attachment molecule Differentiation marker	Up-regulated	10 µl	(Abcam, Cambridge, UK)

2.1.2 Culture of Human umbilical vein endothelial cells (HUVEC)

Human umbilical vein endothelial cells (HUVECs) were obtained from our collaborator (Dr Tony To). To grow these cells, supplemented M199 medium (Gibco) was used and kept at 37 ℃ with 5% CO_2. 1X low serum growth supplement (LSGS) (Gibco) and 10% FBS were added to the medium, which also contained penicillin at a concentration of 100 U/ml and streptomycin at a concentration of 100µg/ml (Gibco). The HUVECs were cultured on a gelatin-coated flask, a T-75 culture flask that was pre-coated with 1% gelatin for 30 minutes at 37 ℃ before use. The cells used in the study belonged to passage 2–8.

2.1.3 Culture of the *Aedes albopictus* clone (C6/36) cell line

The *Aedes albopictus* clone (C6/36) cell line was obtained from the Public Health Diagnostic Centre in HKSAR, China. To grow this cell line, supplemented minimal essential medium (MEM) medium (Gibco) was used and kept at 28 ℃ with 5% CO_2. Ten percent FBS was added to the medium, which also contained penicillin at a concentration of 100 U/ml and streptomycin at a concentration of 100 μg/ml (Gibco). The C6/36 cell line was cultured on a T-75 flask at a cell concentration of 2.5 x 10^6. The cells were split in a ratio of 1:5 every 5–7 days.

2.1.4 Culture of the Vero cell line

The Vero cell line (ATCC Cat. No. CCL-81) was cultured on supplemented minimal essential medium (MEM) medium (Gibco), which was kept at 28 ℃ with 5% CO_2. Ten percent FBS was added to the medium, which also contained penicillin at a concentration of 100 U/ml and streptomycin at a concentration of 100 μg/ml (Gibco). The Vero cell line was cultured on a T-75 flask at a cell concentration of 2 x10^6. The cells were split in a ratio of 1:4 every five days.

2.2 Dengue virus infection

2.2.1 DENV-2 Virus stock preparation

The DENV-2 strain used in this study was isolated from a DF patient in Hong Kong. The virus was propagated in an *Aedes albopictus* mosquito (C6/36) cell line. The 80% monolayer of C6/36 cells were infected with the virus at 0.1 MOI and kept at 28 ℃ with 5% CO_2 for five days. The infected cells were disrupted by freeze-thaw three times and intracellular particles were released into the culture medium. This was then centrifuged for 10 minutes at 1500×g to collect the supernatant. A PEG virus precipitation kit (Biovision Inc., Milpitas, CA, USA) was used to concentrate the supernatant containing the virus according to the manufacturer's instructions. Briefly, the 10 ml of collected supernatant was added to 2.5 ml of PEG solution and centrifuged at 3200×g for 30 minutes at 4 ℃. The white pellet was then collected and the concentrated virus was kept at a temperature of −80 ℃ before use.

2.2.2 DENV-2 viral titer determination

Tissue culture 50% infectious dose assay ($TCID_{50}$ assay) with the Vero cell line was used to determine the titer. The virus was diluted in increasing 10-fold serial dilutions and introduced into a 96-well dish (Thermo Scientific) seeded with a monolayer of Vero cells (~ 2 x 10^4 cells in 100 µl completed culture medium per well) with 90% confluence. Each virus dilution was repeated 10 times (Table 2-2). The cells were then incubated at 37 ℃

with 5% CO_2 for 5 days. After incubation, the number of cells that had died was determined by observing the cytopathic effect (CPE) under a phase contrast microscope (Nikon), with the values noted for each serial dilution of the virus.

To convert the $TCID_{50}$ values to plaque-forming units/ml (pfu/ml), a formula was applied under the assumption that the conditions used for the plaque and TCID assays did not affect the expression of the infectious virus. The calculation for the conversion of $TCID_{50}$ to pfu/ml (multiplicity of infection [MOI]) is given by:

$$pfu/ml = 0.7 * TCID_{50}$$

Table 2-2: $TCID_{50}$ assay virus dilution template

Dilution	**-VE Control**												**% Death**
	1	2	3	4	5	6	7	8	9	10	11	12	
1													
10^{-1}													
10^{-2}													
10^{-3}													
10^{-4}													
10^{-5}													
10^{-6}													
10^{-7}													
10^{-8}													

2.2.3 DENV-2 infection of primary macrophages

The monocyte-derived macrophages were exposed to DENV-2 at an MOI of 1 in MEM (Gibco) (C6/36 cell line medium) containing 2% FBS for 2 hours at 37 ℃ with 5% CO_2 to allow for virus absorption. Cells were then washed with serum-free RPMI 1640 to remove the cell-free virus and cultured at $1–2\times10^6$ cells per ml in RPMI 1640, supplemented with 2% FBS. Infected cells and culture supernatants were collected at days 1, 2 and 3 post-infection. Macrophages cultured in MEM supplemented with 2% FBS without inoculation of DENV-2 virus were included as a mock-infected control. Detection of DENV-2 infection was confirmed by staining with protein E (surface protein) using the immunocytochemical staining method (see section 2.5). The DENV-2 negative strand RNA was also detected to confirm viral replication in the macrophages by SYBR green real-time RT-PCR (see section 2.3), as the negative RNA strand is produced only during DENV replication.

2.2.4 DENV-2 infection of HUVECs

HUVECs were exposed to DENV-2 at an MOI of 10 in M199 containing 2% FBS for 2 hours at 37 ℃ with 5% CO_2 to allow virus absorption. Cells were then washed with serum-free RPMI 1640 to remove cell-free virus, and cultured at $1–2 \times 10^6$ cells per ml in RPMI 1640, supplemented with 2% FBS. Infected HUVECs and culture supernatants were collected at days 1, 2, 3 and 4. HUVECs cultured in M199 supplemented with 2% FBS without inoculation of DENV-2 virus were included as a mock-infected control. Detection

of DENV-2 infection was confirmed by staining with protein E (surface protein) using the immunocytochemical staining method (see section 2.5). To confirm the active production of DENV-2 in HUVECs, viral titers of cell supernatants were measured by using $TCID_{50}$ assay (see section 2.2.2).

2.3 Real-time quantitative RT-PCR

2.3.1 RNA extraction

Infected cells were collected at days 1, 2 and 3 post-infection. Total RNA was prepared from cell lysates using RNeasy kits (Qiagen, Hilden, Germany) following the manufacturer's instructions. Briefly, 1 x 10^7 cells were resuspended in 350 µl RLT buffer. Homogenisation of the cells was achieved by passing the lysates 10 times through 21-gauge needles attached to RNase-free syringes. One volume of 70% ethanol was added to each volume of the cell homogenates and the contents were thoroughly mixed. Samples were then transferred to the spin column and were bound to the membranes by pulse centrifugation at 8000g. Afterwards, 350 µl RW1 buffer was added to the membranes, followed by spinning down for 15 seconds. The membranes were then washed once with 350 µl RPE buffer, and twice with 500 µl 80% ethanol. The RNA was finally eluted with 14 µl RNase-free water. The quality and concentration of the extracted RNA samples were determined by Nanodrop ND-1000 spectrophotometer (ThermoScientific, USA). Those extracted RNA samples with an optical density (OD) A260/A280 ratio and OD A260/A230 ratio close to 2.0 were used for subsequent analysis.

2.3.2 Reverse transcription

For RT-PCR, the RNA was first treated with DNase (TakaraBio, Shiga, Japan), and 1 µg of this treated total RNA was subsequently used in the RT reaction using the SuperScript® III First-Strand Synthesis System (Invitrogen, Thermo Scientific, USA), according to the manufacturer's protocol. Briefly, 1µg of total RNA was mixed with 500ng of random primer, 1µl of 10mM dNTPs (at a temperature of 70℃ for 5 minutes), 4µl of 5× first strand buffer, 2µl of 0.1M dithiothreitol, 1µl of RNaseOUT, and 1µL of Superscript II (at a temperature of 37 ℃ for 50 minutes and 70 ℃ for 15 minutes).

2.3.3 Real-time PCR

The quantitative PCR reaction was performed using SYBR green PCR master mix reagents (Qiagen). Real-time quantitative PCR was performed using the ABI 7500 system (Applied Biosystems, Foster City, CA, USA) according to the following thermal cycling protocol: 95 ℃ for 5 minutes followed by 40 cycles of 95 ℃ for 30 seconds, a specific annealing temperature (Table 2-4) for 30 seconds and extension for 35 seconds. Quantitative PCR was performed using a 20 µl reaction mixture in 96-well reaction plates. All reactions were performed in triplicate. The specificity of each pair of primers was confirmed by melt curve analysis. The housekeeping gene, glyceraldehyde-3-phsophate dehydrogenase (GAPDH), was used as a control for data normalisation. The primer sequences used in this study are presented in Table 2-4.

2.3.4 Real-time PCR data analysis

The primary raw data output from the detector is shown as the threshold cycle value (Ct). The Ct for each gene tested and for the control gene GAPDH, and the difference between their Ct values (ΔCt), were determined. For each gene tested, normalisation was performed against the GAPDH sample as the reference, with its ΔCt value subtracted from the ΔCt value of the uninfected sample to obtain the ΔΔCt value (Livak, 2001). To calculate the fold change in the target gene, taking into account the GADPH endogenous control gene, the following equation was used:

$$\text{Relative fold change} = 2^{-\Delta(\Delta Ct)}$$

where $\Delta Ct = Ct, \text{target} - Ct, \text{GAPDH}$

and $\Delta(\Delta Ct) = \Delta Ct, \text{Time X} - \Delta Ct, \text{uninfected sample}$

2.4 Cell-surface marker studies by flow cytometry

The cell-surface marker expressions were determined by flow cytometry FC500 (Beckman Coulter, CA, USA). Mouse anti-human monoclonal antibodies, anti-CD68-FITC and anti-CD14-PerCP were used in this assay (Table 2-1). After washing in PBS, ~ 3 x 10^5 cells were collected and then resuspended in 100 μl PBS. For each staining reaction, the diluted antibodies were mixed with suspended cells. Cells were kept on ice for 30 minutes for incubation and then washed with cool PBS (4℃) and fixed with 2% (w/v)

paraformaldehyde for 10 minutes at room temperature. Nonspecific binding was monitored using isotypic controls. Non-stained cells were used as negative controls.

2.5 Western blotting analysis

Cells were collected at 1, 2 and 3 days post-infection from uninfected cells, DENV-2-infected cells and cells treated with caspase inhibitors (caspase-1 or caspase-4) prior to DENV-2 infection. The cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Abcam). The entire mixture was centrifuged at 13,000×*g* for 15 minutes at a temperature of 4 ℃ and the resultant supernatant was used for western blot analysis. Protein (50 µg) and sodium dodecyl sulfate (SDS) sample loading buffer were mixed in a 1:1 ratio and separation was performed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% acrylamide gel. After separation, the proteins were electrotransferred to a polyvinylidene difluoride membrane (GE Healthcare UK Ltd.). The membrane was blocked for 60 minutes using 5% non-fat dry milk mixed with tris-buffered saline containing 0.5% Tween-20 (TBST) at room temperature, then incubated with primary antibodies overnight at 4℃ on a rocking platform. The primary antibodies used in the experiment were mouse anti-caspase-1 (BioVision Inc.), rabbit anti-β-actin (Santa Cruz Biotechnology, Dallas TX, USA), rabbit anti-caspase-4 (Cell Signaling Technology, Danvers, MA, USA) and mouse anti-DENV2-E (4G2) (Table 2-3).

After incubation, the membrane was washed three times with TBST. Anti-rabbit HRP-conjugated secondary antibody (Cell Signaling Technology) was then added and incubated for one hour at room temperature. ECL plus chemiluminescent reagents (Amersham, Piscataway, NJ, USA) were used to produce signals. The mixture was subjected to repeated washings with washing buffer before the signals were visualised using the Bio-Rad imaging system PhosphorImager (ChemiDoc TM MP System, Bio-Rad, Hercules, CA, USA). Treatments on the membrane had to be conducted in the dark until visualisation was finished to avoid false-positive or high background signals. The software Image-J 1.48 (NIH, Bethesda, MD, USA) was used to analyse the band intensity, with the band intensity ratio of the specific protein/β-actin band obtained after normalisation being used to determine the variation produced in the different proteins.

Table 2-3: Primary antibodies used in the study for Western blot

Primary antibody	**Clonality**	**Source**	**Dilution**
Anti-DENV2-E	Mouse, monoclonal	4G2	1:2000
Anti-caspase-1	Mouse, monoclonal	Abcam, Cambridge, UK	1:1000
Anti-caspase-4	Rabbit polyclonal	Cell Signaling Technology, Danvers, MA, USA	1:1000
Anti-β-actin	Rabbit polyclonal	Santa Cruz Biotechnology, Dallas TX, USA	1:1000

Table 2-4: Primer sequences used in the study for RT-PCR analysis

Primer		**Sequence (5′ → 3′)**	**Annealing Temp.**	**Product size (bp)**	**Reference**
IL-1β	Forward	ACAGATGAAGTGCTCCTTCCA	58.5 ℃	73	(Giribaldi et al., 2010)
	Reverse	GTCGGAGATTCGTAGCTGGAT			
Caspase-1	Forward	CTCAGCAGCTCCTCAGGCA	60.0 ℃	155	This study
	Reverse	TGCGGCTTGACTTGTCCATT			
Caspase-3	Forward	GAGGCGGTTGTAGAAGTTAAT	54 ℃	221	This study
	Reverse	GAGATGTCATTCCAGTGCTT			
Caspase-4	Forward	CAGACTCTATGCAAGAGAAGCAACGTATGGCAGGA	65.0 ℃	609	(X. Y. Lin, Choi, & Porter, 2000)
	Reverse	CACCTCTGCAGGCCTGGACAATGATGAC			
NALP3	Forward	CTGTTCTCATGGGTTGGGGC	60.0 ℃	260	This study
	Reverse	TGGTCAGGGAATGGCTGGTG			
ASC	Forward	TCTACCTGGAGACCTACGGC	62.5 ℃	144	This study
	Reverse	CTATAAAGTGCAGGCCCTGGTG			
GAPDH	Forward	TGCACCACCAACTGCTTAGC	60.0 ℃	87	(Kovacs et al., 2012)
	Reverse	GGCATGGACTGTGGTCATGAG			
DENV-2	NegS-RT5	GTGCAGCCTGTAGCTCCACC	52.8 ℃	-	This study
DENV-2 (NS-1)	Forward	CGGGAGGCCACAAACCAT	61.1 ℃	59	This study
	Reverse	TCCTCTAACCGCTAGTCCACTAC			
DENV-2 (protein E)	Forward	CTGTAGTCTCACTGGAAGGAC	58.0 ℃	110	(Lai, Hu, King, & Wang, 2008)
	Reverse	CATTCCATTTTCTGGCGTCCT			

2.6 Immunocytochemical staining

Cells from uninfected and DENV-2-infected cells were cultured onto glass coverslips inside 6-well plates, air dried and fixed in 4% paraformaldehyde for 10 minutes at room temperature. The cells were treated with Triton-X100 (Sigma) for 10 minutes at room temperature to disrupt the cell membrane. Cells were then incubated for one hour with a targeted primary antibody at room temperature (Table 2-5). The cells were washed four times with PBS after the primary antibody incubation. Immunochemistry staining was performed with a biotin-free immuno-enzymatic antigen detection system using the mouse- and rabbit-specific HRP/AEC detection IHC kit (Abcam) according to the manufacturer's instructions. Horseradish peroxidase (HRP) was used as a label for the immunochemistry and antibody binding was detected using 3-amino-9-ethyl-carbazole (AEC) (Abcam) as a chromogenic substrate. Red indicated a positive result. Slides were counterstained with Giemsa stain (blue), mounted with glycerol and analysed using a standard light microscope.

Table 2-5: Primary antibodies used in the study for immunocytochemical staining

Primary antibody	**Clonality**	**Source**	**Dilution**
Anti-DENV2-E	Mouse, monoclonal	4G2	1:100

2.8 Detection of cytokines and cytochrome c in cell culture supernatants by ELISA

Culture supernatants were collected from uninfected cells, DENV-2-infected cells and cells treated with caspase inhibitors prior to DENV-2 infection at days 1, 2 and 3 post-infection. All samples were stored at −70℃ until the time of analysis. Measurement of human IL-1β protein in cell culture medium was performed in triplicate using Human IL-1β ELISA MAX Deluxe sets according to the manufacturer's instructions (BioLegend, San Diego). Sandwich ELISA was used in this assay. Briefly, each well of a PVC microtiter plate was coated with capture antibody by overnight incubation. The plate was then washed with PBS to remove any excess coating solution. 50 µl of the samples was added to each well. The plate was then covered with adhesive plastic and incubated for two hours at room temperature. A secondary antibody was added after three washes and further incubated for an hour. After incubation and removal of excess antibody, HRP was added to the wells for signal detection using a TMC substrate. A standard curve was prepared from the data obtained from the serial dilutions, with known concentrations on the X-axis v. absorbance on the Y-axis. Unknown samples were interpolated from the standard curve.

Measurement of human IL-18 protein and cytochrome c in cell culture medium was conducted using Human IL-18 Platinum ELISA sets (eBioscience, Vienna) and Human Cytochrome c Platinum ELISA sets (eBioscience), respectively. The procedures followed those for using the IL-1β ELISA MAX Deluxe sets, except that the microtiter plate had already been coated with the target monoclonal antibody.

2.9 Measurement of caspase-1, -3 and -4 enzyme activity

The capsase-1, -3 and -4 enzyme activity of the cells was measured using the Caspase-1/ICE, Caspase 3/CPP32 and Caspase-4 Fluorometric Assay kits (Biovision Inc.), respectively. These assays are based on the detection of cleavage of the 7-amino-4-trifluoromethylcoumarin (AFC) labelled substrates YVAD-AFC, DEVD-AFC and LEVD-AFC by capsase-1, -3 and -4, respectively. Briefly, 2 x 10^6 cells were collected from uninfected cells, DENV-2-infected cells and cells treated with caspase inhibitors prior to DENV-2 infection at days 1, 2 and 3 post-infection. The cells were resuspended in 50 µl cell lysis buffer on ice for 10 minutes. 50 µl of 2x reaction buffer was added to each sample along with 5 µl of 1mM specific substrate, followed by incubation for two hours at 37 ℃. Upon cleavage of the substrate by the specific caspase, a yellow-green fluorescence is emitted at 505 nm by the free AFC. Samples were read in a fluorometer equipped with a 400 nm excitation filter and 505 nm emission filter. This fluorescence can be quantified using a fluorescence microtiter plate reader. Comparison of the AFC fluorescence in a treated sample (i.e., a sample infected with DENV-2 and/or treated with caspase-1 or caspase-4 inhibitor) to that of an untreated control allowed for the determination of the fold increase in caspase-1, -3 or -4 activity after treatment.

2.10 Evaluation of cell death using lactate dehydrogenase assay

The release of LDH from the macrophages or ECs into the tissue culture media was measured using the Biovision LDH activity detection kit (Biovision Inc.), according to the manufacturer's instructions. The assay was performed in triplicate at

three different time points (days 1, 2 and 3 post-infection). Cell culture supernatants were collected from uninfected cells, DENV-2-infected cells and cells treated with caspase inhibitors prior to DENV-2 infection, with all samples stored at −70°C until the time of analysis. Briefly, 50 µl of cell culture supernatant was added to each well of a 96-well plate, together with 50 µl of assay buffer. A 1:24 diluted substrate was added to each well (samples and controls) and incubated for 30 minutes at room temperature. LDH was quantified using a colorimetric LDH quantification assay in which LDH reduces NAD to NADH, which interacts with a specific probe to produce a colour, with λmax = 450 nm. This colour change was measured at OD 450nm using a microtiter plate reader. Comparison of the colour change in a treated sample (i.e., a sample infected with DENV-2 and/or treated with caspase-1 or caspase-4 inhibitor) to that of an untreated control allowed for the determination of the fold increase in LDH release after treatment.

2.11 Assessment of cell viability

The percentages of viable cells in non-infected and DENV-2-infected samples were determined by the Trypan blue dye exclusion method using a BD Science Automatic Vi-CELL Cell Viability Analyser (BD Biosciences, San Jose, CA, USA). Two hundred microliter cell suspensions were added to the analyser and cell concentration and viability were provided. The assay was performed in triplicate.

2.13 Caspase-4 RNAi knockdown

Macrophages and HUVECs were cultured in 6-well plates to 80% confluence at the time of transfection. siRNA transfection was performed using the SignalSilence® Control siRNA (Santa Cruz Biotechnology), following the manufacturer's instructions. To each well was added 80 pmol of caspase-4 siRNA (Santa Cruz Biotechnology) and 4 µl of siRNA transfection reagents in 200 µl of siRNA transfection medium, with incubation for 6 hours at 37℃ in the presence of 5% CO_2. After incubation, 1 ml of normal growth medium with 2x FBS was added, followed by incubation for another 24 hours, before being used for analysis. The knockdown efficiency was examined by real-time RT-PCR and immunoblotting using anti-caspase-4 (Cell Signaling Technology).

2.14 Endothelial cell permeability assay

The change in permeability of the HUVEC monolayers was determined by measuring the passage of HRP after incubation with supernatants of DENV-infected ECs. Briefly, 2×10^5 HUVECs were seeded in a 24-well collagen and fibronectin-coated transwell (6.5-nm diameter, 0.4-µm pore size, Corning Life Sciences) and cultured in Complete Medium 199 (Cascade Biologics) with LSGS and FBS. On day 2 after plating, the culture media was replaced with mock-infected control, DENV-2 (MOI=10) or DENV-2 (MOI=10) with caspase-1 inhibitor (YVAD) and incubated for two hours at 37 ℃. Before collecting the medium from the lower chamber, 10 µl of fluorescein isothiocyanate-conjugated 70-kDa dextran (500 µg/mL) was introduced in the upper chamber of the transwell and 15 minutes was allowed to elapse before starting the

collection. The lower chamber was analysed for fluorescence at 485nm excitation and 520nm emission after 72 hours at a temperature of 37 ℃, and four cycles were simultaneously run. The permeability of the endothelial monolayer and flux of 70-kDa dextran in the monolayer shared a directly proportional relationship in the transwell. The Graph Pad Prism 6.0 software (GraphPad Software, La Jolla, CA, USA) was used to plot the graph between the concentration *(C)* and time *(t)*, and (dC/dt) was used to denote the first derivative.

2.15 Detection of apoptosis and necrosis

The morphological characteristics of cell death induced by DENV-2 at different MOIs were examined by fluorescein staining. The uninfected and DENV-2-infected cells were stained by fluorescein-labelled FTIC-annexin V (Abcam) and ethidium homodimer III (Abcam). Apoptotic cells were stained green by the fluorescein-labelled annexin V (green fluorescence), which is impermanent in live cells and early apoptotic cells. Pyroptotic and necrotic cells were also detected by staining with ethidium homodimer III (red fluorescence). The fluorescence positive cells were evaluated by phase contrast and fluorescence (470 and 530 nm) microscope. Briefly, 2 x 10^6 cells were washed with PBS once before resuspension in 1 ml PBS buffer. Five microliters of FTIC-annexin V and 5 µl of ethidium homodimer III were added to 100 µl of cell suspension. The mixture was incubated for 15 minutes under dark conditions. After incubation, the cells were washed with PBS buffer and resuspended in 1x binding buffer. Fluorescence was observed using FITC and Texas Red filter set.

2.16 Statistics

The Graph Pad Prism 6.0 software (GraphPad Software) was used to perform the statistical calculations. The Mann-Whitney *U*-test was used to determine the difference in the enzyme activity of caspase-1, -3 and 4, the expression of genes and proteins and production of IL-1β and IL-18, the activity of LDH and cytochrome c release between the infected group and the uninfected group at different time points. This difference was found by comparing the mean values in the non-parametric Kruskal–Wallis test. A *P* value of less than 0.05 was considered statistically significant while a *P* value of less than 0.01 was considered statically highly significant.

Chapter 3: Results

3.1 Human macrophages and ECs as a permissive in-vitro model for studying pyroptosis in DENV-2 infection

3.1.1 High purity of primary macrophages generated from PBMC

Human peripheral blood mononuclear cells (PBMCs) were isolated from the leucocyte-enriched buffy coats by a density gradient created using a Ficoll-plaque plus. The monocytes were separated from all other cell types using the adhesive properties of mononuclear cells. Macrophages were differentiated from monocytes by adding GM-CSF and incubating for 7 days. The morphology of the monocytes was different from the differentiated primary macrophages. The monocytes appeared round and in suspension after purification from PBMC (Figure 3-1A) whereas the primary macrophages became adherent and had an elongated structure (Figure 3-1B). The purity of the monocyte-derived primary macrophages was examined using a flow cytometer. After differentiation, the expression of CD14 was down-regulated and the expression of CD68 was up-regulated. The purity of these cells was more than 80%, as indicated by the change in the expression of the surface markers CD14 and CD68. In total, the experiment comprised three separate experiments. The black line was a non-stained control.

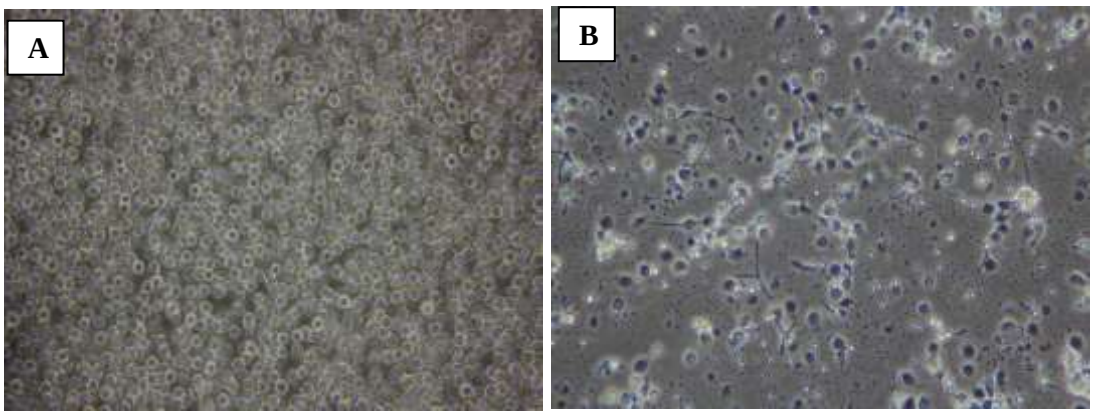


Figure 3-1: Morphology of human primary macrophages generated from human monocytes

Human primary monocyte-derived macrophage morphology: (A) after purification by PBMC; (B) primary macrophages. The cells were observed by a phase-contrast microscope with 400x magnification.

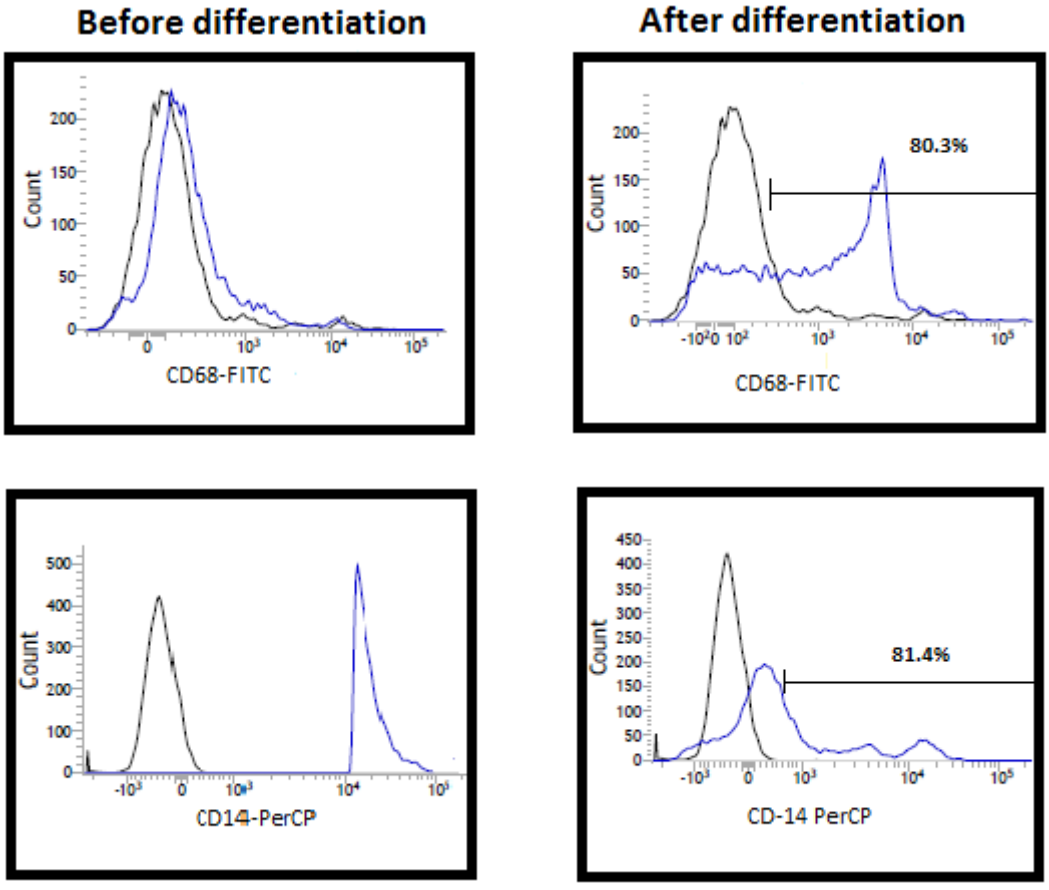


Figure 3-2: Surface marker expression of monocyte-derived primary macrophages before and after differentiation.

A flow cytometer was used to examine the purity of the monocyte-derived primary macrophages after differentiation. The purification and the surface marker expression were analysed using flow cytometer. The experiment comprised three separate experiments. The black line was a non-stained control.

3.1.2 DENV titer determination

The viral titer of DENV after being propagated in C6/36 cell line was determined by a $TCID_{50}$ assay. The assay was performed by diluting the virus in increasing 10-fold serial dilutions in a monolayer of Vero cells (Table 3-1). After incubation, the total number of cells that had died was determined by examining the cytopathic effect (CPE) in each well under a phrase-contrast microscope. After 10^{-6} dilution, a negative CPE was noted (Table 3-1). The value of $TCID_{50}$/ml was converted to MOI. In Table 3-1, the first two columns show the negative controls with no virus added to the wells, proving no contamination occurred.

Table 3-1: The viral titer of DENV-2 by $TCID_{50}$

Dilu-tion	**Control**		**Cytopathic effect (CPE)**										**% death**
	1	2	3	4	5	6	7	8	9	10	11	12	
1	-	-	+	+	+	+	+	+	+	+	+	+	100%
10^{-1}	-	-	+	+	+	+	+	+	+	+	+	+	100%
10^{-2}	-	-	+	+	+	+	+	+	+	+	+	+	100%
10^{-3}	-	-	+	+	+	+	+	+	+	+	+	+	100%
10^{-4}	-	-	+	+	+	+	+	+	+	+	+	+	100%
10^{-5}	-	-	+	+	+	+	+	+	+	+	+	+	100%
10^{-6}	-	-	+	+	-	+	+	+	-	+	+	+	80%
10^{-7}	-	-	+	+	-	-	-	-	+	-	+	-	40%
10^{-8}	-	-	-	-	-	-	-	-	-	-	-	-	0%

Note: The CPE of the Vero cell was examined under a phrase-contrast microscope, with (-) indicating no CPE observed in the well, and (+) indicating that CPE was observed in the well. The percentage death was then calculated based on these results. The first two columns show the negative controls.

Mathematical determination of $TCID_{50}$

1. $TCID_{50}$/ml was calculated using the formula below:

   $TCID_{50}/ml = 10^{(PD+ (-log\ dilution\ greater\ than\ 50\%\ infected))}$/inoculum volume (ml)

2. The proportionate distance (PD) between the two dilutions in between 50% cell death:

   PD = (% next above 50%) – 50% / [(% next above 50%) – (% next below 50%)]

   PD = (80% – 50%) / (80% – 40%) = 30/40 = 0.75

3. The 50% end point, log-lower dilution was equivalent to the dilution in which the position is above 50%:

   Log lower dilution = 10^{-6} = -6

4. Log $TCID_{50}$ was calculated by adding PD with the log of the lower dilution:

   Log $TCID_{50}$ = $10^{-(-6+0.75)}$

   = 1 / 5.62 *10^{6}

5. $TCID_{50}$/ml was then calculated by dividing the millilitres of viral inoculum added to the first row:

   $TCID_{50}/ml = 5.62 *10^{6} / 0.05 = 1.12 *10^{4}$

6. Assuming the conditions used for plaque assay and $TCID_{50}$ assay don't alter the expression of infectious virus, $TCID_{50}$/ml and pfu/ml are related by:

   pfu/ml = 0.7 * $TCID_{50}$ = 0.7 * 5.62 *10^{6}

   = 3.9 *10^{6} pfu/ml

7. MOI is the ratio of virus to the number of cells:

   MOI = 1 = 3.9 *10^{6} viruses infected 3.9 * 10^{6} cells

3.1.3 DENV-2 efficiently infected and replicated in primary macrophages

Immunoperoxidase staining was used to detect viral antigen in infected monocyte-derived macrophages. The DENV-E antigen was detected in macrophages as early as day 1 post-infection with DENV-2 (Figure 3-3). The *de novo* production of negative-stranded DENV-2 RNA demonstrated that the active replication of DENV-2 occurred in primary macrophages. The number of viral gene copies/µl peaked at day 1 post-infection ($p < 0.001$), rapidly declined on day 2 ($p = 0.0398$), and declined further on day 3 ($p = 0.0914$) (see Figure 3-4). These findings suggest that DENV-2 replicated rapidly after infecting human macrophages. The viral genes produced by infected cells decreased over time due to cell death.

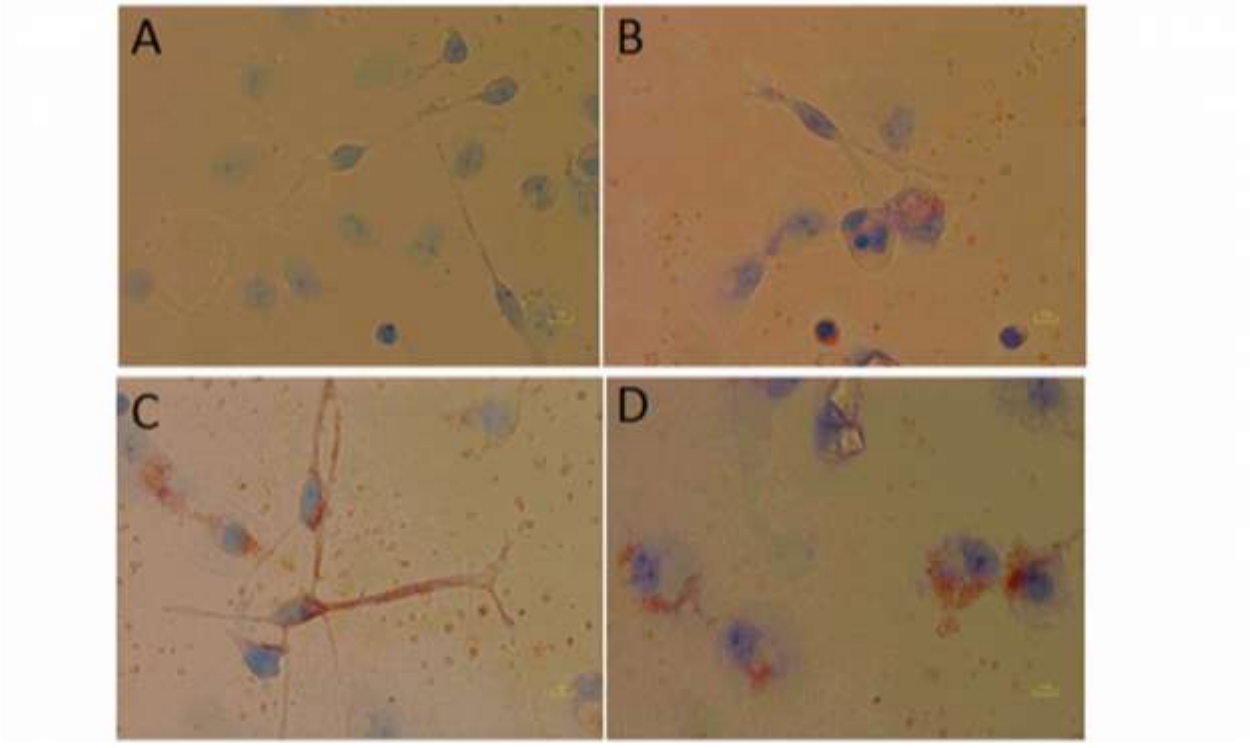


Figure 3-3: DENV-2-infected primary macrophages

Immunocytochemistry staining with an anti-DENV-E monoclonal antibody (clone 4G2) of uninfected and DENV-2-infected primary macrophages at various time points. Red indicates the presence of viral antigen (E protein). Giemsa was used as a counter-stain. Uninfected cells after day 3 of culture (A). DENV-2-infected cells at days 1, 2 and 3 post-infection (B, C and D, respectively). Magnification is ×1000.

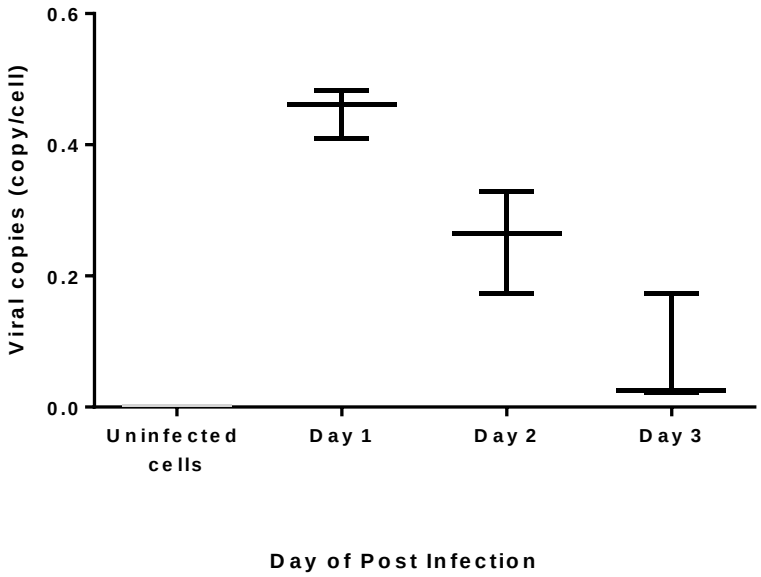


Figure 3-4: DENV-2 infection and replication in primary macrophages

The number of copies of DENV-2 in primary macrophages on days 1, 2 and 3 after DENV-2 infection. An uninfected sample was used as a negative control (n = 3). * $p < 0.05$ (Mann-Whitney *U*-test) v. uninfected cells. Error bars indicate standard error of mean (SEM).

3.1.4 Productive infection of human ECs by DENV-2

In the first experiment, we confirmed the *in-vitro* permissivity of human ECs to DENV-2 infection. Immunoperoxidase staining was used to detect viral antigen in infected cells. HUVECs were used as the cell model of human ECs in this study. Using western blotting, the DENV-E antigen was detected in HUVECs at 3 days post-infection with DENV-2 at an MOI of 10 (Figure 3-5). The results shown in Figure 3-6 demonstrate that 60–70% of HUVECs were infected with DENV-2 after 24 hours post-infection, and almost all cells were infected after 72 hours post-infection (Figure 3-6). Consistent with these results, DENV-E proteins were detected in ECs lysates (see Figure 3-5). The DENV-2 infection of HUVECs rapidly resulted in a titer of 10^5 pfu/ml in cell supernatants, as found using $TCID_{50}$ assay (Figure 3-7). This is similar to the viral replication in C6/36 cells at 3 days post-infection ($p = 0.463$). C6/36 cells are

highly susceptible to DENV infection (Hase, Summers, & Eckels, 1989), and the infected cells were used as the comparative control. These findings demonstrate that DENV-2 efficiently and productively infects ECs, resulting in rapid replication and the release of infectious virions.

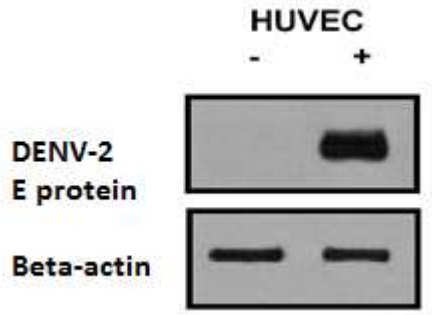


Figure 3-5: DENV-2 E protein synthesis in ECs

Western blotting analysis of DENV2 viral antigens (E protein) in total protein extracted from HUVECs 3 days post-infection. Beta-actin was used as an internal control. +: infected samples; - : uninfected controls.

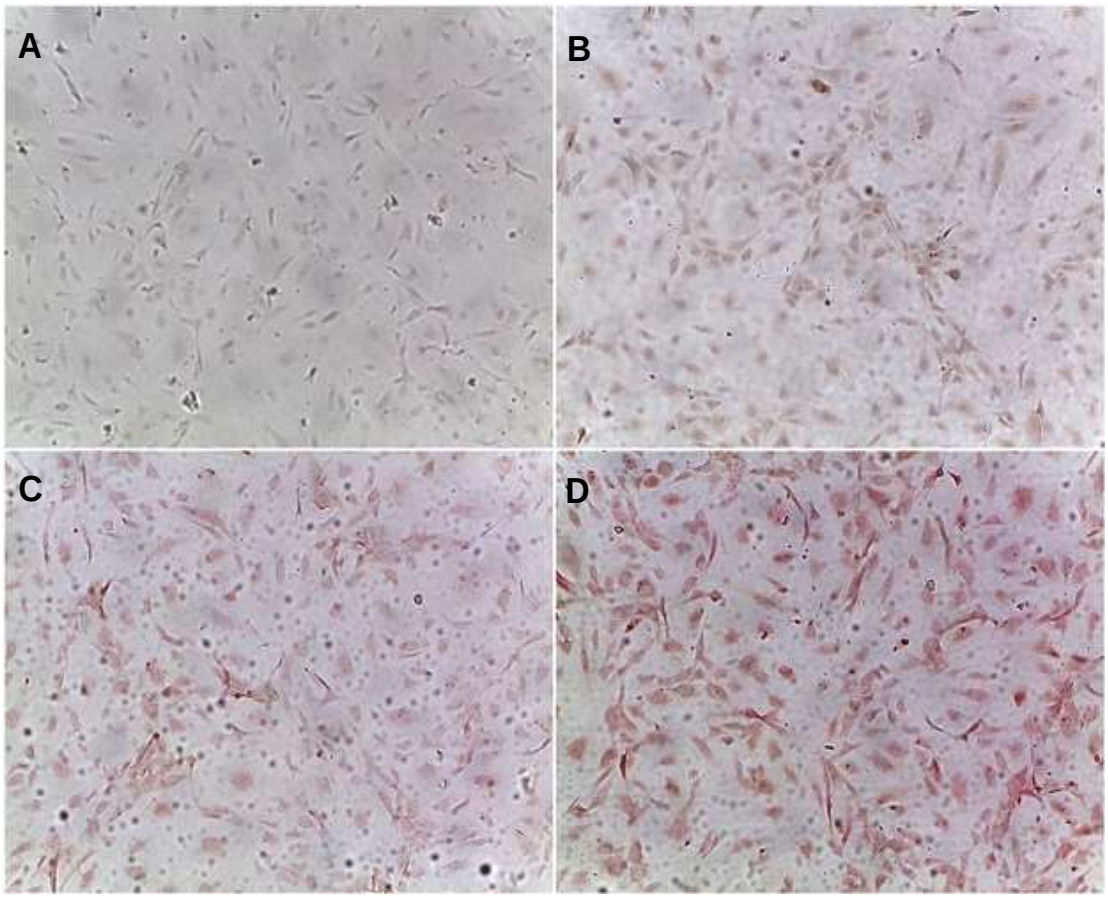


Figure 3-6: Infection of human ECs by DENV-2

Immunocytochemistry staining with an anti-DENV-E monoclonal antibody (clone 4G2) of uninfected and DENV-2-infected HUVECs at different time points. Red indicates the presence of a viral antigen (E protein). Giemsa was used as a counter-stain. Uninfected cells after day 3 of culture (A). DENV-2-infected cells at days 1, 2 and 3 post-infection (B, C and D, respectively). Magnification is ×1000.

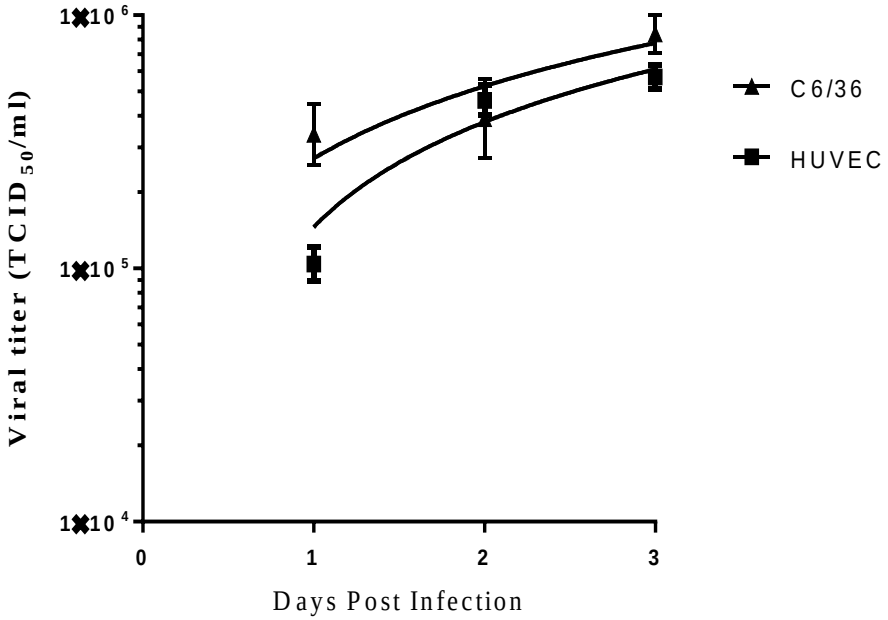


Figure 3-7: Productive infection of primary human ECs by DENV

The titers of DENV present in supernatants of HUVECs and C6/36 cells (1, 2 and 3 days post-infection) were determined on a Vero cell line using a $TCID_{50}$ assay. The Kruskal–Wallis test was applied to compare the trend between the two cell lines in viral titer production within the time (n=3). * $p < 0.05$. Error bars indicate SEM.

3.2 Pyroptosis in primary macrophages during DENV-2 infection

3.2.1 Increased caspase-1 mRNA and protein expression after DENV-2 infection

Caspase-1 plays the central role in pyroptosis. To investigate the activation of caspase-1 during DENV-2 infection, caspase-1 mRNA and protein expressions were evaluated by real-time RT-PCR and western blotting, respectively. The results demonstrated that the expression of caspase-1 gradually increased over time post-infection (Figures 3-8 and 3-9A and B). Using the Mann-Whitney *U*-test, the changes in mRNA levels for caspase-1 at day 1 ($p = 0.013$) and day 3 ($p = 0.027$) post-infection

were significantly different when compared to the uninfected controls (Figure 3-8). However, the values for infected and uninfected macrophages at different time points were not significantly different, as calculated by the Kruskal–Wallis test (p = 0.078). The observed trends in the caspase-1 mRNA expression (Figure 3-8) were similar to those observed for the caspase-1 protein expression (Figure 3-9A and B). Increased caspase-1 transcription and protein expression is an important characteristic of the activation of caspase-1. The findings from the real-time RT-PCR and western blotting data suggest that caspase-1 activity may be enhanced in DENV-2-infected macrophages.

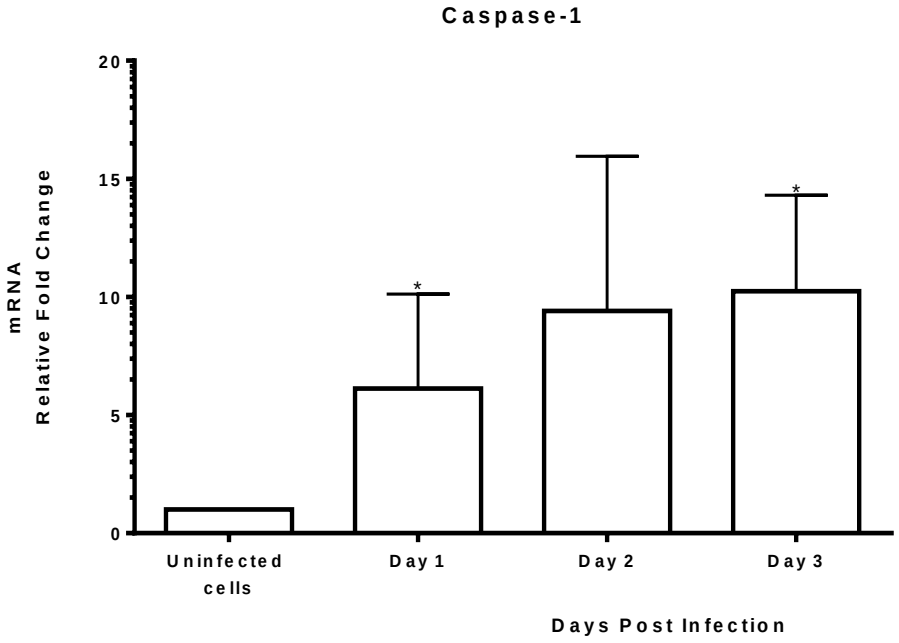


Figure 3-8: Changes in mRNA levels for caspase-1 in primary macrophages at days 1, 2 and 3 post-infection, as measured by real-time RT-PCR

The real-time RT-PCR data were normalised using GAPDH as a housekeeping gene. Error bars indicate SEM. * $p < 0.05$ (Mann-Whitney *U*-test) v. uninfected cells.

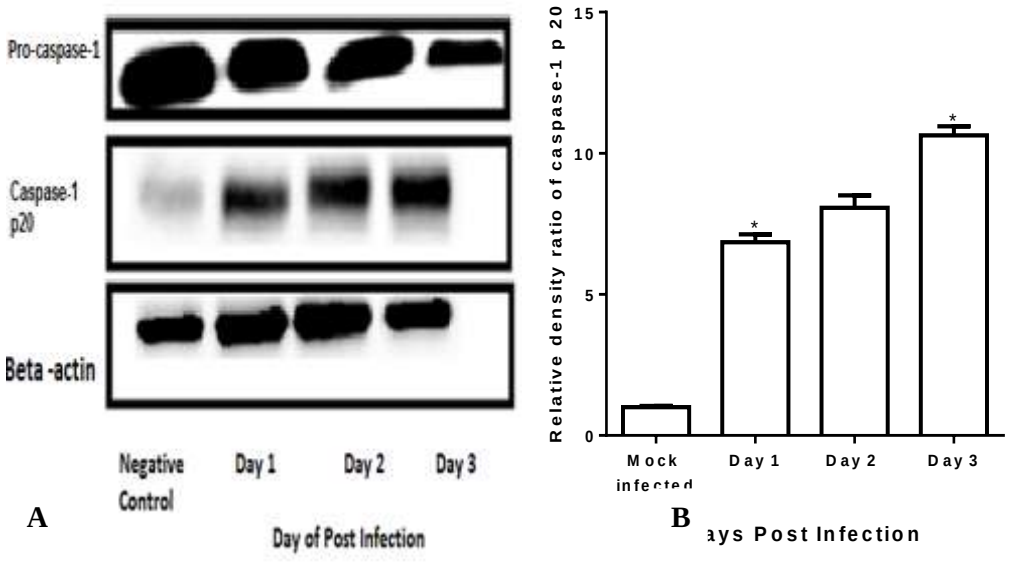


Figure 3-9: Caspase-1 expression induced by DENV-2 infection

The protein expression of caspase-1 in macrophages was determined by western blotting. Caspase-1 was cleaved into two subunits, large (20 kDs) and small (10 kDs), during activation. The expression of the cleaved p20 subunit caspase-1 increased in a time-dependent manner after DENV-2 infection compared with the mock-infected macrophages (n = 3) (A). The relative band density of caspase-1 p20 on western blots was normalised with a β-actin loading control and was quantitated (B). Error bars indicate SEM (n = 3). * $p < 0.05$ (Mann-Whitney *U*-test) vs. uninfected cells (mock infected).

3.2.2 DENV-2 infection induced IL-1β cytokine production

The production of IL-1β and IL-18 has been shown to be one of the major characteristics of pyroptosis (Bergsbaken et al., 2009). ELISA was used to quantify the amount of IL-1β (Figure 3-10) and IL-18 (Figure 3-11) in cell culture supernatants during DENV-2 infection. Samples were collected at 1, 2 and 3 days post-infection. IL-1β and IL-18 were measured independently in triplicate for each sample at each time point. The mean concentrations of IL-1β in the macrophage culture supernatants on days 1, 2 and 3 post-infection were 42.73, 47.35 and 54.79 pg/ml, respectively (Figure 3-10). When compared with uninfected cells, these data reveal an increase in the

expression of IL-1β protein from day 1 post-infection (p = 0.0508). Further, the IL-1β levels increased significantly on day 2 (p = 0.0213) and day 3 (p = 0.0051) post-infection. Uninfected cells were used as controls to account for the effect of macrophage differentiation. These data suggest that DENV-2 infection stimulates the production and release of the mature biologically active IL-1β over time. However, production of IL-18 was not observed to markedly increase in the primary macrophages post-infection (Figure 3-11).

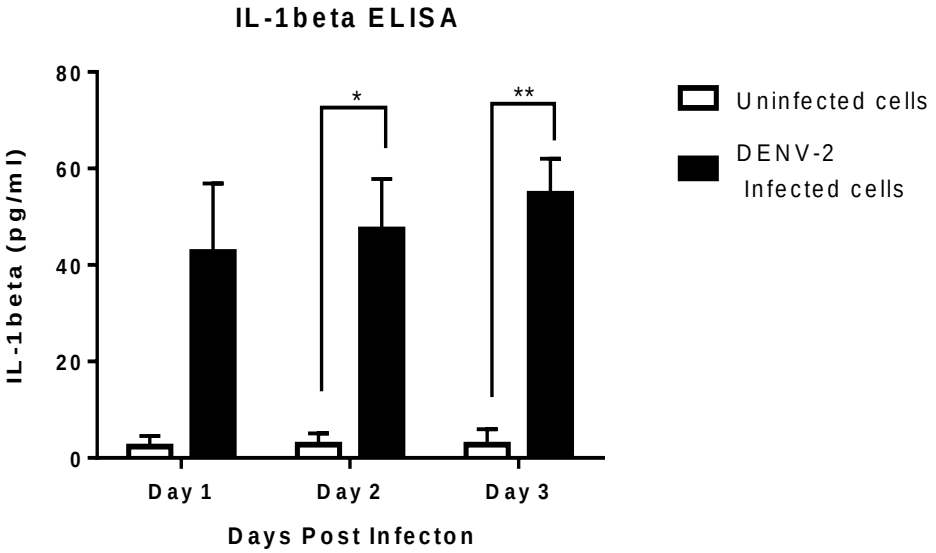


Figure 3-10: IL-1β production during DENV-2 infection

The concentration of IL-1β in culture supernatant on days 1, 2 and 3 post-DENV-2 infections were measured by ELISA. The data are presented as the mean ± SEM (n = 3). Statistical comparisons were made between infected and uninfected macrophages at various time points; * $p < 0.05$, ** $p < 0.01$ (Mann-Whitney *U*-test) vs. uninfected cells.

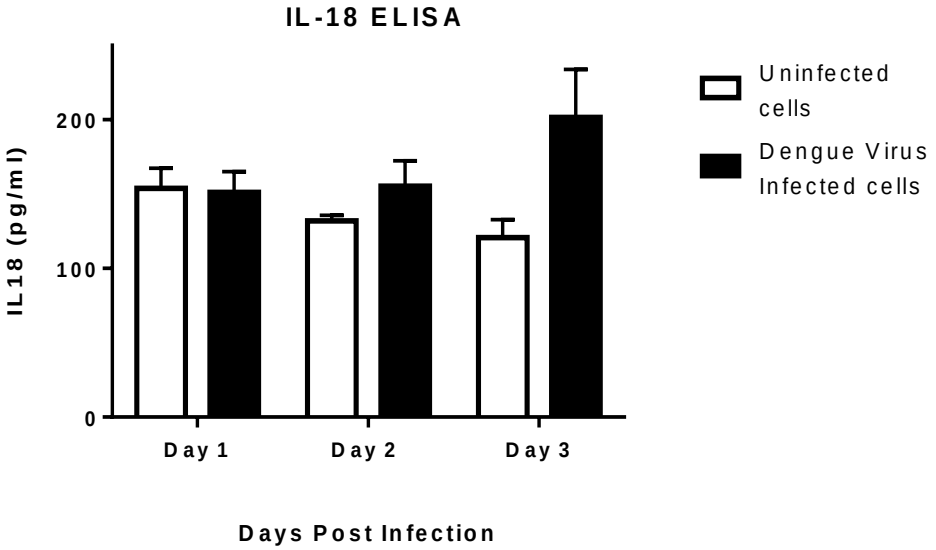


Figure 3-11: IL-18 production during DENV-2 infection

The production of IL-18 after DENV infection at days 1, 2 and 3 in primary macrophages. The concentration of IL-18 in the culture supernatant was measured by ELISA. The data are presented as the mean ± SEM of three individual experiments. Statistical comparisons were made between infected and uninfected macrophages at different time points using the Mann-Whitney *U*-test. * $p < 0.05$, ** $p < 0.01$.

3.2.3 DENV-2 infection increased IL-1β and inflammasome mRNA expression in human primary macrophages

To determine whether the observed IL-1β production was due to *de novo* synthesis of IL-1β or to the release of pre-formed cytokines stored inside the cells, we analysed the IL-1β mRNA levels in DENV-2-infected macrophages. IL-1β mRNA expression was found to be up-regulated during the course of DENV-2 infection in primary macrophages (Figure 3-12A). The expression of IL-1β mRNA increased dramatically on day 2 in the DENV-2-infected cells and continued to increase on day 3 ($p = 0.017$) compared with uninfected controls.

DENV-2 infection increases the levels of the pro-IL-1β transcript, which can be converted into biologically active IL-1β via cleavage by caspase-1 (Ghayur et al., 1997). ASC is the major protein for forming pyroptosome, and it rapidly recruits and activates caspase-1. In this study, we demonstrated that the expression of ASC also peaked on day 2 post-infection and dropped on day 3 post-infection (Figure 3-12B). Previous studies have shown that the pattern recognition receptors, NALP3, were the only receptors for dengue to induce pyroptosis (Tan & Chu, 2013; Wu et al., 2013). This is in line with the finding in this study that the expression of NALP3 mRNA was up-regulated as early as day 1 post-infection, before peaking on day 2 post-infection (Figure 3-12D). The gene expression of IL-1β and NALP3 inflammasome followed the same pattern as capsase-1(Figure 3-8), except for ASC, indicating that inflammasome may also be involved in the activation of the caspase-1 and IL-1β production during DENV-2 infection. These results are consistent with previous studies (Wu et al., 2013).

IL-18 mRNA expression was observed to increase with time post-DENV-2 infection, peaking at day 3 post-infection ($p = 0.028$). However, in our study, the ELISA detected no IL-18 cytokines in the supernatant. One possible explanation would be that the production of IL-18 requires an additional proteolytic step that is controlled by other factors. Thus, the production of IL-1β and IL-18 is regulated in a two-step fashion.

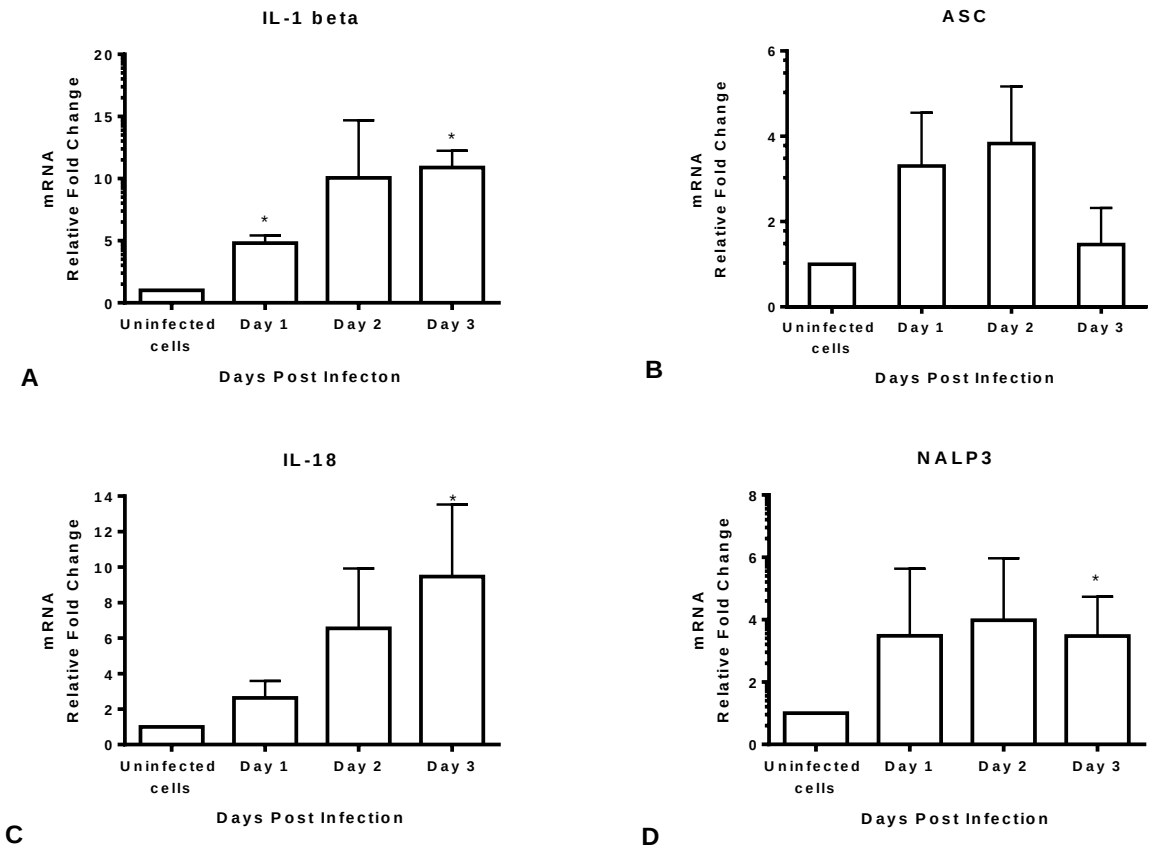


Figure 3-12: Changes in the mRNA levels of IL-1β (A), ASC (B), IL-18 (C) and NALP3 (D) in primary macrophages at days 1, 2 and 3 post-DENV-2 infections, as measured by real-time RT-PCR.

The data were normalised using GAPDH as a housekeeping gene. Error bars indicate SEM (n = 3). Statistical comparisons were made between infected and uninfected macrophages at different time points; * $p < 0.05$ (Mann-Whitney *U*-test).

3.2.5 Decrease in cell viability and LDH release from macrophages after DENV-2 infection

It has been proposed that DENV infection induces cell death by pyroptosis, which is a caspase-1-dependent process that results in cell lysis with LDH release (Legrand et al., 1992). LDH is a stable cytosolic enzyme that is released into the extracellular matrix after cell lysis. The LDH activity in the supernatants from DENV-2-infected macrophages was significantly different from that observed in the supernatants from uninfected cells on day 3 post-infection (p = 0.0248) (Figure 3-13). Moreover, the viability of the DENV-2-infected macrophages was significantly lower than that of the uninfected cells (p = 0.0145) (Figure 3-14). Together with the observed increase in expression of caspase-1 (Figures 3-8 and 3-9) and IL-1β production (Figure 3-10), these findings suggest that the macrophages underwent pyroptosis during DENV-2 infection.

3.2.6 The stimulation of IL-1β production and pyroptosis in primary macrophages in a caspase-1-dependent manner during DENV-2 infection

To address the roles of caspase-1 in the synthesis of IL-1β and pyroptosis during DENV-2 infection, caspase-1 activity (Figure 3-15A), LDH release (Figure 3-15B), and IL-1β production (Figure 3-15C) were evaluated respectively, in the presence of caspase-1 inhibitors. From the results, caspase-1 activity (p = 0.0230), LDH and IL-1β (p = 0.0423) were all increased after DENV-2 infection. The results demonstrate that the caspase-1 activity, IL-1β production and LDH release were reduced to background

levels in the presence of caspase-1 inhibitors. Thus, IL-1β production and pyroptosis in macrophages during DENV-2 infection is controlled by the expression of caspase-1.

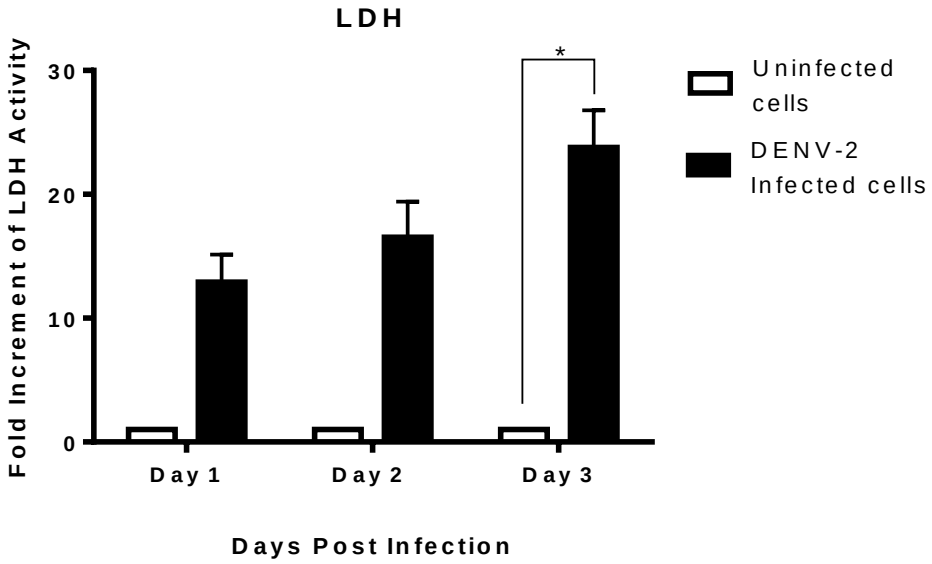


Figure 3-13: LDH release of DENV-2-infected macrophages

The activity of LDH in primary macrophage culture supernatants on days 1, 2 and 3 after DENV-2 infection. The data are presented as the mean ± SEM of three individual experiments. Statistical comparisons were made between infected and uninfected macrophages at different time points using the Mann-Whitney *U*-test. * $p < 0.05$.

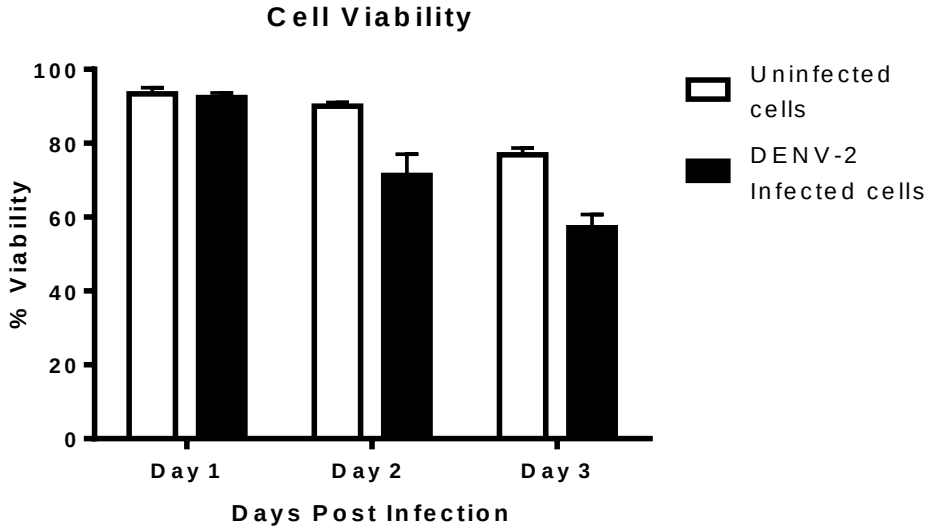
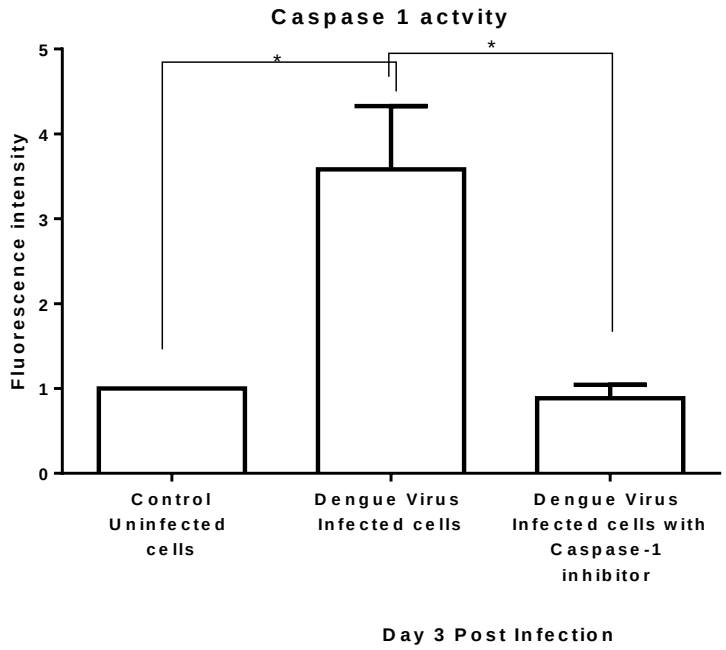


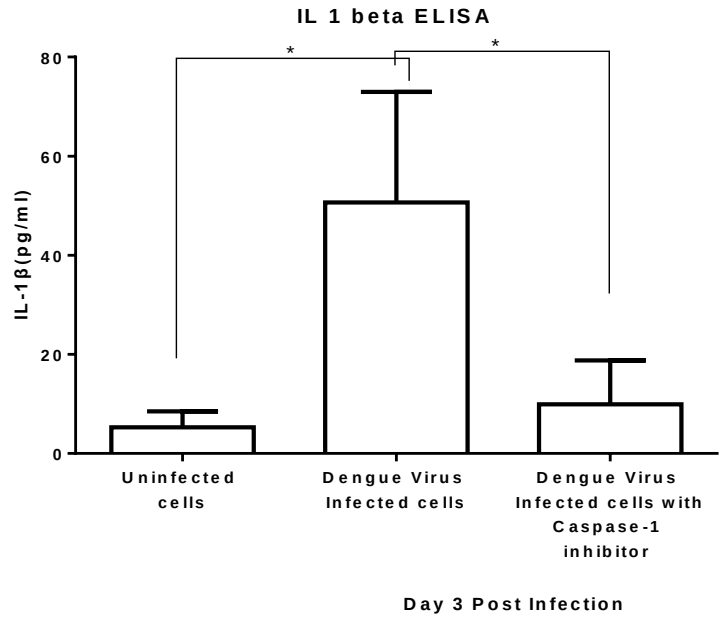
Figure 3-14: Cell viability of DENV-2-infected macrophages

Comparison of the viability of non-infected and DENV-2-infected primary macrophages on days 1, 2 and 3 post-infection. Cell viability was determined by Trypan blue exclusion using an automatic Vi-Cell cell viability analyser. The data are presented as the mean ± SEM of three individual experiments. The data were statistically analysed using the Mann-Whitney *U*-test. * $p < 0.05$.

A


Caspase 1 actvity
Fluorescence intensity
0
1
2
3
4
5
*
*
Control Uninfected cells
Dengue Virus Infected cells
Dengue Virus Infected cells with Caspase-1 inhibitor
Day 3 Post Infection

B


IL 1 beta ELISA
IL-1β(pg/ml)
0
20
40
60
80
*
*
Uninfected cells
Dengue Virus Infected cells
Dengue Virus Infected cells with Caspase-1 inhibitor
Day 3 Post Infection

C

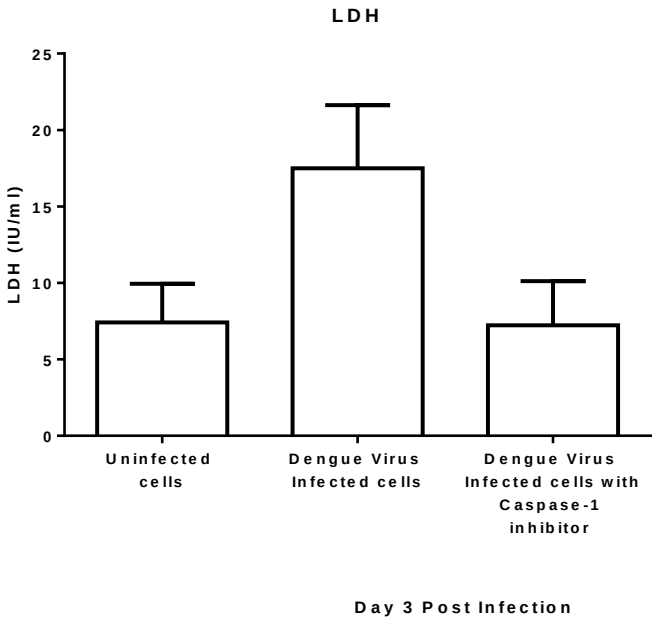


Figure 3-15: Caspase-1 activity, IL-1β production and LDH release with caspase-1 inhibitors during DENV-2 infection

Caspase-1 inhibitors (2µg/ml) were added to the macrophages at DENV-2 infection. Caspase-1 activity (A), LDH release (B) and IL-1β production (C) were measured 72 hours post-infection. The caspase-1 activity, LDH and IL-1β production were reduced to background control levels after the use of inhibitors. The data are presented as the mean ± SEM of three individual experiments. The data were statistically analysed using the Mann-Whitney *U*-test. * $p < 0.05$.

3.3 Pyroptosis in ECs during DENV-2 infection

3.3.1 Increased expression of caspase-1 during DENV-2 infection

Real-time RT-PCR was firstly performed to study the endogenous levels of caspase-1 mRNA in HUVECs after DENV infection. The total RNA was extracted at 24-hour intervals from HUVECs that were inoculated with DENV-2 at an MOI of 10 mock-infected. We found that caspase-1 mRNA increased slowly over time: a remarkable change of 2.15-fold was found by 24-hour after DENV-2 infection (p = 0.0017), indicating up-regulation of caspase-1 transcripts post-infection (Figure 3-16)., We next examined the caspase-1 expression over 72-hour post-infection by using western blotting (Figure 3-17). When infected with DENV-2, pro-caspase-1 levels remained relatively constant for day 1 but start decreasing after 24-hour (Figure 3-17). This result correlates with an increase in activated caspase-1 p20 subunits, as exhibited by a sharply intense band on 48 hours post-infection (Figure 3-17A). The approximately low level of activated caspase-1 p20 was shown on the first 48-hour post-infection. It was suggested that the expression of caspase-1 occurred mainly as a slow response in ECs after DENV-2-infection.

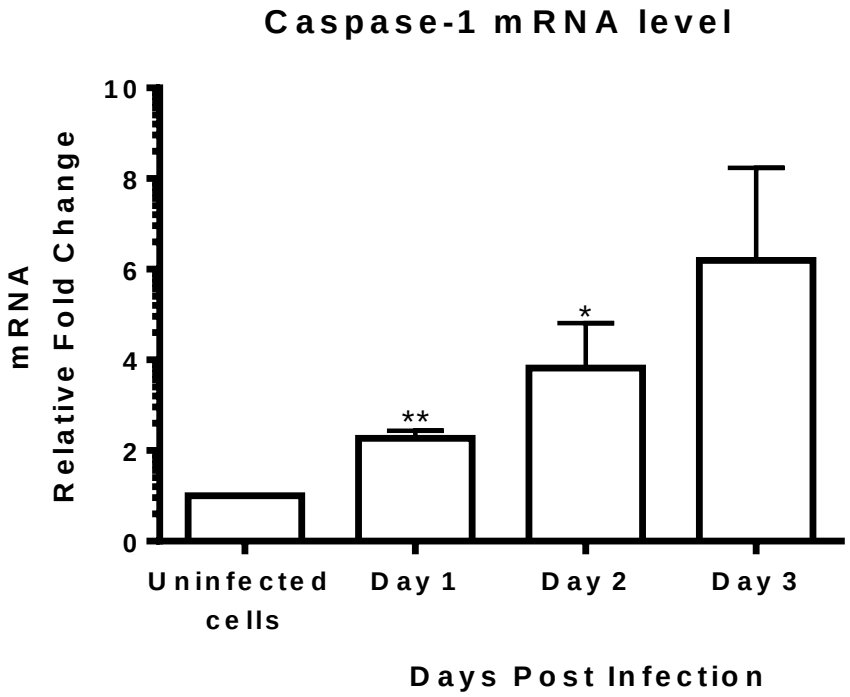


Figure 3-16: Changes in mRNA levels for caspase-1 in HUVECs at days 1, 2 and 3 post-infection, as measured by real-time RT-PCR

Real-time RT-PCR data were normalised using GAPDH as a housekeeping gene. Errors bars indicate SEM. Statistical comparisons were made between infected and uninfected HUVECs at different time points; * $p < 0.05$ (Mann-Whitney *U*-test).

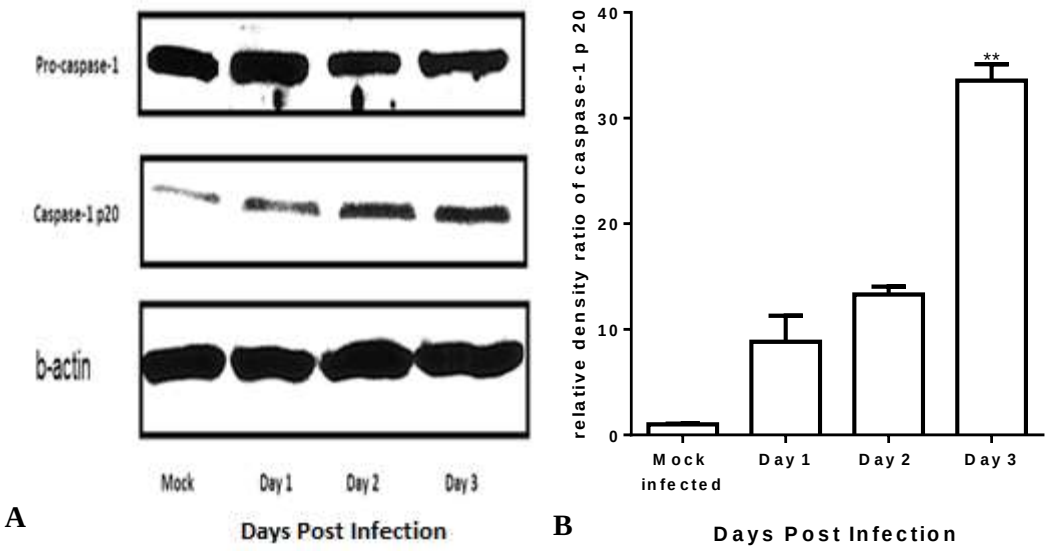


Figure 3-17: Caspase-1 expression in HUVECs induced by DENV-2 infection

The protein expression of caspase-1 in HUVECs was determined by western blotting. Caspase-1 was cleaved into two subunits, large (20 kDs) and small (10 kDs), during activation. In our study, the expression of the cleaved p20 subunit caspase-1 increased in a time-dependent manner after DENV-2 infection (n = 3) (A). The relative band density of caspase-1 p20 on western blots was normalised with a β-actin loading control and was quantitated (B). Error bars indicate SEM (n = 3). * $p < 0.05$, ** $p < 0.01$ (Mann-Whitney U-test) v. uninfected cells.

3.3.2 Induction of IL-1β in DENV-2-infected ECs

The function of caspase-1 is known to cleave pro-IL-1β into thebiologically active form. We examined the secretion of this pro-inflammatory cytokine by ECs during DENV-2 infection using ELISA. The mean concentrations of IL-1β in the HUVEC supernatants on days 1, 2 and 3 post-infection were 12.90, 16.528 and 23.77 pg/ml, respectively (Figure 3-18). We observed that a DENV-2 infection at an MOI of 10 was capable of triggering IL-1β secretion (Figure 3-18). The secretion was significantly enhanced at day 1 post-infection ($p = 0.0403$). The remarkable rise in IL-1β by 24 hours post-infection was an unexpected result since our western blot data showed

a signifcant later activation of caspase-1 after DENV-2-infected ECs (Figure 3-17). Moreover, the ELISA results seem inconsistent with the studies earlier that report that IL-1β secretion could not be produced from ECs in DENV infection (Huang et al., 2000). The phenomenon could be explained by infection with a different MOI. It is suggested that a difference outcome may result in patients suffering from viremia.

To determine whether the observed IL-1β production was due to *de novo* synthesis of IL-1β or to the release of pre-formed cytokine stored inside the HUVECs, we analysed the IL-1β mRNA levels in DENV-2-infected HUVECs. IL-1β mRNA expression was up-regulated during the course of the DENV-2 infection of the HUVECs (Figure 3-19A). The expression of IL-1β mRNA increased dramatically as early as on day 1 in the DENV-2-infected cells and continued to increase on day 2 compared with uninfected controls. The results concerning IL-1β mRNA expression correlated with the IL-1β production detected by ELISA. Interestingly, the mRNA expression of IL-1β declined on day 3 post-infection, but the level of IL-1β protein continued to increase. This can be explained by a cumulative effect. However, it can also be suggested that the *de novo* synthesis of IL-1β is not only controlled by the caspase-1 enzyme, but that other factors also stimulate the *de novo* synthesis of IL-1β during DENV-2 infection. More experiments are needed to arrive at a conclusion.

The mRNA expression of ASC and NALP3 was also determined. Results demonstrated that the expression of NALP3 peaked on day 2 post-infection, before dropping slightly on day 3 post-infection (Figure 3-19C). Interestingly, the mRNA expression of ASC and NALP3 started being up-regulated on day 2 post-infection (Figure 3-19B and C). Further, ASC and NALP3 inflammasome showed delayed

expression compared with IL1β and caspase-1. It is thus suggested that caspase-1 activation may be controlled by other factors.

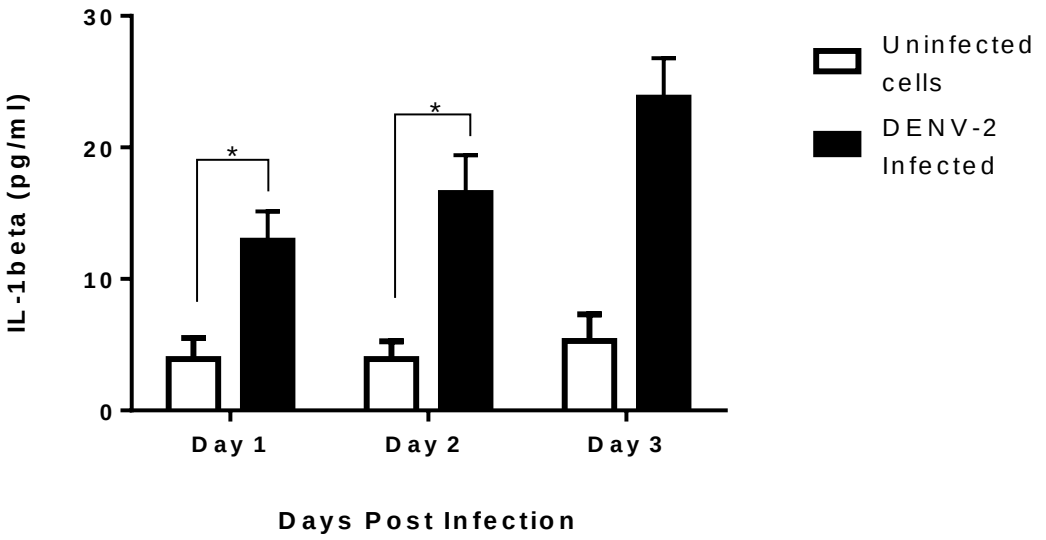


Figure 3-18: IL-1β production by ECs during DENV-2 infection

The production of IL-1β by HUVECs on days 1, 2 and 3 of DENV-2 infection. The concentration of IL-1β in the supernatant was measured by ELISA. The data are presented as the mean ± SEM (n = 3). Statistical comparisons were made between infected and uninfected macrophages at various time points; * $p < 0.05$, ** $p < 0.01$ (Mann-Whitney *U*-test).

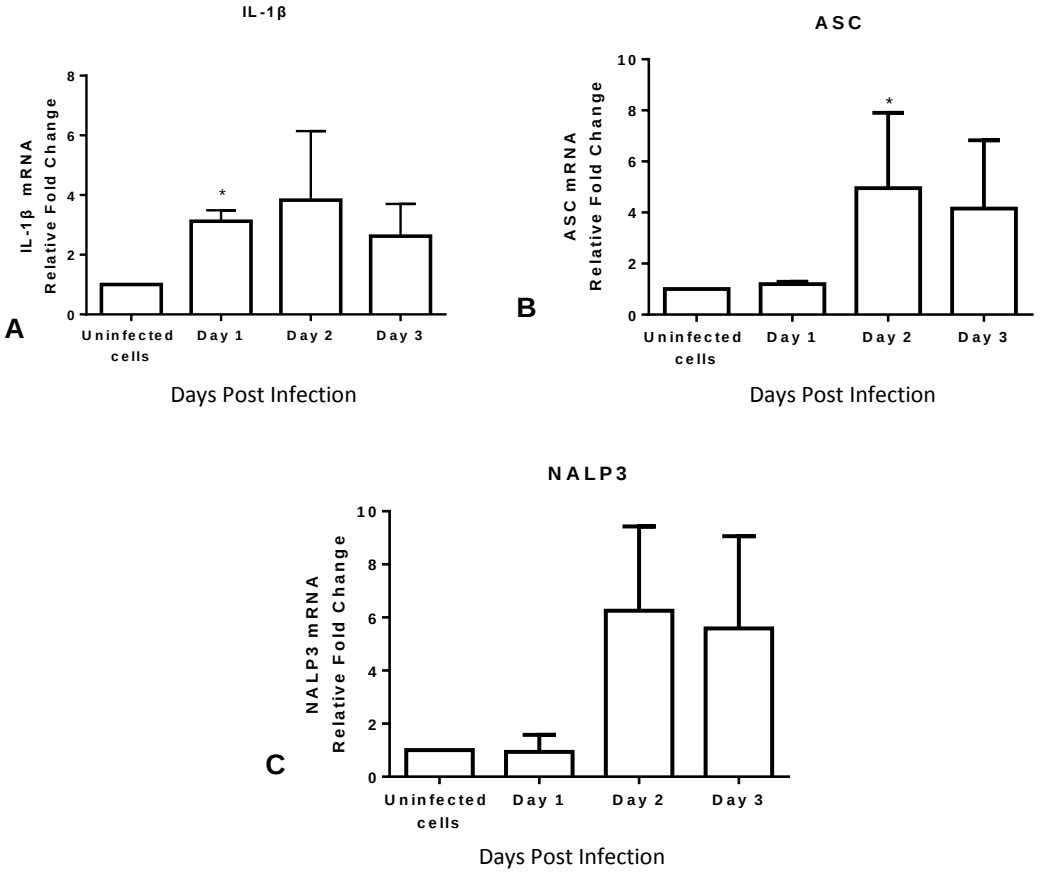


Figure 3-19: Changes in mRNA levels of IL-1β (A), ASC (B) and NALP3 (C) in HUVECS on days 1, 2 and 3 post-infection, as measured by RT-PCR

The data were normalised using GAPDH as a housekeeping gene. Error bars indicate SEM (n = 3). Statistical comparisons were made between infected and uninfected macrophages at different time points; * $p < 0.05$ (Mann-Whitney *U*-test).

3.3.3 LDH released from infected ECs after DENV-2 infection

To explore the occurrence of pyroptosis in ECs after DENV-2- infection, the release of LDH from HUVECs was monitored after infection with DENV-2. LDH levels were found to increase with time. The LDH level in the supernatants from DENV-2-infected HUVECs was significantly different from that observed in the supernatants from uninfected cells on day 3 post-infection ($p = 0.0418$) (Figure 3-20).

To further confirm that infection of ECs with DENV-2 induced pyroptosis, caspase-1 inhibitor treatment was incorporated into LDH release analysis. This can provide addition evidence for us to detect the presence of DENV-2-induced pyroptotic cell death specifically via two feactures of pyroptosis; caspase-1 dependency and LDH release. Based our results, it shown that the caspase-1 activity inhibition caused a 2- and 4-fold reduction in the release of LDH after DENV-2 infection on days 1 and 2 post-infection, respectively, with a further significant decrease in release on day 3 post-infection (p = 0.028) (Figure 3-20). Moreover, the viability of the DENV-2-infected HUVECs was significantly lower than that of the uninfected cells (p = 0.0042) at day 3 post-infection (Figure 3-21). Since pyroptosis is the only form of cell death suggested to be dependent on caspase-1 activity as well as the release LDH by cell lysis, to our knowledge this result is the first evidence that pyroptosis in human ECs was indueced by DENV-2 infection.

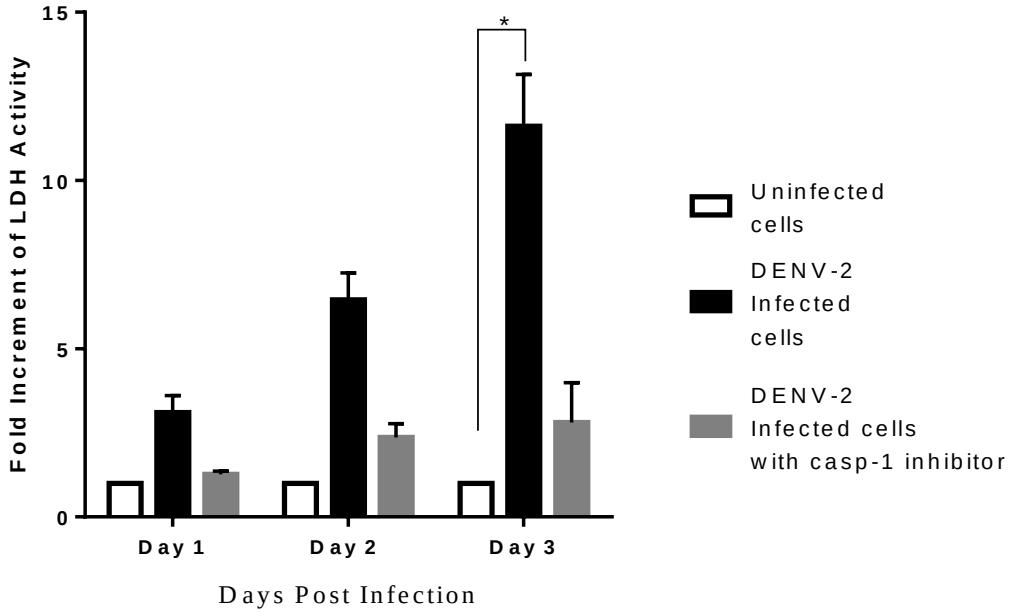


Figure 3-20: LDH release of DENV-2-infected HUVECs

The data are presented as the mean ± SEM of three individual experiments. Caspase-1 inhibitors (2µg/ml) were added to the HUVECs before DENV-2 infection, resulting in the reduction of LDH release to background control levels at day 1 post-infection. Statistical comparisons were made between infected and uninfected HUVECs at different time points; * $p < 0.05$ (Mann-Whitney *U*-test).

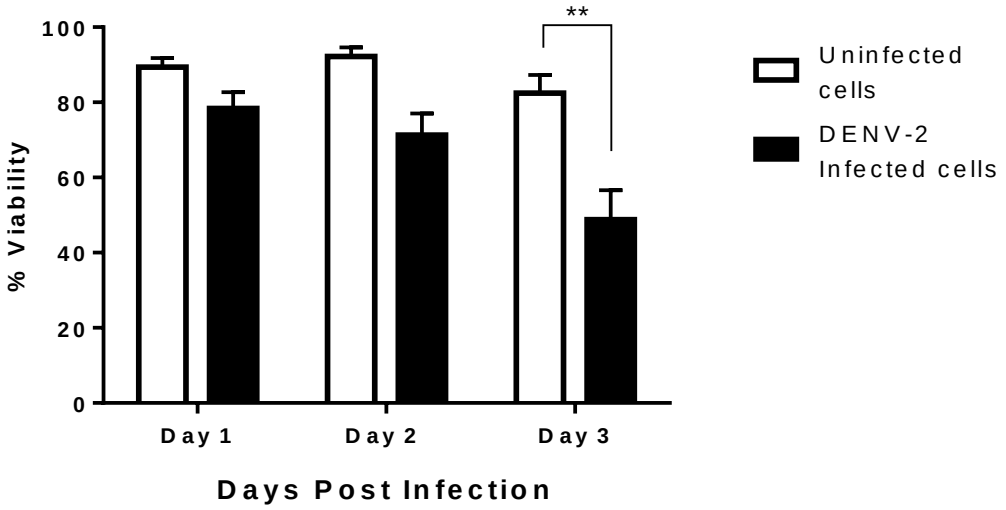


Figure 3-21: Cell viability of DENV-2-infected HUVECs

Comparison of the viability of uninfected and DENV-2-infected HUVECs on days 1, 2 and 3 post-infection. Cell viability was determined by Trypan blue exclusion using an automatic Vi-Cell cell viability analyser. The data are presented as the mean ± SEM of three individual experiments. The data were statistically analysed using the Mann-Whitney *U*-test. * $p < 0.05$.

3.3.4 Pyroptosis-enhanced endothelium hyperpermeability during DENV-2 infection in a caspase-1-dependent manner

To examine the effects of caspase-1-dependent pyroptosis on endothelial permeability, we treated HUVECs with DENV-2 with an MOI of 10 or as mock-exposed for a 24-hour interval before measuring permeability in a transwell assay. To show evidence of a caspase-1-dependent process, we also performed a caspase-1 inhibitor treatment. From the results, DENV-2 infection caused an increase ($p = 0.0016$) in the permeability of HUVECs, which was blocked by the caspase-1 inhibitor (Figure

3-22). These results indicate that pyroptosis in ECs induced by DENV-2 infection increases membrane permeability. This is the first evidence that pyroptosis in ECs contributes to the mechanism of vascular leakage during DENV infection.

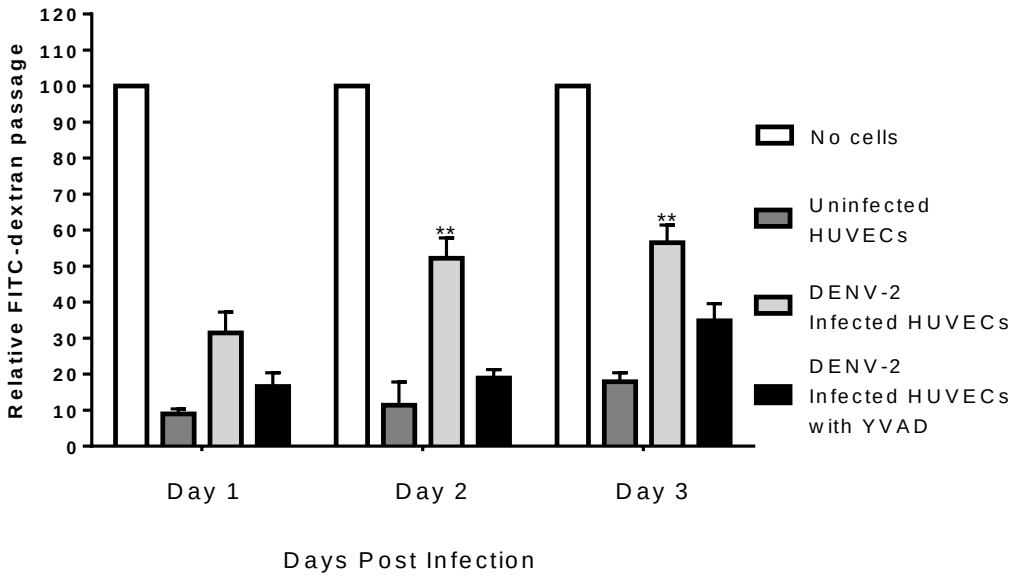


Figure 3-22: Endothelial permeability in DENV-2 infection

To determine permeability, FITC fluorescence dextran was measured at 485nm excitation and 520nm emission in 24-hour intervals. The FITC-dextran transit across the ECs was significantly enhanced after DENV-2 infection; an effect suppressed by the caspase-1 inhibitor (Z-YVAD-FMK). Results are shown from three independent experiments. Error bars indicate SEM. The data were statistically analysed among each group using the Kruskal–Wallis test. * $p < 0.05$, ** $p < 0.01$.

3.4 Involvement of capsase-4 in pyroptosis and IL-1β production in both macrophages and ECs during DENV-2 infection

3.4.1 Up-regulation of caspase-4 coincides with the up-regulation of caspase-1

To address the roles of caspase-4 in the synthesis of IL-1β and pyroptosis during DENV-2 infection in primary macrophages, caspase-4 mRNA and protein expression were evaluated by real-time RT-PCR and western blotting, respectively. We observed

that the mRNA expression of caspase-4 gradually increased over time post-infection in both macrophages ($p = 0.0258$) and HUVECs ($p < 0.001$) and was significantly increased for both at day 3 post-infection ($p = 0.0318$ and $p = 0.0128$, respectively) (see Figures 3-23 and 3-24). The protein expression of caspase-4 in macrophages and EC was determined by western blotting and found to increase in a time-dependent manner after DENV-2 infection. The observed trends in caspase-4 expression were similar to those observed for caspase-1 (Figures 3-8, 3-9A and B, 3-16 and 3-17). Our results demonstrate that caspase-4 expression is closely associated with the activation of caspase-1, the production of IL-1β and pyroptosis in human macrophages and ECs after DENV-2 infection.

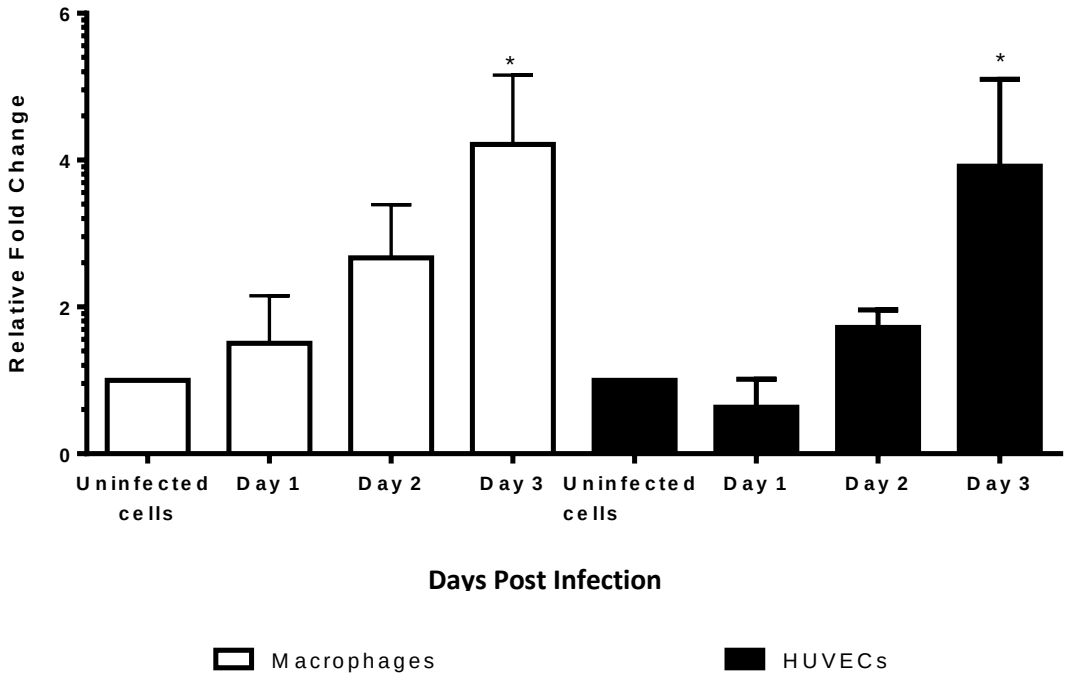


Figure 3-23: Changes in mRNA levels for caspase-4 in primary macrophages and HUVECs at days 1, 2 and 3 post-infection, as measured by real-time RT-PCR

The data were normalised using GAPDH as a housekeeping gene. Errors bars indicate SEM. * $p < 0.05$, ** $p < 0.01$ (Mann-Whitney *U*-test) v. uninfected cells. The difference between infected and uninfected macrophages at different time points was calculated using the Kruskal–Wallis test. * $p < 0.05$.

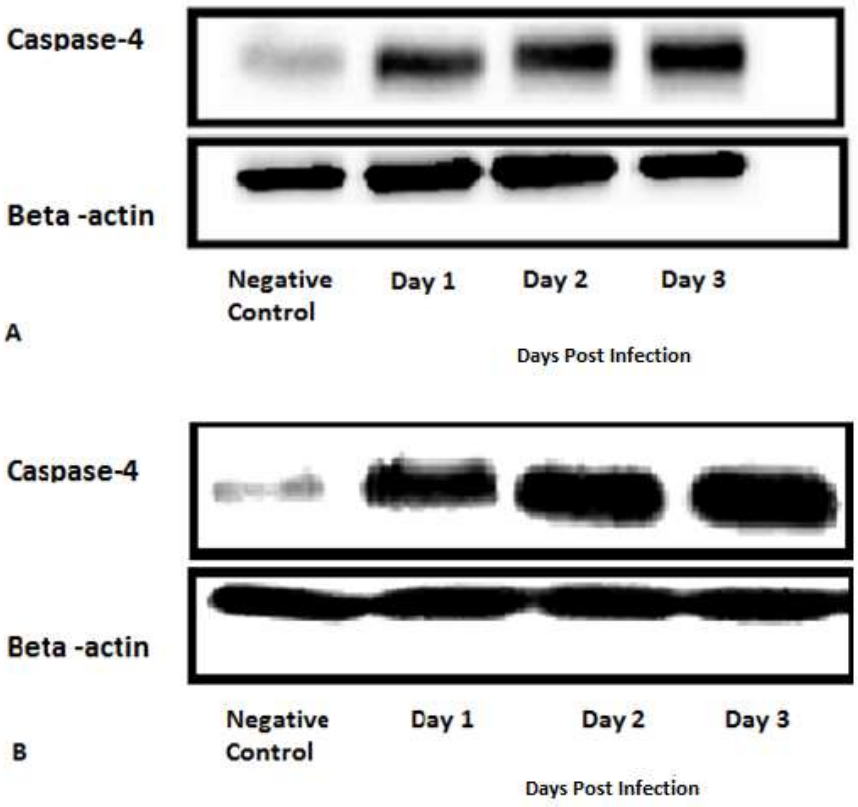


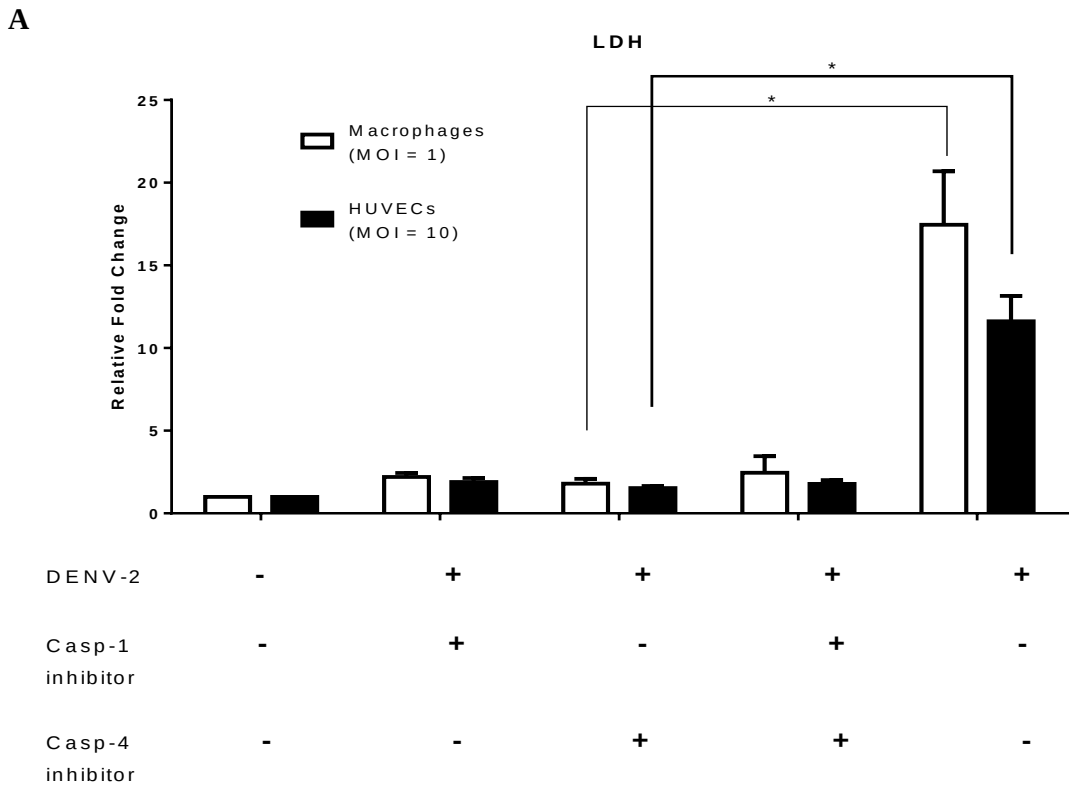
Figure 3-24: Expression of caspase-4 induced by DENV-2 infection

The protein expression of caspase-4 in macrophages (A) and HUVECs (B) was determined by western blotting. Caspase-4 expression increased in a time-dependent manner after DENV-2 infection (n = 3). B-actin was used as a loading control.

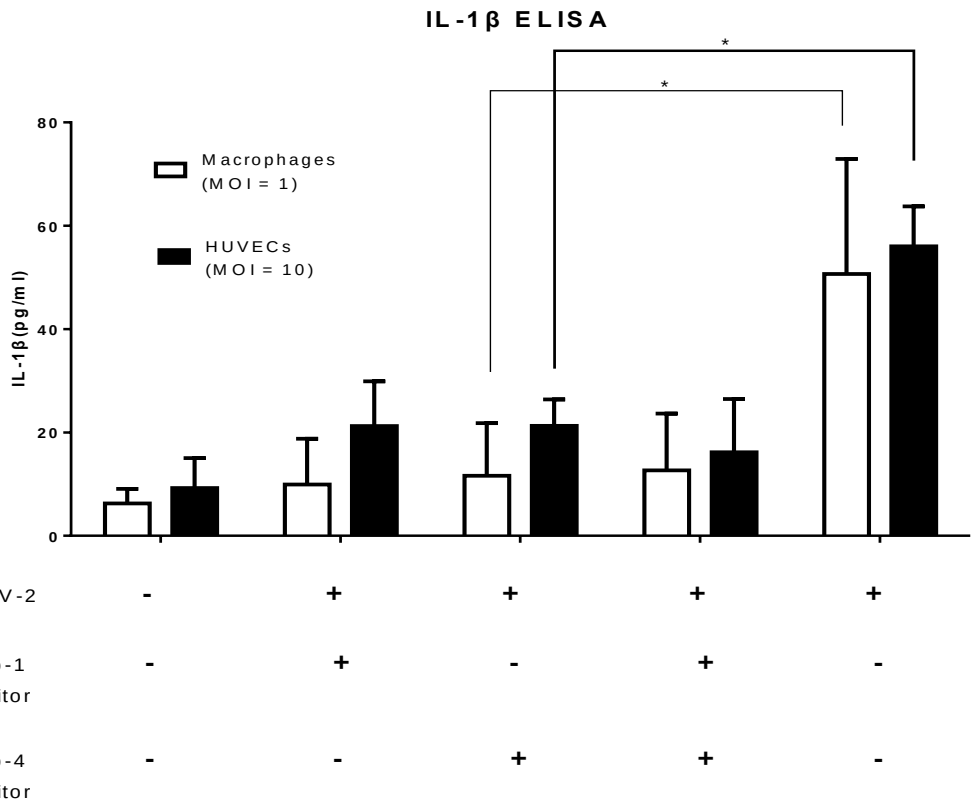
3.4.2 Caspase-4 regulates caspase-1 activity during the stimulation of IL-1β production and pyroptosis in macrophages and ECs during DENV-2 infection

To determine the effects of DENV-2 infection on pro-inflammatory caspase activation, the catalytic activity of caspase-1 and -4 were assessed. These were found to increase approximately 4-fold in macrophages and 5-fold in HUVECs after DENV-2 infection (Figure 3-25C and 3-25D). To further investigate the hierarchical relationship between caspase-1 and -4, the activity of these proteins was evaluated in the presence of caspase-1 and -4 inhibitors. As shown in Figure 3-25A, LDH production was reduced to background levels in the presence of caspase-1 and/or -4 inhibitors. Similar patterns

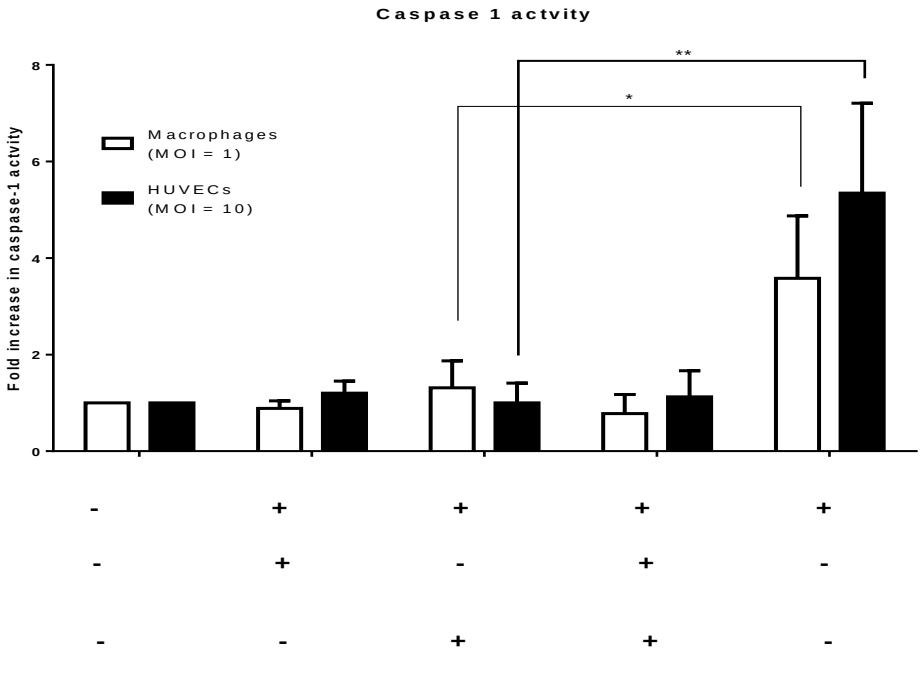
were observed for IL-1β activity in the culture supernatants (Figure 3-25B). The results demonstrate that caspase-1 activity was inhibited by both caspase-1- and -4-specific inhibitors (Figure 3-25C); however, caspase-4 activity was suppressed only by the caspase-4 inhibitor (Figure 3-25D). Further, the short interfering RNA (siRNA) knockdown of caspase-4 strongly inhibited DENV-2-induced pyroptosis in macrophages and HUVECs (Figures 3-26B and 3-26D). The secretion of IL-1β (Figure 3-26A) and caspase-1 activity (Figure 3-26C) also declined in the presence of capsase-4 siRNA. This finding suggests that caspase-4 is upstream of caspase-1 in the pathway that regulates pyroptosis and IL-1β synthesis in macrophages during DENV-2 infection.



B


IL-1β ELISA
IL-1β(pg/ml)
80
60
40
20
0
Macrophages
(MOI = 1)
HUVECs
(MOI = 10)
*
*
DENV-2 - + + + +
Casp-1 inhibitor - + - + -
Casp-4 inhibitor - - + + -

C


Caspase 1 actvity
Fold increase in caspase-1 actvity
8
6
4
2
0
Macrophages
(MOI = 1)
HUVECs
(MOI = 10)
**
*
DENV-2 - + + + +
Casp-1 inhibitor - + - + -
Casp-4 inhibitor - - + + -

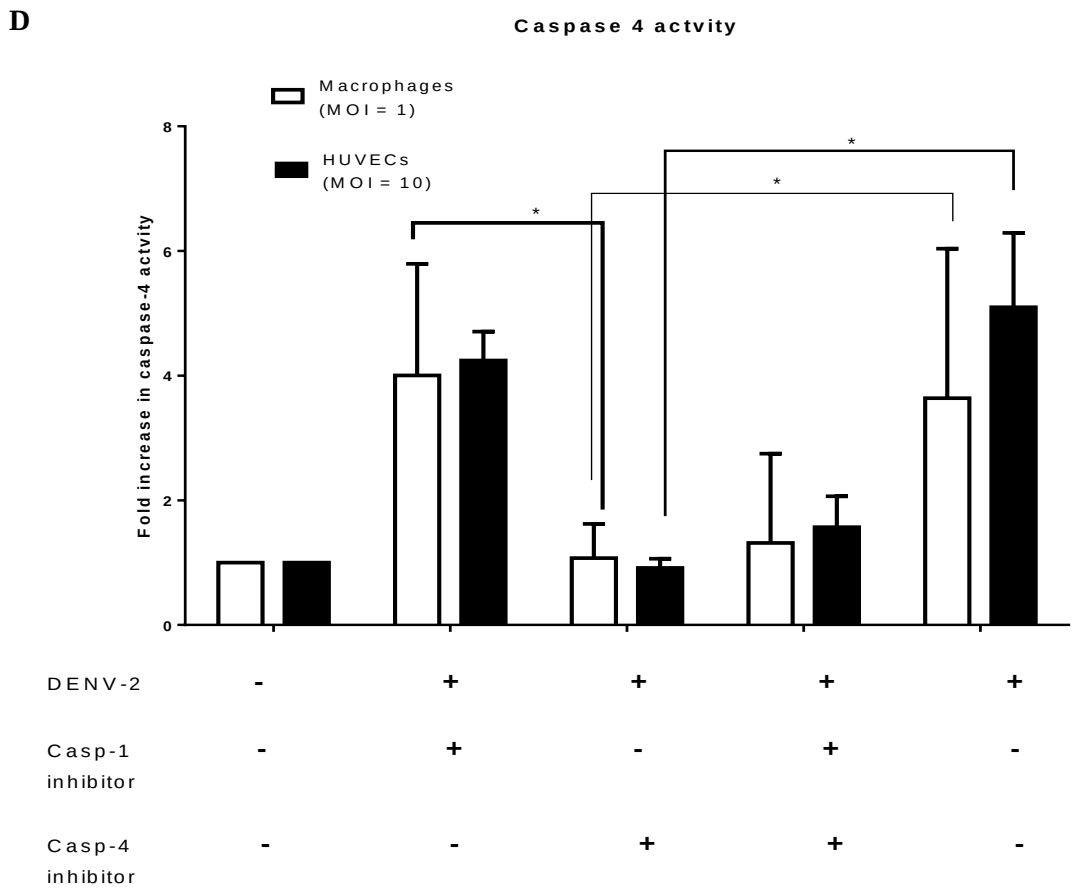
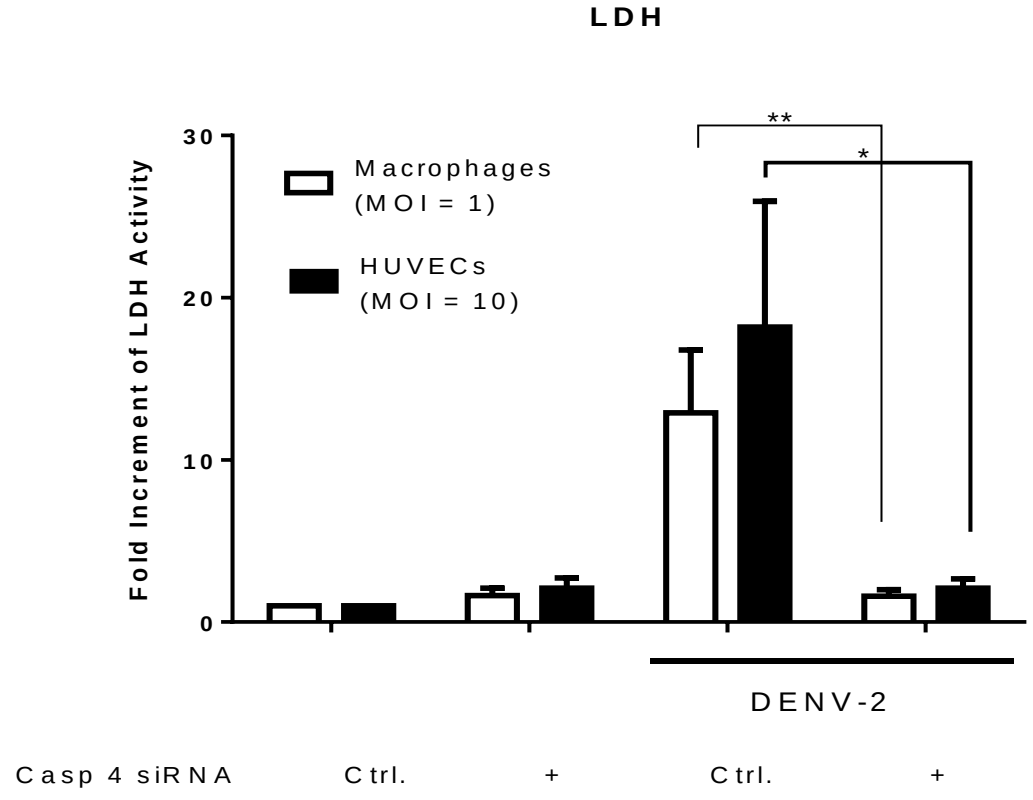


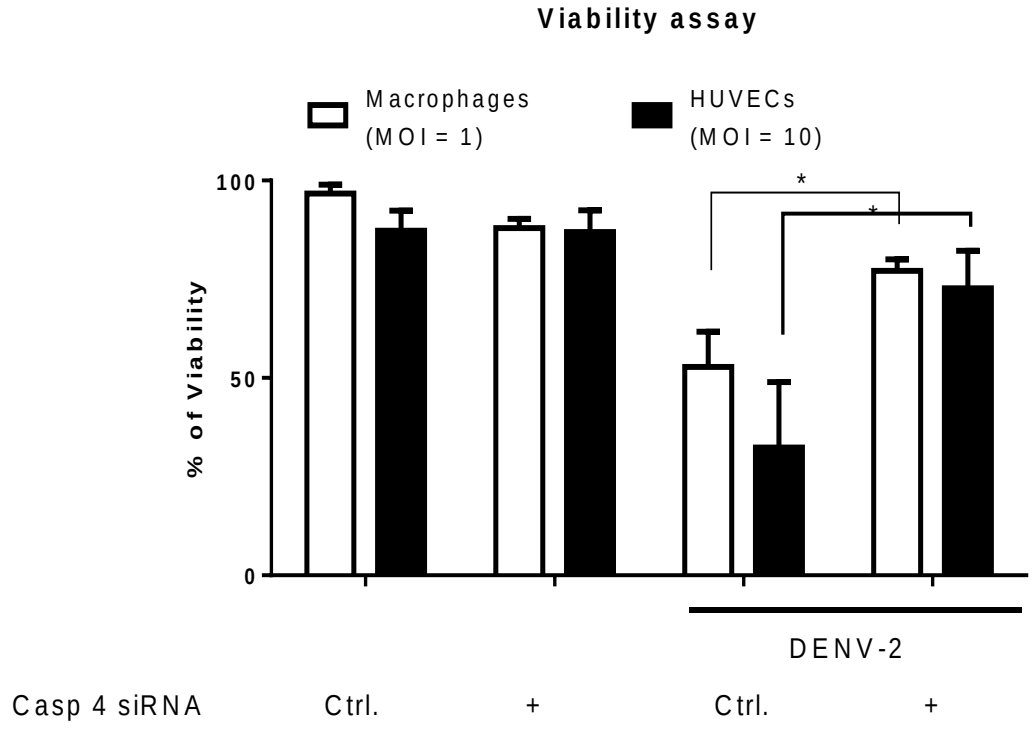
Figure 3-25: Enzymatic activity of caspsase-1 and -4 in the presence of caspase inhibitors

The role of pro-inflammatory caspases in the production of IL-1β by macrophages and HUVECs during DENV infection was investigated. Caspase-1 and -4 inhibitors (2 µg/ml) were added to the macrophages before DENV-2 infection. LDH (A), IL-1β (B), caspase-1 (C) and caspase-4 (D) activity were measured 72 hours post-infection. Error bars indicate SEM (n=3). Statistical comparisons were made between infected and uninfected cells at various time points; * $p < 0.05$, ** $p < 0.01$ (Mann-Whitney *U*-test).

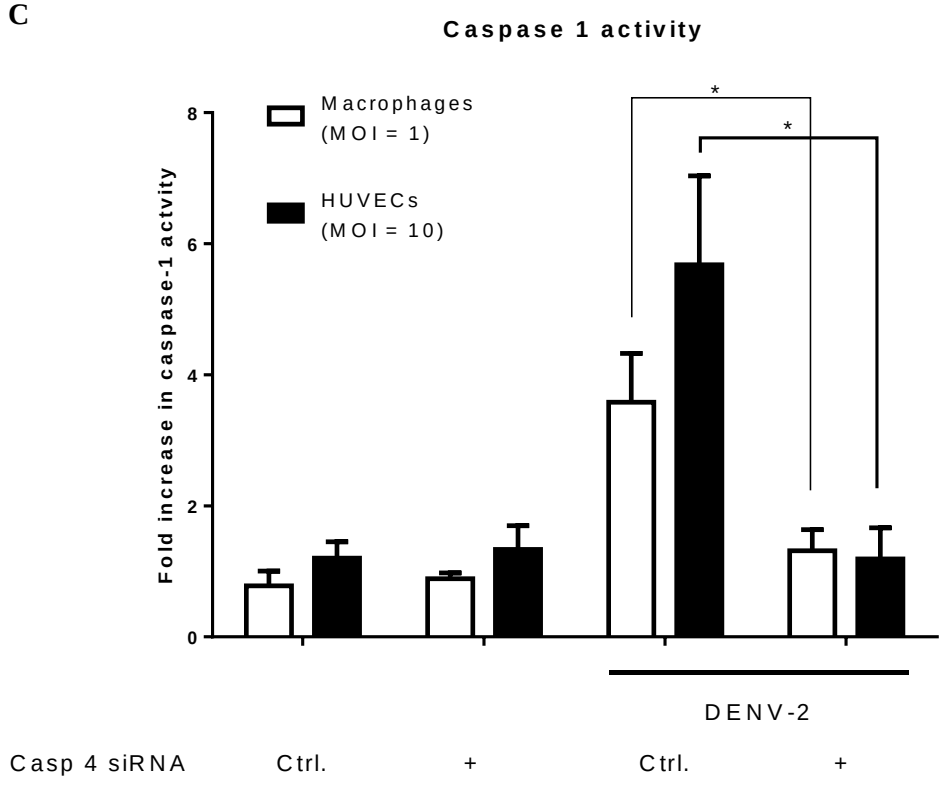
A


LDH
Fold Increment of LDH Activity
30
20
10
0
Macrophages
(MOI = 1)
HUVECs
(MOI = 10)
**
*
DENV-2
Casp 4 siRNA
Ctrl.
+
Ctrl.
+

B


Viability assay
Macrophages
(MOI = 1)
HUVECs
(MOI = 10)
% of Viability
100
50
0
*
*
DENV-2
Casp 4 siRNA
Ctrl.
+
Ctrl.
+

C



D

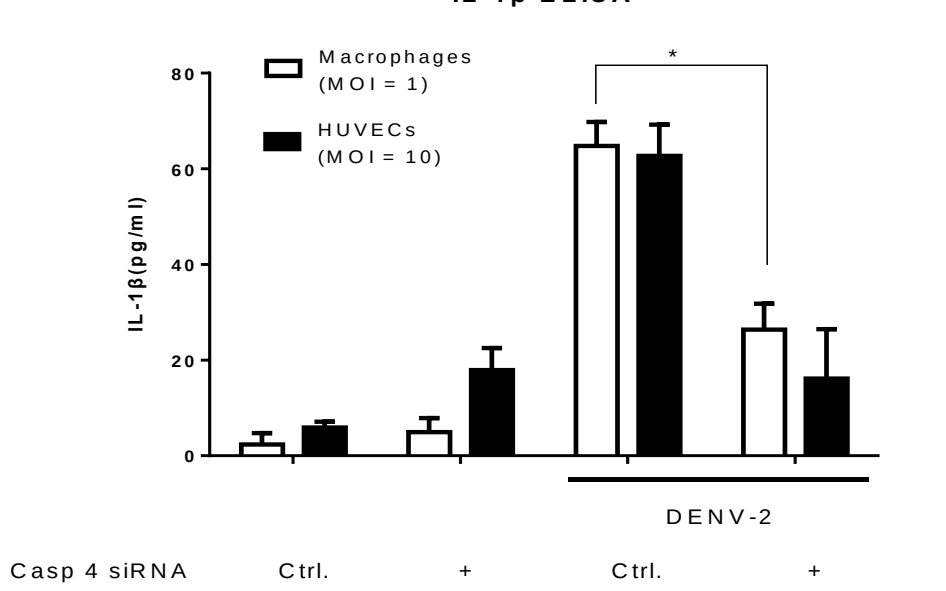


Figure 3-26: Caspase-4 siRNA suppresses the activity of caspase-1, IL-1β production and pyroptosis after DENV-2 infection

The siRNA knockdown of caspase-4 expression strongly inhibited DENV-2-induced cell death (B) and LDH release (A) in macrophages and HUVECs. The activity of caspase-1 (C) and IL-1β secretion (D) after DENV-2 infection was also suppressed under siRNA knockdown. Statistical comparisons were made between infected and uninfected cells treated with caspase-4 siRNA; * $p < 0.05$ (Mann-Whitney *U*-test).

3.5 DENV activated both apoptotic and pyroptotic cell death in a dose-dependent manner

3.5.1 Cell viability of primary macrophages and ECs infected with DENV-2 at different MOIs

To investigate the effect of an infectious dose of DENV on pyroptotic cell death in the two study models (primary macrophages and HUVECs), the cells were infected with DENV-2 at MOIs of 0.1, 1, 10 and 100 (Figure 3-27). Cell viability was analysed at 24-hour intervals. In DENV-2-infected cells with lower MOIs (e.g., MOI = 0.1 or 1), the onset of cell death was delayed. At day 1 post-infection, cell viability was approximately 80–95%, decreasing to about 70% by 3 days post-infection. For ECs infected with DENV-2 at high MOIs (10 or 100), cell viability was less than 30% after 3 days post-infection (Figure 3-27). From the results, the initial infectious dose of DENV-2 was proportionally related to the degree of cell death post-infection in macrophages ($p = 0.0048$) and HUVECs ($p = 0.002$) (Figure 3-27). The number of viable cells were shown persistently constant throughout the experiment in the mock-infected cells.

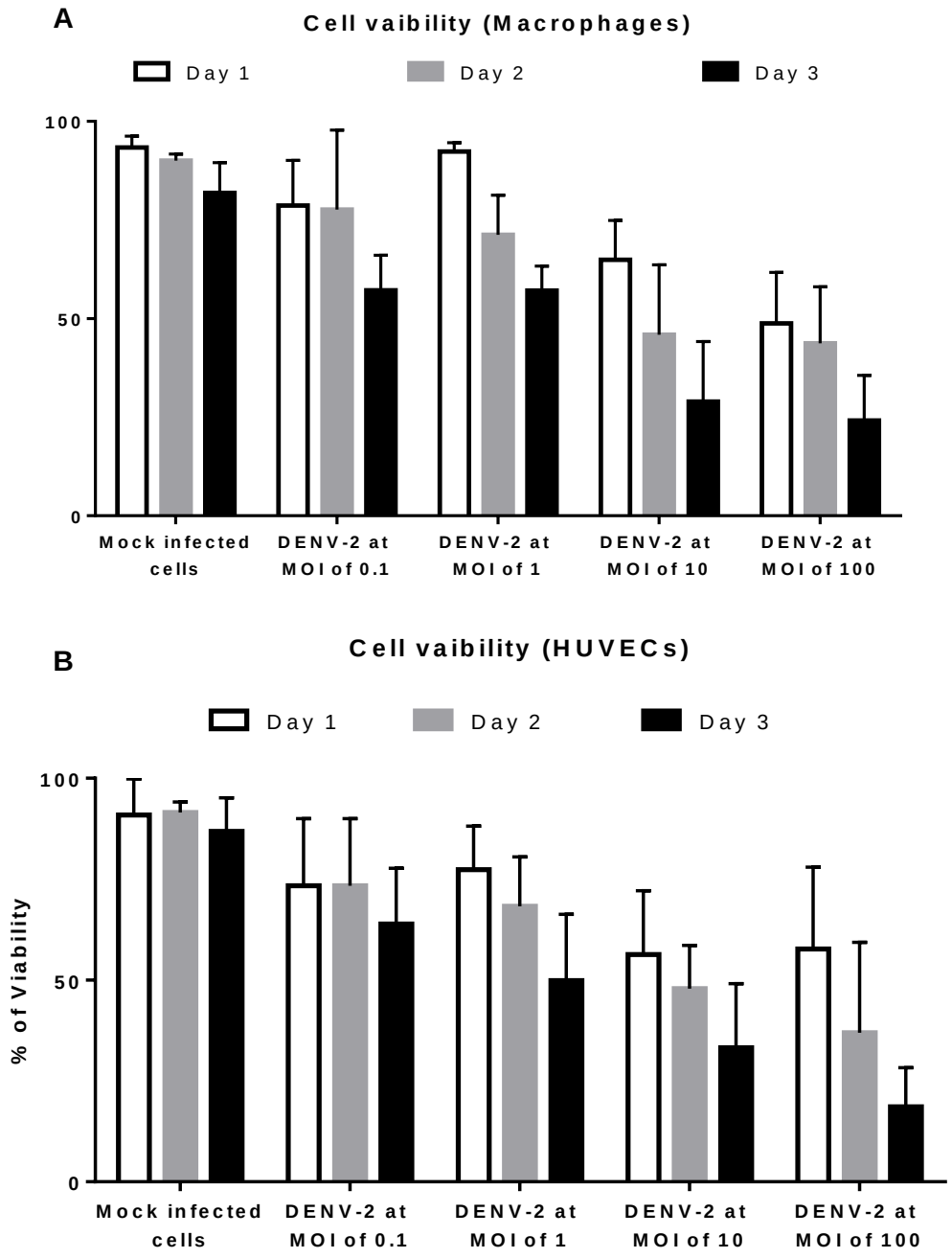


Figure 3-27: The effects of different infectious doses of DENV-2 virus on primary macrophages and HUVECs

Both primary macrophages (A) and HUVECs (B) were infected with DENV-2 at MOIs of 0.1, 1, 10 and 100. At the indicated time intervals, cells were collected and cell viability was determined by Trypan blue exclusion using an automatic Vi-Cell cell viability analyser. Results from three independent experiments were plotted as the mean ± SEM. Statistical comparisons were made between different infectious doses; * $p < 0.05$ and ** $p < 0.01$ (Kruskal–Wallis test).

3.5.2 Release of LDH from DENV-2-infected primary macrophages at different MOIs

To confirm that infection of primary macrophages with DENV-2 at high infectious doses (MOI>10) induced pyroptosis, LDH activity was assessed. Figure 3-28 shows that there was a distinguised increase in the level of LDH activity, generally a 3- to 4-fold increase by 24 hours post-infection at high MOIs (MOI = 10 and 100). In contrast, there was only a small increase in released LDH activity in macrophages, about 1.2-fold by 24 hours post-infection (Figure 3-28A), rising to a 2- to 5-fold increase after 72 hours post-infection. The release of LDH from primary macrophages infected with DENV-2 was significantly different for the different MOIs on day 2 post-infection in macrophages ($p = 0.026$). The change in LDH activity in DENV-2-infected HUVECs was similar to that in macrophages. The release of LDH from HUVECs infected with DENV-2 was significantly proportional to the difference in MOI ($p = 0.013$) (Figure 3-28B).

Previous studies have suggested that caspase-1 activity is another specific marker for pyroptosis (Bergsbaken et al., 2009; S. L. Fink & Cookson, 2005). The caspase-1 enzyme executes rapid pyroptosis after microbial infection. To further confirm that infection of primary macrophages with DENV-2 at high infectious doses (MOI>10) induces pyroptosis, caspase-1 catalytic activity was assessed from cell lysates. This activity was found to approximately double in macrophages after DENV-2 infection at MOIs of 10 and 100 at 3 days post-infection (Figure 3-29A). Activity was much lower in primary macrophages after DENV-2 infection at MOIs of 0.1 and 1 at 3 days post-infection (Figure 3-29A). The caspase-1 activity in HUVECs was only significantly increased at higher MOIs (10 and 100) at day 2 and 3 post-infection (see

Figure 3-29B): an 18.2-fold increase by day 3 post-infection. Thus, for both macrophages and HUVECs, both showed high capsase-1 activity at an MOI of 10 or 100.

Together with the observed increased activity of LDH (Figure 3-28A and B), these findings suggest that primary macrophages and ECs underwent pyroptosis during DENV-2 infection at high MOIs.

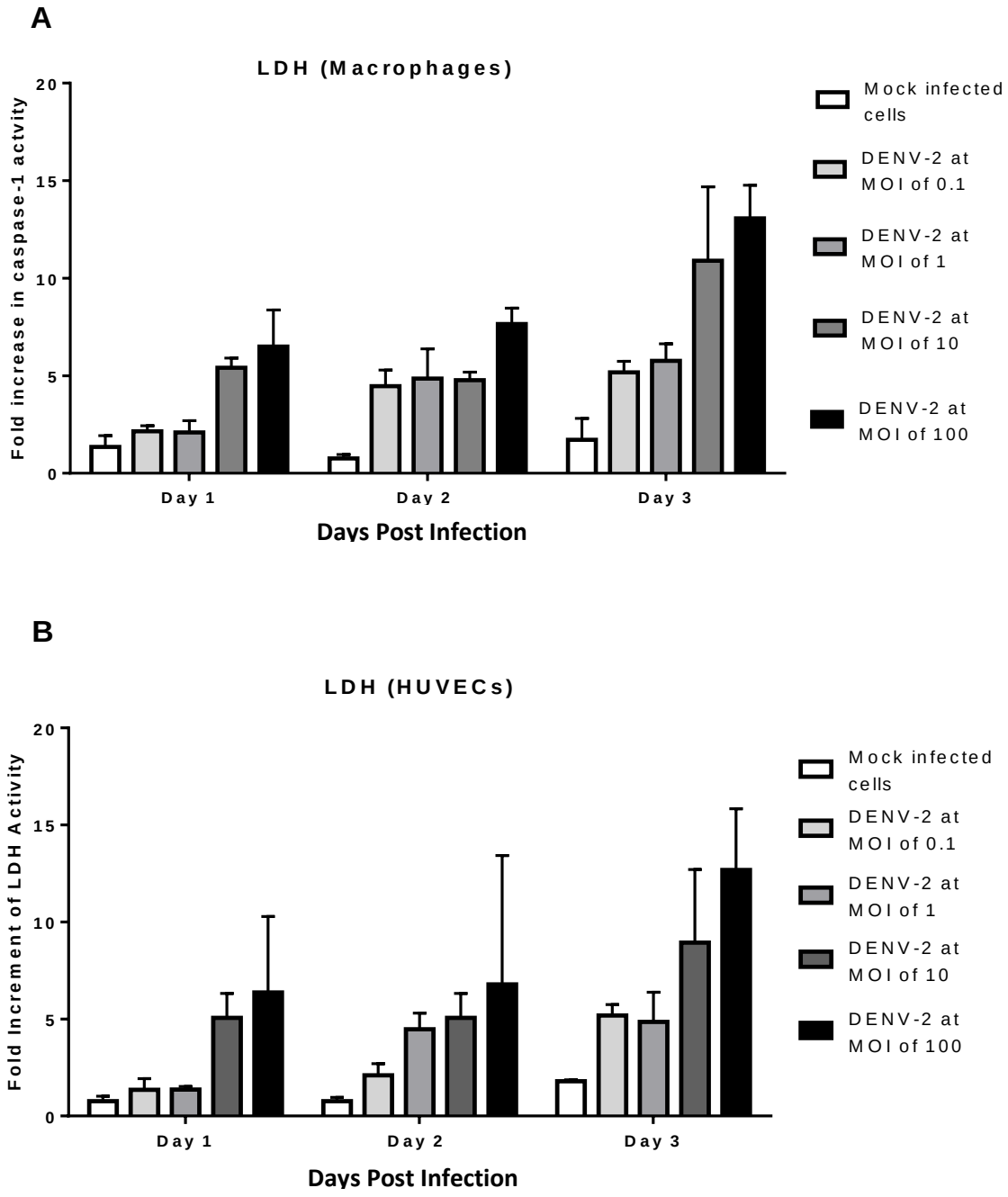


Figure 3-28: LDH activity in DENV-2-infected primary macrophages (A) and HUVECs (B) at different MOIs

The activity of LDH in culture supernatants at days 1, 2 and 3 post-DENV-2 infections at MOIs of 0.1, 1, 10 and 100. The level of activity peaks at over a 12-fold increase at 72 hours post-infection. In contrast, the primary macrophages that were infected with DENV-2 at an MOI of 0.1 only showed a slight increase in LDH activity. Results from three independent experiments are plotted as the mean ± SEM. Statistical comparisons were made between different infectious doses; * $p < 0.05$ (Kruskal-Wallis test).

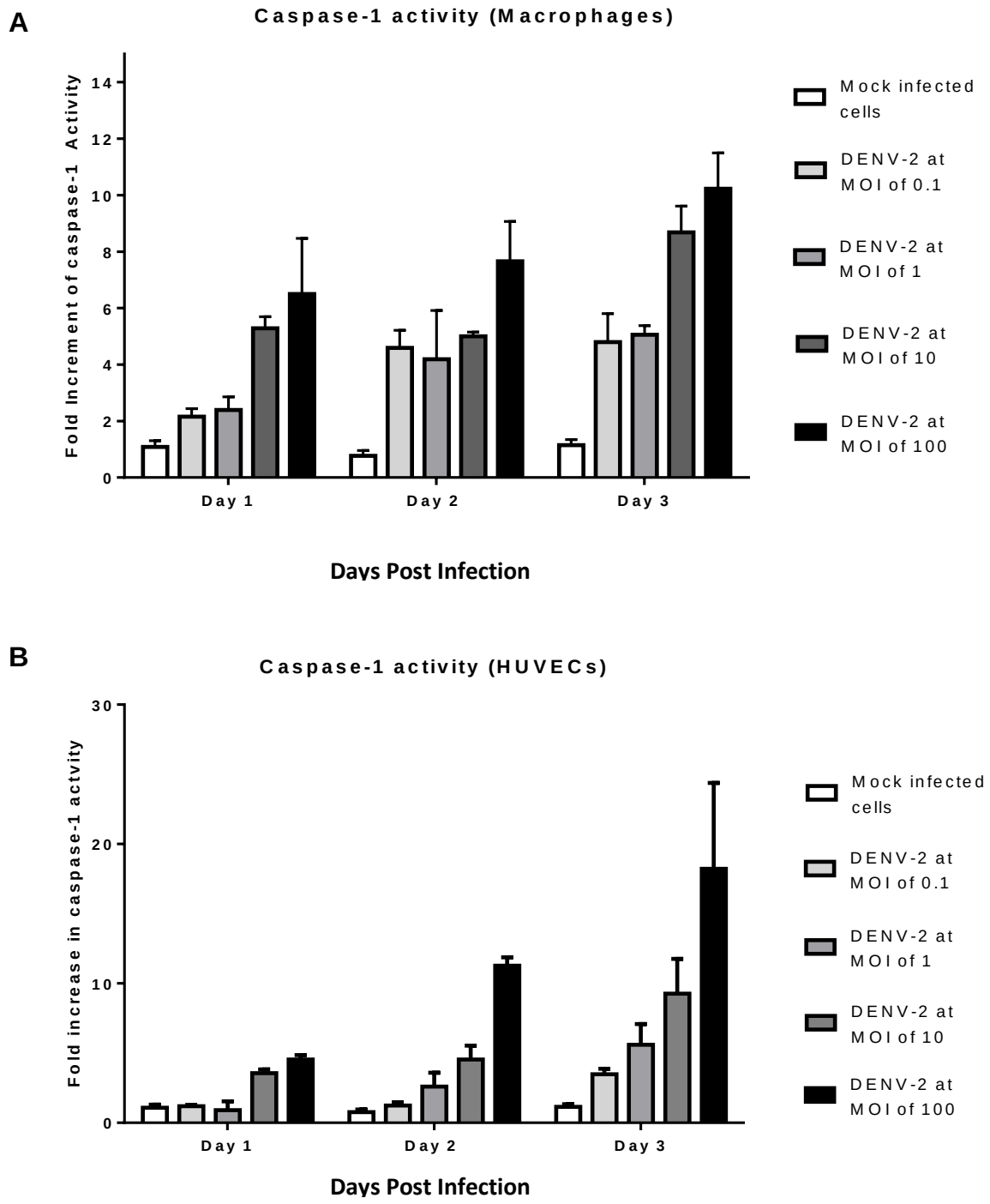


Figure 3-29: Enzymatic activity of capsase-1 in DENV-2-infected primary macrophages (A) and HUVECs (B) at different MOIs

The enzymatic activity of caspase-1 was measured by using the caspase-1/ICE Fluorometric assay kits (Biovision). Whole cell lysates were collected from non-infected and DENV-2-infected cells. These assays are based on the detection of cleavage of the 7-amino-4-trifluoromethylcoumarin (AFC) labelled substrates by capsase-1. Comparison of the AFC fluorescence in the cell lysates was against non-infected (mock) and DENV-2-infected cells. Error bars indicate SEM. Statistical comparisons were made between different infectious doses; * $p < 0.05$ (Kruskal–Wallis test).

3.5.3 Release of cytochrome c from DENV-2-infected primary macrophages at different MOIs

An experiment was performed to study the relevant apoptosis signal in DENV-2-infected cells at different MOIs. Cytochrome c is a small protein found loosely to the outer surface of the inner mitochondrial membrane whose release from mitochondria is conserved in all mammalian cells undergoing apoptosis (S. L. Fink & Cookson, 2005; Jiang & Wang, 2004). The released cytochrome from the mitochondria react to adaptor proteins procaspase-9 and Apaf-1 to form a cytosolic apoptosome complex, inducing a number of biochemical reactions and subsequent caspase cleavage and activation, eventually resulting in apoptotic cell death. The cytochrome c released from mitochondria into the cytosol of DENV-2-infected primary macrophages at different MOIs was determined by ELISA. Figure 3-30 shows that the cytochrome c was detected as early as day 1 post-infection at lower MOIs (0.1) in macrophages. Thereafter, there was a remarkable increase in the level of cytochrome c in the cytosol lysates, approximately a 3- to 4-fold increase by 3 days post-infection at lower MOIs. In contrast, there was only a slight increase in the amount of released cytochrome c at high MOIs: about 1.2-fold by 3 days post-infection (Figure 3-30), a level less than that for lower MOIs at 2 days post-infection (Figure 3-30). The release of cytochrome c was thus inversely proportional to the infectious dose (Figure 3-30A). The release of cytochrome c in HUVECs showed a remarkable increase at day 2 post- infection, although with a delay in expression when compared with macrophages. In general, high levels of cytochrome c were associated with lower MOIs (Figure 3-30B). Primary macrophages and HUVECs favoured apoptotic cell death during DENV-2 infection at low MOIs.

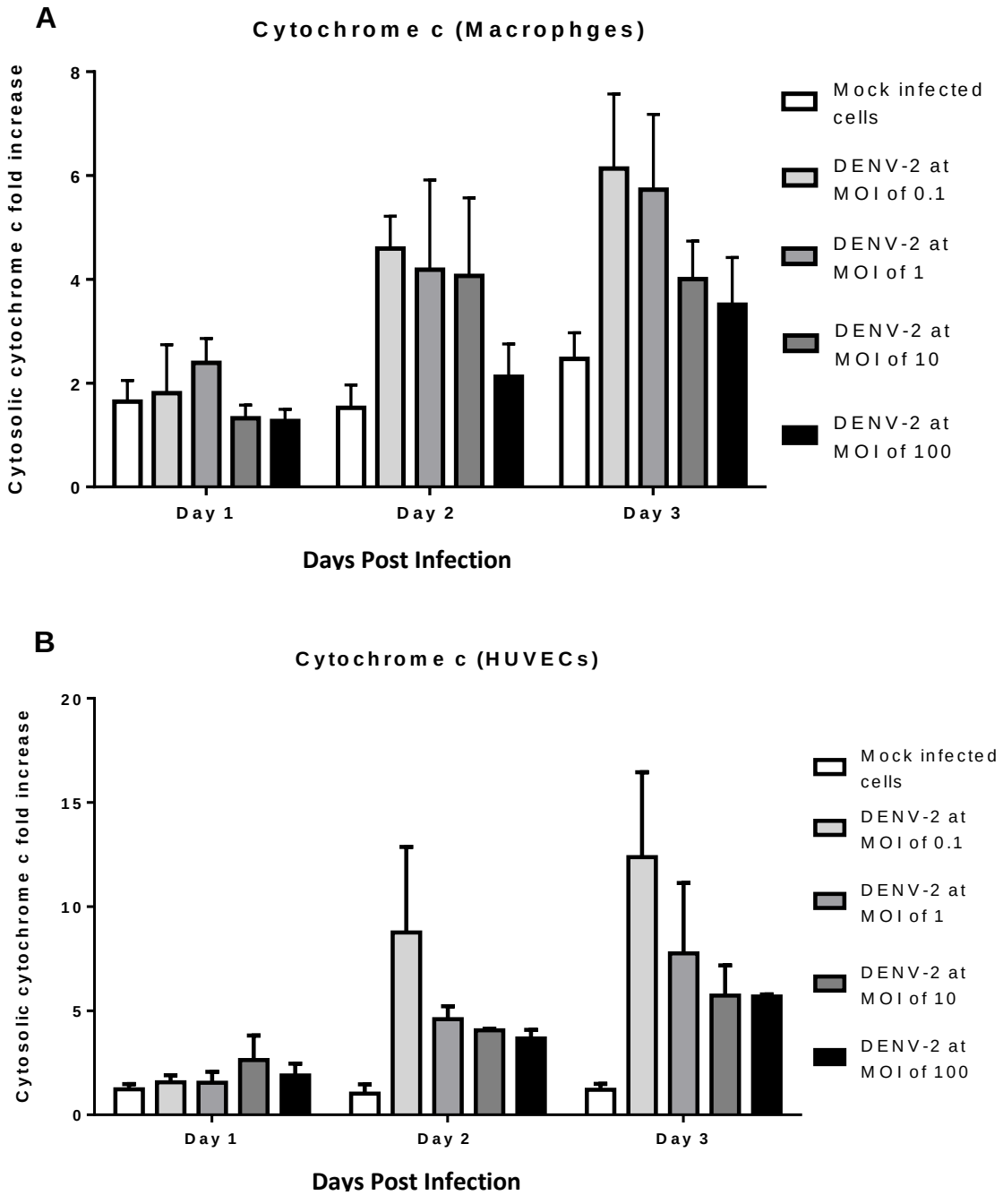


Figure 3-30: The release of cytochrome c from mitochondria into cytosol of DENV-2-infected primary macrophages (A) and HUVECs (B) at different MOIs, as determined by ELISA (ENZO)

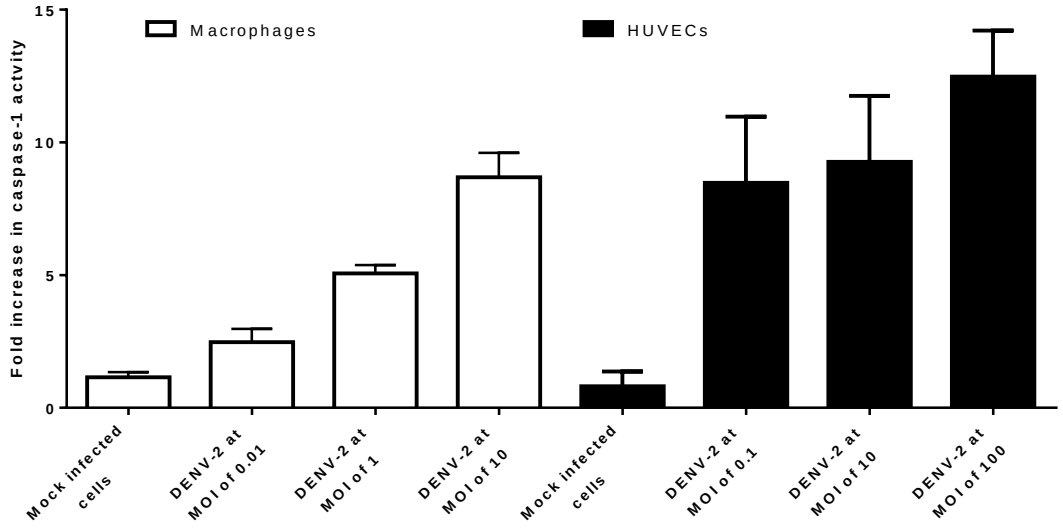
The cytosolic fraction of the proteins from the cell lysates was isolated with digitonin cell permeabilization buffers. The amount of cytochrome c released from the cytosol was determined by measuring the optical density (OD) of the sample, which is directly proportional to the concentration of cytochrome c. The comparison was performed between the OD for non-infected (mock) and DENV-2-infected cells with different MOIs. Error bars indicate SEM. Statistical comparisons were made between different infectious doses; * $p < 0.05$ (Kruskal–Wallis test)

3.5.3 Expression and activation of caspase-1, -3 and -4

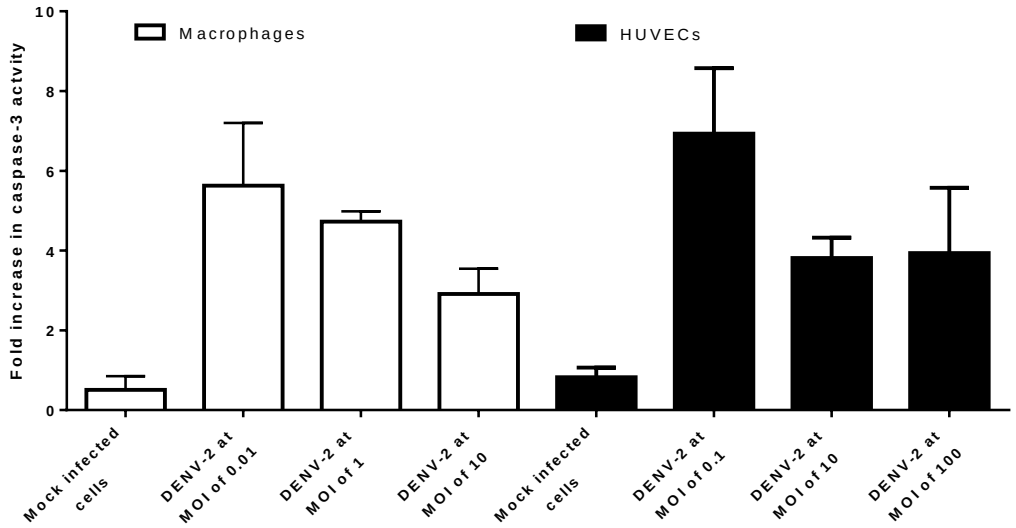
Caspases actavtion in cells plays an important role in the mediation of apoptosis and pyroptosis in mammalian cells. The upstream caspase is Caspase-8 and the downstream effector of apoptosis is caspase-3. It is participated in the execution of apoptosis and induces the cells to undergo apoptotic cell death. Conversely, caspase-1 is classified as an initiator of pyroptosis. In this study, we have demonstrated that caspase-4 is involved in the activation of caspase-1 and pyroptosis in primary macrophages after DENV-2 infection. To study the role of the different caspases on observed cell death with different MOIs, the caspase activation was investigated in DENV-2-infected primary macrophages at MOIs of 0.01, 1 and 10 and HUVECs at MOIs of 0.1, 1, 10 and 100. The cell lysates from infected and mock-infected cells were measured for caspase-3 and -4 activity using a fluorometric assay. Caspase-1 activity was already studied as part of this research (Figures 3-29 and 3-31A). A significant increase was found in caspase-3 activity in macrophages (p = 0.004) and HUVECs (p = 0.0069) at lower MOIs (MOI=>0.1) (Figure 3-31B). The activation of caspase-3 in macrophages and HUVECs was suppressed when compared with higher MOIs (MOI = or 0.1) (p = 0.005) (see Figure 3-31B). The activity of caspase-4 continually increased with MOI in primary macrophages (p < 0.001) and HUVECs (p = 0.0028) (Figure 3-31C). Interestingly, the observed trend in caspase-4 activity was similar to that observed for caspase-1 in both macrophages and HUVECs (Figures 3-29 and 3-31C). This finding corresponds with caspase-4 activity being closely associated with the activation of caspase-1 and pyroptosis in primary macrophages after DENV-2 infection.

To determine whether the observed caspase activity was due to *de novo* synthesis, we analysed the mRNA levels of caspase-1 (Figure 3-31D), -3 (Figure 3-31E) and -4 (Figure 3-31F) in both infected and mock-infected cells. Caspase-1 mRNA expression was up-regulated at higher infectious doses (MOI = 10 or 100) during the course of DENV-2 infection (Figure 3-31D). The expression of caspase-3 mRNA increased dramatically in the DENV-2-infected cells at lower infectious doses (MOI = 0.01 or 1) (Figure 3-31E) but was suppressed at higher MOIs (MOI =10 or 100) (Figure 3-30E). The results for caspase-4 mRNA expression correlated with those for caspase-1 mRNA expression (Figure 3-31F). Likewise, the mRNA expression results were consistent with those for caspase activity. In general, the mRNA expression of caspase-3 and -4 in HUVECs was lower than that in macrophages and ECs.

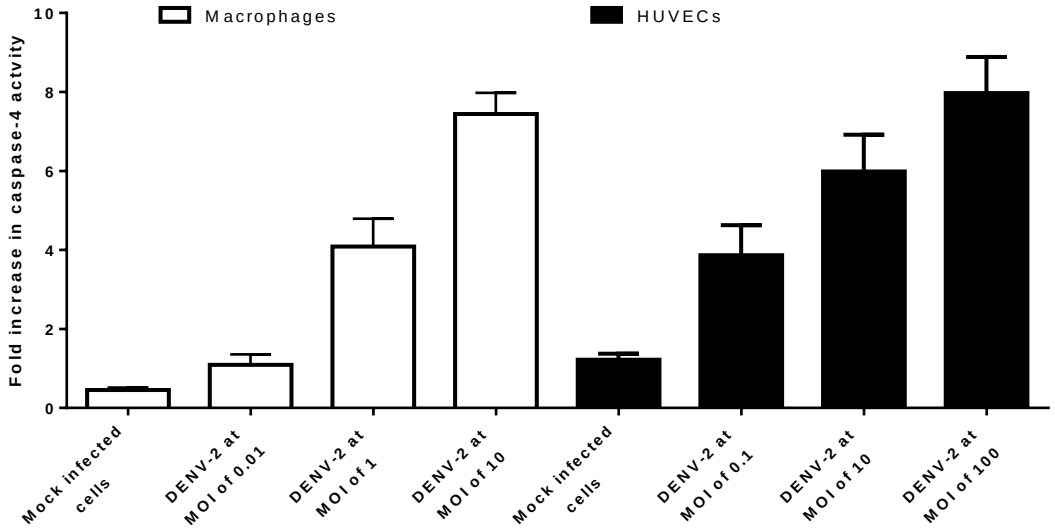
A


Macrophages
HUVECs
Fold increase in caspase-1 actvity
15
10
5
0
Mock infected cells
DENV-2 at MOI of 0.01
DENV-2 at MOI of 1
DENV-2 at MOI of 10
Mock infected cells
DENV-2 at MOI of 0.1
DENV-2 at MOI of 10
DENV-2 at MOI of 100

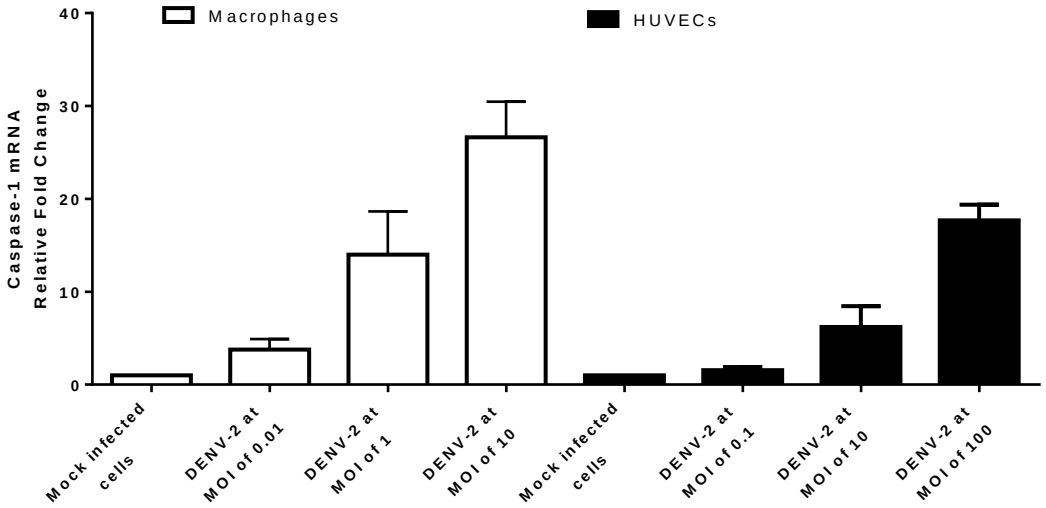
B


Macrophages
HUVECs
Fold increase in caspase-3 actvity
10
8
6
4
2
0
Mock infected cells
DENV-2 at MOI of 0.01
DENV-2 at MOI of 1
DENV-2 at MOI of 10
Mock infected cells
DENV-2 at MOI of 0.1
DENV-2 at MOI of 10
DENV-2 at MOI of 100

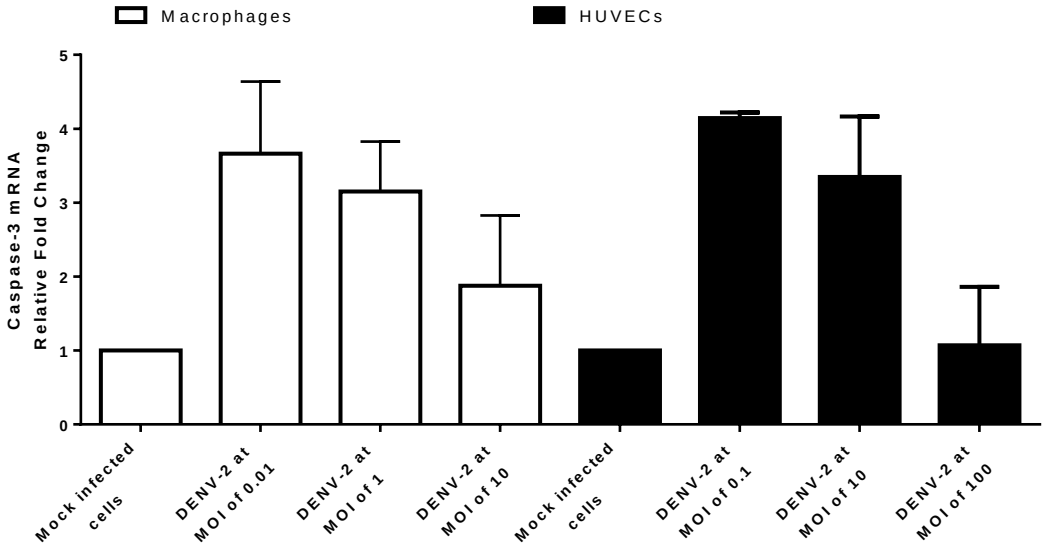
C


Macrophages
HUVECs
Fold increase in caspase-4 actvity
10
8
6
4
2
0
Mock infected cells
DENV-2 at MOI of 0.01
DENV-2 at MOI of 1
DENV-2 at MOI of 10
Mock infected cells
DENV-2 at MOI of 0.1
DENV-2 at MOI of 10
DENV-2 at MOI of 100

D


Macrophages
HUVECs
Caspase-1 mRNA
Relative Fold Change
40
30
20
10
0
Mock infected cells
DENV-2 at MOI of 0.01
DENV-2 at MOI of 1
DENV-2 at MOI of 10
Mock infected cells
DENV-2 at MOI of 0.1
DENV-2 at MOI of 10
DENV-2 at MOI of 100

E


Macrophages
HUVECs
Caspase-3 mRNA
Relative Fold Change
5
4
3
2
1
0
Mock infected cells
DENV-2 at MOI of 0.01
DENV-2 at MOI of 1
DENV-2 at MOI of 10
Mock infected cells
DENV-2 at MOI of 0.1
DENV-2 at MOI of 10
DENV-2 at MOI of 100

F

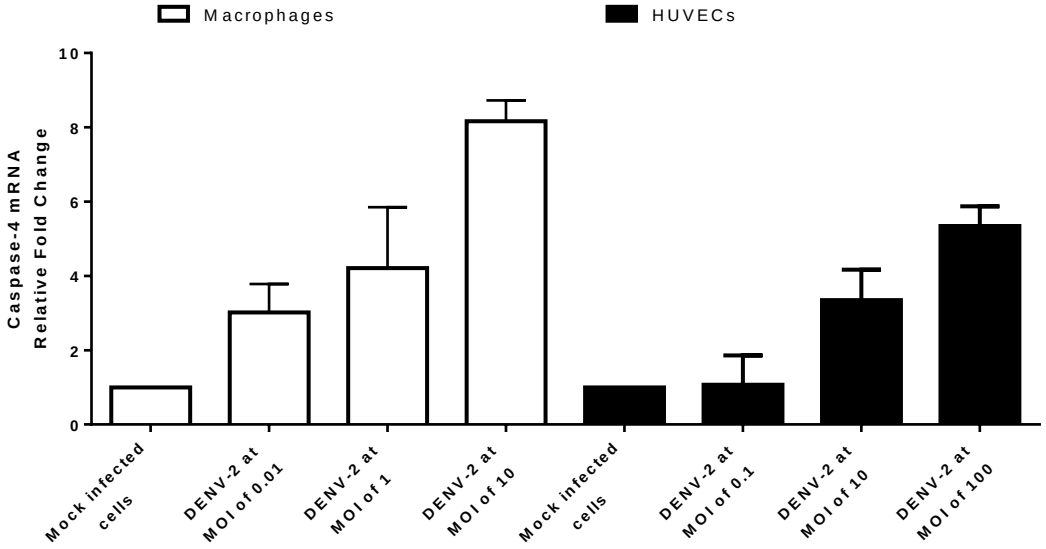

Macrophages
HUVECs
Caspase-4 mRNA
Relative Fold Change
10
8
6
4
2
0
Mock infected cells
DENV-2 at MOI of 0.01
DENV-2 at MOI of 1
DENV-2 at MOI of 10
Mock infected cells
DENV-2 at MOI of 0.1
DENV-2 at MOI of 10
DENV-2 at MOI of 100

Figure 3-31: Enzymatic activity and mRNA expression of capsase-1, -3 and -4 in DENV-2-infected primary macrophages at different MOIs

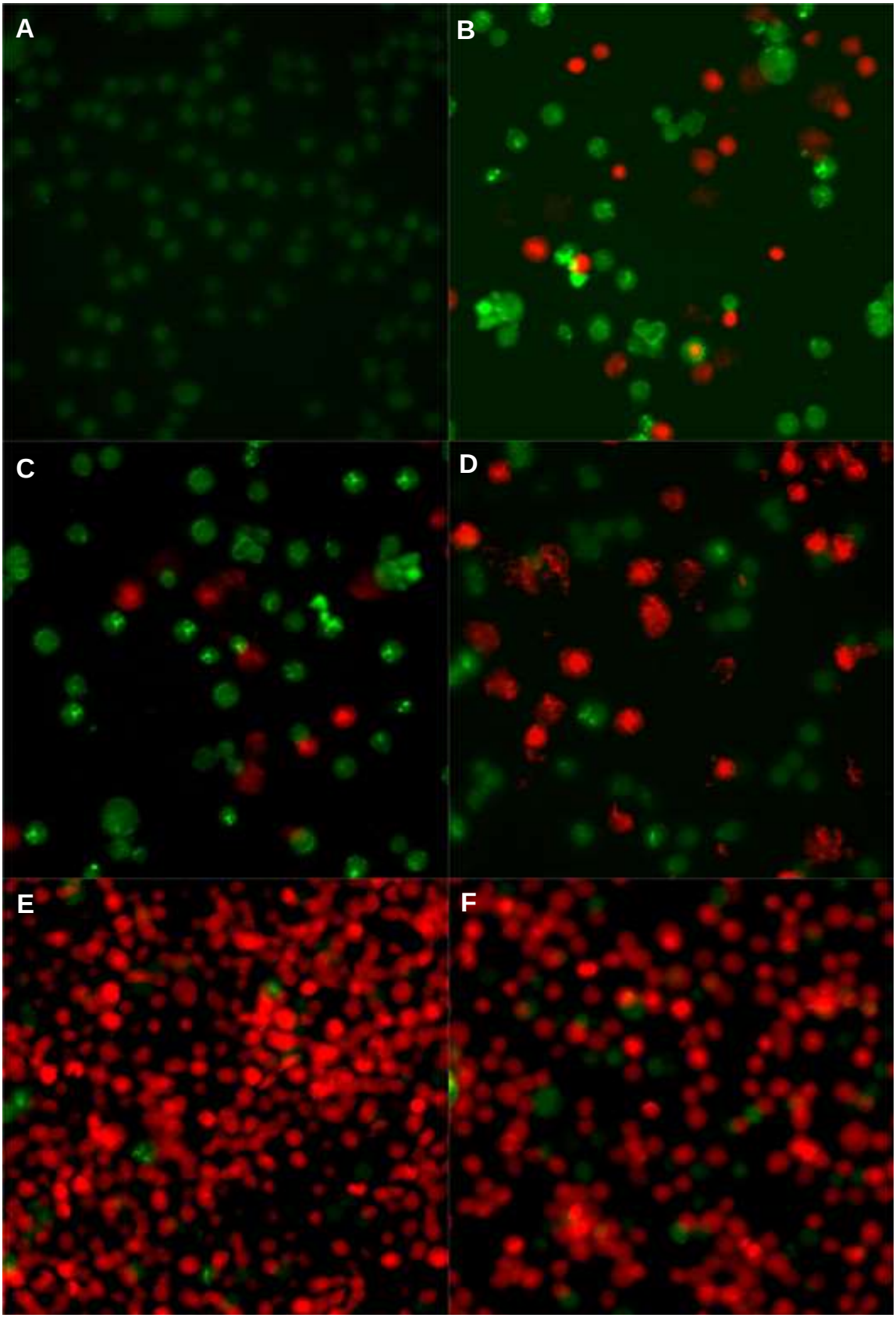
The enzymatic activity of caspase-1(A), -3 (B) and -4 (C) was measured by using the caspase-1/ice, caspase-3 and caspase-4 fluorometric assay kits (Biovision Inc.). Whole cell lysates were collected from mock-infected and DENV-2-infected cells and their AFC fluorescence were compared. Error bars indicate SEM (n=3).

Changes in mRNA levels for caspase-1 (D), -3 (E) and -4 (F) in primary macrophages post-infection at different infectious doses (MOI = 0.01, 1 and 10 in macrophages; MOI= 0.1, 10 and 100 in HUVECs) were measured by real-time RT-PCR. Data were normalised using GAPDH as a housekeeping gene. Errors bars indicate SEM. Statistical comparisons were made between different infectious doses; * $p < 0.05$, ** $p < 0.01$ (Kruskal–Wallis test)

3.5.4 Apoptosis observed in primary macrophages infected with DENV-2 at lower MOIs

To further confirm our results, morphological characteristics of cell death induced by DENV-2 at different MOIs were examined. The infected cells were stained by fluorescein-labelled annexin V and ethidium homodimer III. Apoptotic cells were stained green by the fluorescein-labelled annexin V (green fluorescence), which is impermanent in live cells and early apoptotic cells. Further, pyroptotic and necrotic cells were detected by staining with ethidium homodimer III (red fluorescence). The fluorescence positive cells were evaluated by a phase contrast and fluorescence (470 and 530 nm) microscope (Figure 3-32A–F). In the mock-infected cells, the percentages of annexin-V stained apoptotic cells and ethidium homodimer III-stained pyroptotic and necrotic cells were 2.4% and 1.2%, respectively (Figure 3-32G). Compared to the mock-infected cells, the apoptotic cells in DENV-2 infection at MOIs of 0.01 and 0.1 saw a significant increase ($p = 0.0003$) (Figure 3-32G). However, there were significantly fewer apoptotic cells in DENV-2 infection at MOIs of 10 or 100. These

results indicate that DENV-2 infection induced primary macrophage cell apoptosis at lower infectious doses, but that apoptosis was suppressed at higher infectious doses.



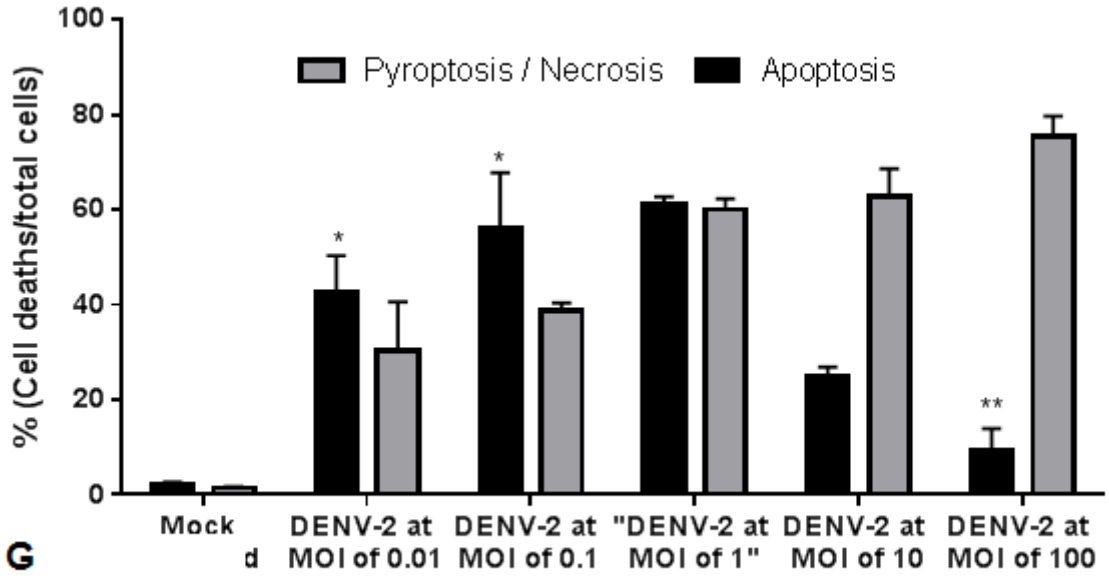


Figure 3-32: Effect of cell death in primary macrophages infected with DENV-2 at different MOIs

The number of apoptotic and pyroptotic/necrotic cells was detected by using an apoptotic/necrotic cell detection kit. The apoptotic cells were stained green by fluorescein-labelled annexin V (green fluorescence) and the pyroptotic/necrotic cells were stained red by ethidium homodimer III. The primary macrophages were mock-infected cells (A) or DENV-2 infected at MOIs of 0.01 (B), 0.1 (C), 1 (D), 10 (E) and 100 (F). The graph indicates the percentage of apoptotic and necrotic cells at the different MOIs. The number of apoptotic or pyroptotic/necrotic cells were counted and related to the total number of cells (G).

Chapter 4: Discussion

DF is a significant public health concern, especially in the tropics and subtropics. This disease is caused by DENV, which is a mosquito-borne virus of the Flaviviridae family. DENV has five serotypes: DENV-1, -2, -3, -4 and -5) (Guzman et al., 2010; M. S. Mustafa et al., 2015). DENV causes DF, which is a self-limiting illness; however, DF can also progress to more severe forms of the disease (DHF and DSS), especially during secondary infection with a different DENV serotype. DHF and DSS are severe febrile illnesses that can progress to hypovolemic shock with characteristic hemostatic abnormalities and increased capillary leakage (Neeraja et al., 2013). The outcome of DENV infection depends on viral and host factors. The host cells are thought to respond to viral infection by initiation of cell death or secretion of stimulating factors to the host, like cytokines. Apart from apoptosis, which is the most well-known cellular self-destruction in response to DENV, pyroptosis has recently been demonstrated in DENV-infected cells (Wu et al., 2013). Pyroptosis is a process of programmed cell death in response against intracellular pathogens, especially microbial infections. It is an important innate immune response to viral infection and has mainly been described in cells of the mononuclear phagocyte lineage. IL-1β are secreted in the process of pyroptosis. It has been reported that serum levels of IL-1β correlate with disease severity in dengue patients (Srikiatkhachorn & Green, 2010). Much effort has been invested in trying to elucidate the pathogenesis of DENV, including the role of pro-inflammatory cytokine production and direct cellular damage leading to plasma leakage during DENV infection (Chaturvedi et al., 2000; Espada-Murao & Morita, 2011).

An evidence of EC damage in DHF patients has been shown, but the mechanism by which this occurs remains unclear (Beatty et al., 2015; Cardier et al., 2006). Apoptosis of dengue-infected ECs has been reported *in-vitro* (Lin et al., 2002). However, the mechanisms involved in the cell death causing plasma leakage and production of these cytokines also remain unclear. In this study, macrophages and ECs were used as *in-vitro* cell models to demonstrate that pyroptosis could be an alternative process of programmed cell death in DENV infection. We hypothesised that both macrophages and ECs were able to undergo pyroptosis and be the sources of IL-1β production during DENV-2 infection. This could explain both the immune response to and the pathogenicity of DENV infection. Moreover, we suggested that pyroptotic cell death in ECs during DENV infection could be the mechanism of vascular leakage in DHF and DSS. Since DENV-2 has been suggested to result in more severe forms of dengue disease compared with other serotypes (Fried et al., 2010), it was used in this study to demonstrate the effect of DENV on both macrophages and ECs.

4.1 DENV infection induces pyroptosis and IL-1β production in macrophages

DENV can infect a number of cell types, including peripheral leukocytes, macrophages, dendritic cells, liver cells and ECs, both in patients and *in-vitro* (Chaturvedi et al., 1999; Ho et al., 2001; Huang et al., 2000; Y. L. Lin et al., 2000). Macrophages are a major target for DENV replication and are also the major source of the pro-inflammatory cytokines IL-1β and IL-18 (Table 1-2) (S. J. Wu et al., 2000). These cells generate inflammatory mediators and promote the dissemination of the virus during the initial phase of the disease (Y. C. Chen et al., 1999). Macrophages play a role

in homeostatic, immune and inflammatory responses. They are also considered the first line of host defence against invading microorganisms. Triggering pyroptosis in macrophages can destroy the site of replication of DENV and increase the production of IL-1β to trigger an immune response. Therefore, pyroptosis in primary macrophages could promote the clearance of DENV and be a key innate response of a host to eliminate DENV inside the body.

In this study, we firstly demonstrated that macrophages are permissive to DENV, which replicates inside the macrophage shortly after infection (within 24 hours). Our results show that the rapid decline in viral copies on day 2 post-infection was due to the increase in cell death. As in other studies, we also found that DENV-infected macrophages replicated inside the cells (Kyle et al., 2007; Wu et al., 2013) and initiated cellular responses.

Pyroptosis could promote the clearance of intracellular pathogens and the immunity of the host to those pathogens. While one study mentioned that pyroptosis occurred in DENV infection (Wu et al., 2013), the role of this type of cell death in the pathogenesis of DENV infection remained unclear. Many microorganisms, including DENV, are able to replicate in macrophages and increase their numbers in the host. During the process of pyroptosis, pores are formed on the cell membrane leading to cell lysis, destroying the site of replication. The intracellular pathogens are then released before the completion of replication, which can minimise the number of pathogens produced (Bergsbaken et al., 2009; S. L. Fink & Cookson, 2006; Rajan, Rodriguez, Miao, & Aderem, 2011). Pyroptosis can also initiate the innate immune response and contribute to the recruitment of immune cells after DENV infection by the release of the

pro-inflammatory cytokines IL-1β and IL-18 (van de Veerdonk, Netea, Dinarello, & Joosten, 2011).

A number of pro-inflammatory cytokines, including interleukin IL-1β, IL-8 and tumor necrosis factor-alpha (TNFα), are elevated in DF patients (see Table 1-2) (Tang et al., 2010). These cytokines have been reported to influence the severity of DENV infection (Bandyopadhyay et al., 2006; L. C. Chen et al., 2006; Priyadarshini et al., 2010; Tang et al., 2010). IL-1β belongs to the IL-1 cytokine family and is an important mediator of the inflammatory response. This cytokine is also involved in a number of cellular activities, including proliferation, differentiation and apoptosis (Ankarcrona et al., 1994; Chung et al., 2009; X. Wang et al., 2007). IL-1β is an endogenous pyrogen during the febrile phase of DF. This cytokine is also associated with different clinical manifestations of DF, such as coagulopathy and thrombocytopenia (Nguyen et al., 2004; Suharti et al., 2002). It has been reported that increased levels of IL-1β in serum from DF patients are correlated with disease severity (Srikiatkhachorn & Green, 2010).

Macrophages are believed to be the primary source of IL-1β and the target of DENV infection (Wati, Li, Burrell, & Carr, 2007); however, the molecular mechanism that underlies IL-1β production in these cells remains unclear. Several studies have reported that increased levels of IL-1β and IL-18 are observed in DENV-infected patients (Hober et al., 1998; A. S. Mustafa et al., 2001). Our results suggested that DENV-2 infection stimulated the production and release of the mature, biologically active IL-1β. The *in-vitro* increased level of IL-1β can provide supportive evidence of the occurrence of pyroptosis in DENV-infected patients. IL-1β mRNA expression was up-regulated during the course of DENV-2 infection in monocyte-derived macrophages.

These results are evidence of *de novo* synthesis from DENV-infected cells instead of a release of pre-formed cytokines stored inside cells. We also showed that IL-1β production increased during DENV-2 infection in a time-dependent manner. In summary, we demonstrated *in-vitro* increased levels of IL-1β in macrophages, supporting the occurrence of pyroptosis in DENV infection.

Interestingly, the production of IL-18 was not observed to markedly increase in the macrophages post-infection, although the mRNA expression of IL-18 was up-regulated during DENV-2 infection. Similar results were observed in other studies. For example, one study demonstrated increased production of IL-1β but no IL-18 secretion after the activation of caspase-1 by LPS (Puren, Fantuzzi, & Dinarello, 1999). The Sendai virus induces the production of IL-1β but not IL-18 after caspase-1 activation in macrophages (Pirhonen, Sareneva, Kurimoto, Julkunen, & Matikainen, 1999), whereas IL-1β and IL-18 can be produced after influenza virus (Pirhonen et al., 1999). Therefore, it is possible that the secretion of IL-1β and IL-18 via the caspase-1-dependent pathway may depend on the type of virus infection. Another possible explanation is that the production of IL-18 requires an additional proteolytic step that is controlled by other factors. Thus, the production of IL-1β and IL-18 is regulated in a two-step fashion (Dinarello, 2012). Further study is required to the low level of IL-18 production after caspase-1 activation by DENV-2.

Caspase-1 plays the central role in pyroptosis, and it is also considered a distinct feature of pyroptosis. Caspases are cysteinyl aspartate-specific proteinases that play essential roles in apoptosis and cytokine maturation. These proteins are classified as inflammatory caspases (group 1) or apoptotic caspases (group 2) (Lamkanfi, Declercq,

Kalai, Saelens, & Vandenabeele, 2002). Group 1 caspases include caspases-1, -4, -5 and -12. The genes encoding all known inflammatory caspases are located together on the human chromosome 11q22.2–q22.3 (Sollberger et al., 2012). It is reported that caspase-1 activity is important for host defence against microbial infection (McIntire et al., 2009). In this study, the expression of caspase-1 gradually increased over time post-infection. The observed trends in caspase-1 mRNA expression were similar to those observed for caspase-1 protein expression in macrophages during DENV-2 infection. Studies have shown that the suppression of caspase-1 activity could increase the fatal rate of bacteria infected animals (Winkler & Rosen-Wolff, 2015). In our study, we also showed that the activity of capsase-1 was co-related with the cell viability of the macrophages. In another study, caspase-1 suppression was associated with hepatic lipid accumulation and inflammation in the liver (Dixon, Berk, Thapaliya, Papouchado, & Feldstein, 2012). Therefore, caspase-1 activation is important in innate immunity and inflammation of the host.

Pyroptosis is a form of programmed cell death that is dependent on the activation of caspase-1 via inflammasomes (Y. Yang et al., 2015). The inflammasome is a protein complex that consists of the inactive form of caspase-1, the NLR family protein NALP3 and ASC. The release of active caspase-1 occurs after the inflammasome has been assembled and stimulated by inflammatory stimuli, such as PAMPs or DAMPs (M. S. Kim et al., 2007; Petrilli, Dostert, Muruve, & Tschopp, 2007). Activated caspase-1 subsequently mediates the activation of pro-inflammatory cytokines, such as IL-1β and IL-18 (Agostini et al., 2004; Davis et al., 2011). The function of NALP3 inflammasome in mediating responses to bacterial pathogens has been investigated by several groups (Hwang, Park, Hong, Kim, & Yu, 2012; Krishnan et al., 2013), but is as yet not completely understood.

Several studies recently demonstrated that the inflammasome can be activated by both DNA and RNA viruses (Burdette et al., 2012; Delaloye et al., 2009; Pontillo et al., 2012; Rajan et al., 2011). However, to date, little information is available concerning the mechanisms by which DENV stimulates the inflammasome and the events that occur downstream. Previous studies have shown that the pattern recognition receptor NALP3 inflammasome is the only receptor for DENV to induce pyroptosis (Tan & Chu, 2013; Wu et al., 2013). NALP3 inflammasome plays an important role in antiviral immunity (Masters et al., 2010). It is involved in the activation of caspase-1 and production of IL-1β with ASC that forms pyroptosome and which rapidly recruits and activates caspase-1 after being initiated by NALP3 inflammasome.

In this study, we demonstrated that the expression of ASC also peaked on day 2 post-infection and dropped slightly on day 3 post-infection. Similarly, expression of NALP3 mRNA was up-regulated as early as day 1 post-infection and peaked on day 2 post-infection. The gene expression of ASC and NALP3 inflammasome followed the same pattern as capsase-1 on the first 2 days post-infection only, while the mRNA expression of caspase-1 continued to increase on day 3. The results indicate that inflammasome may be involved in the activation of caspase-1 and IL-1β production during DENV-2 infection at the early stage of pyroptosis and that NALP3 inflammasome may be involved in the activation of caspase-1 during DENV-2 infection. Our data are consistent with previous studies (Wu et al., 2013). Taken together, increased formation of NALP3, ASC, IL-1β and caspase-1 post-infection could support that inflammatory activity is induced by DENV infection in macrophages.

It is possible that the reduced ASC mRNA expression on day 3 post-infection is not a factor of the activity of capsase-1 activation. If so, reduced ASC synthesis may not affect the activation of caspase-1. Our data suggest that the activation of caspase-1 may also be controlled by other factors, like caspase-4. In this study, we demonstrated that the production of IL-1β in DENV-2-infected macrophages involves the activation of caspase-1 and the up-regulation of NALP3. We also showed that the expression of ASC was up-regulated during DENV-2 infection, suggesting that the NALP3 inflammasome complex is an essential member of the pyroptotic cascade in the early phase of pyroptosis.

Pyroptosis is different from other forms of cell death because the process is characterised by features of both apoptosis and necrosis (Rayamajhi, Zhang, & Miao, 2013). During the process of pyroptosis, pores are formed on the cell membrane, leading to cell lysis and the release of cellular contents (S. L. Fink & Cookson, 2006). The activation of caspase-1 can induce the formation of pores of between 1.1 and 2.4 nm in diameter on the cell membrane in pyroptotic cells (S. L. Fink & Cookson, 2006). To show that DENV induces pyroptotic cell death in monocyte-derived macrophages, the viability of infected macrophages was investigated, with the results revealing a significant reduction in viability with time post-infection. Evidence of the occurrence of pyroptosis was further demonstrated by LDH assay in culture supernatants. During cell death, the cell loses membrane integrity and lysis, resulting in the release of LDH, which is normally maintained within the cell cytosol. By measuring the LDH in the culture supernatant, the extent of release of LDH due to cell lysis and damage of the cell membrane can be determined. The assay measured the NADH level, which is directly correlated with the LDH activity of damaged cells (G. Wang et al., 2012). In this study, the LDH activity in the supernatants from DENV-2-infected macrophages was

significantly higher than that observed in supernatants from uninfected cells. Combined with the data on the increased expression of caspase-1 and IL-1β production, these findings suggest that monocyte-derived macrophages underwent pyroptosis with cell lysis during DENV-2 infection. The increased level of LDH in serum was found in DHF and DSS patients. The level of LDH was reported as a predictor of severe DENV infection or DHF and DSS with plasma leakage (Sirikutt & Kalayanarooj, 2014). Therefore, the *in-vitro* increase of LDH in our study can provide a possible explanation of the source of LDH release in DENV patients. In turn, pyroptosis in DENV-infected cells can support the mechanism of pathogeneses in DENV infection.

In summary, our study has demonstrated that the increased expression of caspase-1 was found in DENV-2-infected macrophages and resulted in pyroptosis and the production of the pro-inflammatory cytokine IL-1β. Our results showed an increase in expression of NALP3, ASC and caspase-1. It is suggested that DENV triggers the activation of caspase-1 through the sensor NALP3 and induces the production of the pro-inflammatory cytokine IL-1β in macrophages. Elevated LDH activity provides evidence of cell lysis and demonstrates that cell death occurs after DENV infection. Therefore, we can conclude that the activation of pyroptosis in macrophages could be triggered by DENV infection *in-vitro*.

4.2 DENV infection induces pyroptosis and IL-1β production in ECs

Besides macrophages, ECs also become infected by DENV, and the virus replicates insides the cells (Ho et al., 2001; Huang et al., 2000; King et al., 2000). The endothelium is the primary fluid barrier of the vasculature. The ability of DENV to infect ECs provides a direct means for DENV to alter capillary permeability, permit virus replication and induce responses that recruit immune cells to the ECs. One of the major explanations of the vascular leakage that occurs in DHF or DSS is EC death induced by DENV. It has been reported that DENV directly induces apoptosis in ECs (Avirutnan et al., 1998).

By infecting a cell, DENV could also indirectly induce the production of mediators that induce EC cell death, like TNF-α, which is a key modulator of EC apoptosis (H. C. Chen et al., 2007). In addition, DENV-induced responses resulting in edema or hemorrhagic disease ultimately cause changes in EC permeability. Studies have shown that antibodies from DENV patients cross-reacted with ECs and induced damage (C. F. Lin et al., 2003). In addition, the release of inflammatory cytokines by DENV-infected ECs has been reported and is also thought to trigger vascular leakage *in-vitro* (Beatty et al., 2015). Infected HUVECs and HMEC-1 monolayers generate the inflammatory cytokines IL-6 and IL-8, which are also capable of inducing vascular permeability (Huang et al., 2000; Talavera et al., 2004).

The vascular leakage associated with DHF and DSS may not result from one single mechanism but from multiple interactions between DENV and host cells. The

apoptosis of DENV-infected ECs leads to vascular leakage syndrome, as observed in patients with DHF and DSS (Avirutnan et al., 1998; H. C. Chen et al., 2007; Liew & Chow, 2004). As mentioned, DENV could induce other forms of cell death, like pyroptosis. Hence, we hypothesised that pyroptosis in DENV-infected ECs could be an alternative explanation of the vascular leakage syndrome observed in DENV-infected patients.

In our study, we focused on the direct effect of DENV on the death of ECs in vascular leakage. Recent studies focusing on the DENV infection of primary ECs have demonstrated that ECs are efficiently infected, rapidly produce viral progeny and elicit immune enhancing cytokine responses that may contribute to pathogenesis (Brown et al., 2011; Dalrymple & Mackow, 2011; Huang et al., 2000). Due to the lack of an adequate animal model of DHF and DSS and the difficulty in performing vascular leakage studies in humans, researchers have relied on the use of cultured human ECs to elucidate the molecular mechanisms of DENV infection. More problematically, many conclusions have been derived from studies using the ECV304 cell line (Avirutnan et al., 1998; Bosch et al., 2002; Liew & Chow, 2006), which was found not to derive from the endothelium (Kiessling et al., 1999).

In the first of our experiments, we confirmed the *in-vitro* permissivity of ECs to DENV-2 infection. The cell model of human ECs in this study was HUVECS, which is important as a model system for the study of the regulation of the function of ECs and the role of ECs in the response of stimuli (Park et al., 2006). DENV-E antigens were detected in HUVECs at 3 days post-infection with DENV-2 at an MOI of 10. Further, almost all cells were infected at 3 days post-infection. DENV-E proteins were also

detected in EC-infected lysates. Our results suggest that DENV-2 infection of human ECs actively and rapidly produces DENV in cell supernatants which, in a highly permissive cell line like C6/36, is similar to viral replication. In summary, our findings demonstrate that, when the infectious dose is high, DENV-2 efficiently infects ECs, where it rapidly replicates, and releasing infectious virions.

Interestingly, we could not detect the DENV-E antigen on HUVECs at lower MOIs (MOI=0.1 and 1) (data not shown). Using high MOI in HUVECs is due to lower permissiveness compared with macrophages. Therefore, the higher viral dosages were used in HUVECs of the study to balance the effect. The reason for this could be that the receptor expression on ECs is different from other cell types (e.g., monocytes and macrophages). Previous studies have demonstrated that ECs express heparin sulfate, mannose receptor, HSP70 and HSP90, which are the important receptors for DENV entry (Wei, Jiang, Fang, & Guo, 2003). The level of receptor expression on target cells may influence the permissivity of the cells. The expression levels of those receptors across different cells types should be investigated.

Although the role of DENV infection of ECs in pathogenesis remains unclear, the presence of DENV-infected ECs in patients suggests their likely role in DHF and DSS via several potential mechanisms (Basu & Chaturvedi, 2008; Noisakran & Perng, 2008; Oishi et al., 2007). Apoptosis of DENV-infected ECs has been shown to be a mechanism that results in the loss of vascular endothelial barrier integrity, leading to vascular leakage syndrome. However, various features of apoptotic cell death, including DNA fragmentation and plasma membrane permeabilization, are also characteristic of other programmed cell death pathways, such as pyroptosis. It has been shown that under

dyslipidemia and inflammatory environments, caspase-1 in ECs becomes activated and induces pyroptosis (Lopez-Pastrana et al., 2015). The ECs that line the inner surface of the vessel wall are the first cells exposed to different danger signals in the circulatory system, including viruses, cytokines secreted by T cells and metabolite-related endogenous danger signals (Libby, Ridker, & Hansson, 2011; Mestas & Ley, 2008). However, the underlying mechanism of caspase-1 activation in DENV infection and the pathogenesis of EC damage in DENV remains poorly defined. In the present study, we have shown that EC caspase-1 activation occurs in DENV-2 infection. Moreover, we found that caspase-1 is responsible for IL-1β secretion and pyroptosis in DENV-2-infected ECs.

As mentioned, serum levels of IL-1β correlate with disease severity in dengue patients (Srikiatkhachorn & Green, 2010). Although macrophages and monocytes are the major sources of pro-inflammatory cytokines, IL-1β can also come from other sources (S. J. Wu et al., 2000). In the present study, we show that ECs are able to produce IL-1β in response to DENV-2 infection. Our ressult shows that DENV-2 infection at an MOI of 10 was able to trigger IL-1β secretion. The secretion observed was significant on day 1 post-infection. Based on this result, we conclude that ECs can secret IL-1β after DENV-2 infection although our ELISA results are not consistent with previous studies that report that IL-1β secretion cannot be produced from ECs in DENV infection (Huang et al., 2000). In addition, the remarkable rise in IL-1β by 24 hours post-infection was implusively given that the western blot data showed a predominantly later caspase-1 activation after DENV-2 infection. The phenomenon could be proved by infection with a different MOIs, as the same serotype and cell model were used in both studies. This suggests that the viremia of patients may result in different outcomes.

To explain the early secretion of IL-1β on day 1 post-infection, we determined the IL-1β mRNA levels in DENV-2-infected ECs. The expression of IL-1β mRNA increased dramatically as early as day 1 in the DENV-2-infected cells and continued to increase on day 2, compared with uninfected controls. These results correlated with those for IL-1β production detected by ELISA. Taken together, this suggests that the early production of IL-1β with delayed caspase-1 activation in ECs after DENV-2 infection could be controlled by other factors or even other caspases. This phenomenon requires further investigation.

This study sought to show that pyroptotic cell death occurs in ECs after DENV-2 infection. Further, it investigated the occurrence of pyroptosis in DENV-2-infected ECs and monitored the release of LDH from ECs after infection with DENV-2. As mentioned above, LDH was released from damaged cells after pyroptosis through pores formed on the membrane. The activity of LDH increased with time post-infection. On day 3 post-infection, the LDH level in the supernatants from DENV-2-infected HUVECs was significantly different from that observed in the supernatants from uninfected cells. Moreover, the viability of the DENV-2-infected HUVECs was significantly lower than that of the uninfected cells. To summarise, our data offers the first evidence that DENV-2 infection can induce pyroptosis in human ECs.

A recent study showed that the activity of caspase-1 correlated with angiogenic impairment and prognosis of ischemia (Lopez-Pastrana et al., 2015). Inhibition of caspase-1 in ECs can improve the survival of these cells as mediated by vascular endothelial growth factor 2 (VEGF-2). For the role of caspase-1 in pyroptosis in ECs,

we found that caspase-1 mRNA increased gradually over time, indicating up-regulation of caspase-1 transcripts post-infection. We next discovered that pro-caspase-1 levels remained relatively constant for day 1 but decline on day 2. This shows correlation with an increase in activated caspase-1 p20 subunits, as proved by a observable band on day 2 post-infection. The approximately low level of activated caspase-1 p20 found in the first 48-hours post-infection proved that the induction of caspase-1 activity occurred predominantly as a late response in DENV-2-infected ECs.

To further confirm that infection of ECs with DENV-2 induced pyroptosis, we incorporated a caspase-1 inhibitor Z-YVAD-FMK treatment into the LDH release analysis. This allowed us to specifically detect pyroptotic cell death in the presence of DENV-2 via two hallmarks of pyroptosis; namely, caspase-1 dependency and LDH release, and the supernatants were evaluated to check for the presence of LDH. From our results, we observed that caspase-1 activity inhibition sharply reduced the release of LDH from ECs (by more than 50%) after DENV-2 infection on days 1, 2 and 3 post-infection. Conversely, the inhibition of caspase-1 can enhance the viability of ECs as pyroptosis is the only form of cell death that is suggested to be dependent on caspase-1 activity as well as the release of LDH by cell lysis. The activity of caspase-1 in ECs is thus highly important for their survival.

Severe dengue disease is characterised by the onset of vascular leakage syndrome. The onset of vascular permeability occurs well beyond 72 hours post-infection and has been attributed to different mechanisms. One of the major suggested causes is EC damage, with one study showing evidence of EC damage in DHF patients, although the mechanism behind this was not clear (Cardier et al., 2006). Apoptosis in

DENV-infected ECs has been reported *in-vitro* (Lin et al., 2002), and has been shown as the mechanism causing loss of vascular endothelial barrier integrity and vascular leakage syndrome (Avirutnan et al., 1998; H. C. Chen et al., 2007; Liew & Chow, 2004). Another study suggested that ECs can become activated and induce pyroptosis (Lopez-Pastrana et al., 2015). Combining these findings with those from our study, the DENV-2 infection can induce pyroptosis in human ECs by the activation caspase-1. However, the underlying mechanism between caspase-1-dependent pyroptotic cell death in ECs after DENV infection and the pathogenesis of plasma leakage in DENV infection remains poorly defined.

To examine the effects of caspase-1-dependent pyroptosis on endothelial permeability, we treated HUVECs with DENV-2 at 24-hour intervals before measuring permeability in a transwell assay. Further, to show evidence of a caspase-1-dependent process, we incorporated the caspase-1 inhibitor Z-YVAD-FMK into the treatment. From the results, DENV-2 infection caused an increase in permeability of those HUVECs blocked by the caspase-1 inhibitor. These results indicated that pyroptosis in ECs induced by DENV-2 infection increased membrane permeability. This is the first time that pyroptosis in ECs has been shown to contribute to the mechanism of vascular leakage during DENV infection. Our results prove that pyroptotic changes in ECs may be the important consequences for the endothelium and are reasoning of pathogenic changes associated with vascular leakage, as seen in DENV disease. DENV-2 infection can ameliorate hyperpermeability through caspase-1-dependent pyroptosis.

Our study observed caspase-1-dependent pyroptosis in both macrophages and ECs. In both cell models, pro-inflammatory cytokine IL-1β was produced during DENV infection. From the results, we observed a time delay in the activation of caspase-1 and

IL-1β production in the ECs, even though the infectious dose was higher than in the macrophages. The level of mRNA expression peaked at day 2 post-infection in the macrophages but was not seen in the ECs. The same pattern was noticed for IL-1β production. Interestingly, the patterns of cell viability and LDH release for the two cell models were similar. This suggests that pyroptotic cell death in the two models may not be significantly different. Time of expression and activation of caspase-1 and IL-1β production may be affected by DENV doses, although further study is required.

In summary, our study demonstrated that caspase-1 was activated by DENV infection in ECs. This resulted in pyroptosis and the production of the pro-inflammatory cytokine IL-1β. This is the first support that pyroptosis in human ECs can be induced by DENV-2. Further, pyroptosis in ECs induced by DENV-2 infection was shown to increase membrane permeability. This is the first time that pyroptosis in ECs has been linked with the mechanism of vascular leakage during DENV infection. However, more experiments are required to elucidate the underlying molecular mechanism, including how DENV induces pyroptotic cell death in ECs and how this pyroptotic change in ECs alters membrane permeability. An *in-vivo* study in an animal model would provide beneficial evidence. The mechanism of pyroptosis in ECs is important to understanding the pathophysiology of the disease.

4.3 The involvement of Caspase-4 in the production of IL1β and pyroptosis during dengue virus infection

Pyroptosis and mature IL-1β production are both caspase-1-dependent processes. However, one study has suggested that inflammasome can also be activated by other caspases, such as caspase-4 (Sollberger et al., 2012). Unfortunately, human caspase-4 and -5 are poorly characterised. While human capase-4 and -5 are highly homologous to caspase-11 in mice, their functions are not well known. Even less is known about the role of caspase-4 and -5 in infections. Caspase-4 is believed to be an inflammatory caspase because its encoding gene is located in the same chromosomal locus as the caspase-1 gene (Sollberger et al., 2012). Caspase-4 has been reported to respond to several bacterial infections, including *Shigella flexneri, S. typhimurium* and enteropathogenic *E. coli*, in human intestinal epithelial cells (Knodler et al., 2014; Kobayashi et al., 2013). However, few data are available concerning the relationship between these two caspases in the induction of pyroptosis and IL-1β production during DENV infection. Here, we demonstrated that the production of IL-1β during DENV-2 infection is mediated by caspase-1 and regulated by caspase-4, with caspase-1 being activated by caspase-4. Studies have previously demonstrated that caspase-4 regulates caspase-1 during the activation of IL-1β (Sollberger et al., 2012); however, the function of caspase-4 is not fully understood in DENV infection.

In this study, we reported that caspase-1 was highly expressed during DENV-2 infection and was required for the production of IL-1β. Further, caspase-4 was highly expressed in both macrophages and HUVECs. Hence, we hypothesised that caspase-4 was involved in the activation of caspase-1 in macrophages and HUVECs during DENV

infection. To test this hypothesis, we used the caspase-4 inhibitor Z-LEVD-FMK and caspase-4 siRNA to inhibit the activity of caspase-4. Caspase-1 activity was suppressed after DENV-2 infection with either the caspase-4 inhibitor or caspase-4 siRNA. The secretion of IL-1β was also attenuated in DENV-2 infection by the suppression of caspase-4 activity. The results suggest that the activation of caspase-4 is required for the activation of caspase-1 in macrophages and ECs and the production of mature IL-1β during DENV-2 infection. In contrast, caspase-4 activity was not affected by caspase-1-specific inhibition. This finding suggests that in macrophages during DENV infection caspase-4 is upstream of caspase-1 in the IL-1β activation and pyroptosis pathway.

In this study, the siRNA knockdown of caspase-4 and capase-4 inhibition strongly attenuated DENV-2-induced cell death and LDH release in macrophages and HUVECs. This suggests that pyroptosis in DENV-2-infected macrophages is controlled by the activity of capsase-4. We demonstrate that caspase-4 is involved in the process of IL-1β production during DENV-2 infection. We also suggest that caspase-4 is upstream in the signalling pathway that controls caspase-1 activation during DENV infection. These findings point to the role of caspase-4 in the pathogenesis of DF.

In summary, our study showed that caspase-1 and -4 were induced by dengue viral RNA infection with the mediation of NALP3 inflammasome. This resulted in pyroptosis and the production of the pro-inflammatory cytokine IL-1β in monocyte-derived macrophages and also ECs. This demonstrated that caspase-1-dependent pyroptosis is an efficient effector mechanism to restrict dengue viral growth and dissemination; it also enhances innate immune responses. Further, we showed that caspase-4 is upstream in the signalling pathway controlling caspase-1 activation during

DENV infection. The mechanism of pyroptosis is important to understanding the immune response to DENV infection and the pathogenesis of the disease.

4.4 The effect of viral dose on controlling cell death from DENV

Several studies have found viremia titers in patient sera to correlate with DENV disease severity and the presented symptoms (Vaughn et al., 1997; Vaughn et al., 2000). Macrophages play a key role in homeostatic, inflammatory and immunological processes and are also considered the major target cells in DENV infection, as they create room for intracellular viral replication (Halstead, 1989; Honda et al., 2009). ECs are also a target for DENV, and DENV-infected ECs are a major component of immunopathogenesis in DENV infection (Brown et al., 2011; Lin et al., 2002; Peyrefitte et al., 2006). As mentioned, the initial infectious dose of DENV may change the outcome or prognosis of the disease due to the differential effect on the release of DENV in patients. For this reason, further study on the DENV dose effect in ECs could offer valuable insights into vascular leakage in DHF and DSS patients. Although caspase-1-dependent pyroptosis is an efficient effector mechanism in restricting dengue viral growth and dissemination, in addition to enhancing innate immune responses, it is also the fastest way of releasing DENV during infection, by cell lysis. The different form of cell death in DENV-infected cells could affect the release of DENV, which in turn would affect the viremia titers of serum in patients and the levels of pro-inflammatory cytokines.

Our study found that caspase-1 and -4 could be induced by DENV-2, resulting in pyroptosis and the production of the pro-inflammatory cytokine IL-1β. However, the virus can use virulence factors to decrease either caspase-1 or -4 activation by performing another form of cell death, such as apoptosis or autophagy. This means that competition exists between the host and the virus to regulate the activation of these two caspases, with the outcome dictating the fate 'alive or death' of the host. It remains to be investigated whether the inflammasome complex can resolve the fate of cells that have active caspase-1 and -4, and how the cells determine their fate after they are infected with DENV. It also remains unknown whether the process of pyroptosis undergone by DENV-targeted macrophages and ECs the following infection by DENV is controlled by time of infection, the rate of infection, initial infectious dose or the cell itself.

In the present study, we have demonstrated the effect of DENV infectious dose on controlling cell death. The unique feature of pyroptosis is the release of pro-inflammatory cytokines, like IL-1β, and other intracellular matrix elements after cell lysis. These can trigger an inflammatory reaction that can result in extreme tissue damage. In contrast, apoptosis is a form of silent cell death displaying hallmarks such as cytoplasmic shrinkage, chromatin condensation and cell fragmentation into an apoptotic body that phagocytized by leukocyte without provoking any inflammatory response. The fates of DENV-infected cells undergoing either pyroptosis or apoptosis at different initial infectious doses was of interest to this study.

To investigate the effect of a DENV infectious dose on cell death in monocyte-derived macrophages and also ECs, the cells were infected with DENV-2 at different MOIs (i.e., 0.1, 1, 10 and 100). The results showed that initial infectious dose was

directly proportional to the percentage of cell death that occurred post-infection. In the mock-infected controls, the number of viable cells contiuously were constant throughout the experiment. Interestingly, the onset of cell death was delayed when monocyte-derived macrophages were infected with DENV-2 at lower MOIs (e.g., MOI=0.1). One possible explanation for this could be that DENV-2 induced another form of cell death (e.g., autophagy or apoptosis) that was not detected by the viability assay. It is known that DENV can activate autophagy and apoptosis (Ghosh Roy, Sadigh, Datan, Lockshin, & Zakeri, 2014), and autophagy can temporarily spare infected cells and allow a longer period for the reproduction of the virus, which further protects the cell against other stresses, such as reactive oxygen species. However, this requires further investigation. For HUVECs, the cell viability was proportional to the DENV-2 infectious dose. It is suggested that higher infectious doses can enhance cell death in ECs during DENV-2 infection.

Infections exhibiting pyroptosis are often accompanied by an inflammatory response. During DENV infection in humans, inflammation has been observed in several locations of the body (Priyadarshini et al., 2010). To confirm that infection of macrophages and HUVECs with DENV-2 at high infectious doses (MOI>10) induced pyroptosis, LDH activity was assessed. A previous study reported that DENV infection in Vero cells at a high infectious dose (MOI>10) resulted in the release of LDH in these cells (Despres, Frenkiel, Ceccaldi, Duarte Dos Santos, & Deubel, 1998). The LDH release was attributed to the cytotoxic effect resulting from the accumulation of DENV particles at high infectious doses. From our results, the release of LDH from primary macrophages infected with DENV-2 was significantly different on day 2 post-infection for different MOIs. This was consistent with the data on cell viability. LDH release in

HUVECs also increased with infectious dose, following a trend similar to that for primary macrophages.

To further confirm that infection of macrophages and HUVECs with DENV-2 at high infectious doses (MOI >10) induces pyroptosis, caspase-1 catalytic activity was assessed in cell lysates at different infectious doses of DENV. From the results, caspase-1 activity increased at higher MOIs (MOI=10 or 100). Moreover, activity levels at lower, as compared to higher, infectious doses were still lower by 3 days post-infection. Together with the data on LDH activity, these findings suggest that monocyte-derived macrophages underwent pyroptosis during DENV-2 infection at higher infectious doses.

DENV may induce apoptosis, allowing the dissemination of its viral progenies to neighbour cells through phagocytized apoptotic bodies. In addition, it may act to inhibit cell death to ensure virus survival and replication. Apoptosis is norammly induced by an extrinsic single transduction triggered by death receptors, such as TNF receptors although the apoptotic process can also be triggered by endogenous events, especially when the cell has already been infected and the virus has replicated inside the cell. It is well known that cytochrome c is the key signalling molecule of apoptosis. Cytochrome c is a small protein that is found loosely to the outer surface of the inner mitochondrial membrane. Its precursor, apocytochrome c, is produced on a free ribosome in the cytoplasm and spontaneously inserts into the mitochondrial outer membrane. The released cytochrome from the mitochondria can bind to adaptor proteins procaspase-9 and Apaf-1 to form a cytosolic apoptosome complex, inducing a number of biochemical reactions and subsequent caspase cleavage and activation, eventually resulting in apoptotic cell death. The release of cytochrome c from the mitochondria is

conserved in mammalian cells undergoing apoptosis (S. L. Fink & Cookson, 2005; Jiang & Wang, 2004). The cytochrome c released from the mitochondria into the cytosol of DENV-2-infected macrophages and HUVECs at different MOIs was determined by ELISA in this study. The results showed a much higher release of cytochrome c in the cytosol lysates from DENV-2-infected macrophages and HUVECs at lower MOIs (0.01 or 0.1) compared to macrophages and HUVECs infected with higher MOIs (10 or 100). This suggests that macrophages favoured apoptotic cell death during DENV-2 infection at low MOIs.

The caspases are a group of genes that are important for maintaining homeostasis through regulating cell death and inflammation. They are endoproteases that hydrolyze peptide bonds that depends on catalytic cysteine residues in the caspase active site and occurs only in the presence of specific residues in the substrate. Generally, caspases are classified by their known roles in apoptosis and inflammation. Caspase activation plays an important role in the mediation of both apoptosis and pyroptosis in mammalian cells. Caspase-8 is the upstream caspase while caspase-3 is the downstream effector of apoptosis, involved in the execution of apoptosis and induction of the cells to undergo apoptotic cell death. Conversely, caspase-1 is classified as the initiator of pyroptosis. In this study, we demonstrated that caspase-4 is involved in the activation of caspase-1 and pyroptosis in primary macrophages after DENV-2 infection. Our study revealed the role of the different caspases on different observed processes of cell death. The cascade of caspase activation was investigated in DENV-2-infected macrophages at MOIs of 0.01, 1 and 10 and in HUVECs at MOIs of 0.1, 1, 10 and 100. According to our results, capsase-3 activity was suppressed when macrophages were infected at higher MOIs (i.e., 10 in macrophages and 100 in HUVECs). In contrast, caspase-1 and -4 activity increased in macrophages and HUVECs after DENV-2

infection at higher MOIs (i.e., 10 and 100). The mRNA expression on those caspases was consistent with the enzyme activity in the cell lysates. The activation of caspase-3 in macrophages infected with DENV-2 at lower MOIs further supports that macrophages and HUVECs favoured apoptotic cell death during DENV-2 infection at low MOIs. Interestingly, the observed trend in caspase-4 activity was similar to that observed for caspase-1 in macrophages and HUVECs at different MOIs. This finding can further support our previous results that caspase-4 activity is closely associated with the activation of caspase-1 and pyroptosis in macrophages after DENV-2 infection.

To further support our findings, the morphological characteristics of cell death induced by DENV-2 at different MOIs were examined. As compared with the mock-infected controls, a significant increase was observed in apoptotic cells in DENV-2 infection at MOIs of 0.01 and 0.1. Conversely, significantly fewer apoptotic cells were observed in DENV-2 infection at MOIs of 10 or 100. These results indicate that DENV-2 infection induced macrophage cell apoptosis at lower infectious doses, but suppressed it at higher infectious doses.

In summary, we have demonstrated that the mitochondrial-mediated apoptotic pathway was initiated in macrophages and HUVECs infected with DENV at lower infectious doses (MOI<1). This contrasts with the findings for pyroptosis observed in macrophages infected with DENV at higher infectious doses (MOI>10). These results suggest that initial infectious dose is a factor controlling the fate of DENV-infected cells in undergoing either pyroptosis or apoptosis, and may change the outcome or prognosis of the disease.

Chapter 5: Conclusion

Pyroptosis has recently been identified as a pathway of cell death that is response to a range of microbial infections (e.g., *Legionella* and *Salmonella*, *Francisella*) and non-infectious stimuli, such as host factors, produced during myocardial infarction (Bergsbaken et al., 2009). Given that pyroptosis can promote pathogen clearance by introducing an alarm signal that stimulate immune cells to the site of infection, a more aggressive approach than silencing cell death is required (e.g., apoptosis and autophagy). In addition, the production of pro-inflammatory cytokines during pyroptosis could induce a host's antiviral immune response, although the mechanism by which this occurs in DENV infection remains unclear, especially for ECs. Pyroptosis is also not well described for other flaviviruses such as West Nile virus (Ghosh Roy et al., 2014). In the present study, the genes expression of members of the pyroptotic pathway were studied, with the results showing an increase in expression of NALP3, ASC and caspase-1 during DENV-2 infection. It was suggested that DENV triggers the activation of caspase-1 through the sensor NALP3 and induces the production of the pro-inflammatory cytokines IL-1β and IL-18. Finally, elevated LDH activity provided evidence of cell lysis, demonstrating that cell death occurred after DENV infection. This allows for the conclusion that the activation of pyroptosis in macrophages and ECs can be triggered by DENV infection *in-vitro*. However, the molecular mechanism responsible for the recognition of the dengue infection by NALP3 remains unclear, especially in ECs. The triggering molecules could be viral RNA or virion components, and this should be further studied. The present study can be regarded as a pilot study in investigating pyroptosis as triggered by DENV via NALP3 inflammasome.

In addition, this study showed that, at higher MOIs, apoptoses is not a major type of cell death induced by DENV infection in macrophages and ECs, but that it is at lower MOIs. We studied the DENV dose effect in macrophages, as macrophages are the major target of DENV. The fate of DENV-infected macrophages could affect the level of DENV and pro-inflammatory cytokines in patients, in turn affecting severity. For a future study, investigating the DENV dose effect in the EC model could offer valuable insights into vascular leakage in DHF and DSS patients. A further finding of this study was that the production of IL-1β and activation of pyroptosis during DENV-2 infection were regulated not only by caspase-1 but also by caspase-4, and that caspase-4 was upstream of caspase-1 in the activation pathway. Caspase-4 thus appears to be a key mediator of inflammation and pyroptosis in macrophages during DENV-2 infection. Additional studies are required to further elucidate the molecular mechanisms involved.

Plasma leakage is the hallmark of DHF, which is associated with a sudden increase in systemic vascular permeability (Avirutnan et al., 2006). In patients with severe dengue infection, increased plasma leakage and complications of DHF such as encephalopathy may be associated with a higher mortality rate (Shresta et al. 2006). In the present study, pyroptosis and IL-1β production in ECs were observed after DENV-2 infection. Our results indicated that pyroptosis in ECs induced by DENV-2 infection increased membrane permeability. This is the first evidence that pyroptosis in ECs contributes to the mechanism of vascular leakage during dengue virus infection.

To date, there is a limited number of licensed dengue vaccine is available on the market (Guy, Briand, Lang, Saville, & Jackson, 2015), and there is also no specific therapy to treat dengue disease. Treatment of severe dengue infection mainly depends

on supportive fluid replacement therapy (Alam et al., 2004). From the present study, it is understood that DENV infection may trigger the activation of caspase-1 and the production of IL-1β. The secreted IL-1β could induce an immune response, inflammatory response and vascular leakage (Puhlmann et al., 2005). As mentioned previously, the virus can limit caspase-1 activation by performing other forms of cell death, such as apoptosis and autophagy (S. L. Fink & Cookson, 2005; Limonta et al., 2007). In the present study, we demonstrated that initial infectious dose is one factor controlling the fate of DENV-infected cells in either undergoing pyroptosis or apoptosis. The infectious doses of DENV can possibly change the outcome or prognosis of the disease. Thus, the results of the present study provide an in-depth understanding of pyroptosis as an alternative process of programmed cell death, and of caspase-1 and -4 activations during DENV infection. Our results shed light on the pathogenesis of DENV infection and may also provide new knowledge for the future development of therapeutic targets that may help to develop better management of severe dengue disease.

Chapter 6: Future works

6.1 Relationship between pro-caspase-1, ASC and NALP3 in inflammasome complex during DENV-2 infection

Several studies recently demonstrated that inflammasome can be activated by both DNA and RNA viruses (Burdette et al., 2012; Delaloye et al., 2009; Pontillo et al., 2012; Rajan et al., 2011). Inflammasome is involved in the activation of pro-caspase-1 and the production of IL-1β with ASC. Previous studies have shown that the pattern recognition receptor, NALP3 inflammasome, is one of the receptors for DENV to induce pyroptosis (Tan & Chu, 2013; Wu et al., 2013). However, little information is available concerning the mechanisms by which DENV stimulates inflammasome and the events that occur downstream. In this study, we demonstrated that the production of IL-1β in DENV-2-infected macrophages involves the activation of caspase-1 and the up-regulation of NALP3. However, the relationship between these three proteins is still not completely clear. A gene knockdown study on each component may provide a clearer picture regarding how these proteins interact with one another.

6.2 IL-18 production in macrophages and ECs

Several studies have reported an increased level of IL-1β and IL-18 in DENV-infected patients (Hober et al., 1998; A. S. Mustafa et al., 2001). Our present study could only demonstrate the production of IL-1β, not IL-18, in both macrophages and ECs after DENV-2 infection. The gene expression of IL-18 was up-regulated, but no

active IL-18 cytokines were detected. One possible explanation would be that the production of IL-18 requires an additional proteolytic step that is controlled by other factors. Therefore, the production of IL-1β and IL-18 is regulated in a two-step fashion (Dinarello, 2012). Thus, the low level of IL-18 production after caspase-1 activation by DENV-2 may require further study. siRNA knockdown experiments on the TLRs may be needed to provide a deeper understanding of the production pathway of these inflammatory cytokines.

6.3 DENV-2 induces autophagy in macrophages and ECs

The delayed onset of cell death was apparent in DENV-2-infected cells at lower MOIs. One possible explanation for this could be that DENV-2 induces autophagy or apoptosis at lower MOIs. Autophagy was reported on DENV-2-infected cells. To confirm further whether infectious doses of DENV can alter different forms of programmed cell death, including autophagy, LC3 conversion at different time points and different infectious doses is needed. To provide an even clearer picture, it is suggested to use an autophagy inhibitor 3 methyladenine or autophagy inducer rapamycin at different time points and different infectious doses.

6.4 Compare the virus yield in the presence of caspase inhibitor with control group

Doitsh et al reported that a clinically safety drug (VX-675, a caspase-1 inhibitor) could block pyroptosis by HIV-1 (Doitsh et al., 2014), raising the possibility of a new class of antiviral therapeutic targeting the host caspase-1. By comparing the virus yield of dengue in the presence of caspase-1 inhibitor, the potential use of caspase-1 inhibitor would be demonstrated and it would definitely improve the significance of the study.

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