INDUCTION OF HO-1 ON ENDOTHELIAL CELLS VIA PI3K SIGNALING PATHWAY BY ANTI-NS1 ANTIBODIES IN DENGUE VIRUS INFECTED PATIENTS

PUJI RAHAYU

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Giessen 2010

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Prof. Dr. med. Gregor Bein

Date of the Defense: November 3rd, 2010

Declarations

I declare that I have completed this dissertation without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Giessen,

Puji Rahayu

ABBREVIATIONS

ADE	antibody-dependent enhancement
BCA	bicinchoninic acid
C	core
CFR	case fatality rate
CO	carbon monoxide
DF	dengue fever
DHF	dengue haemorrhagic fever
DMEM	dulbecco's modified eagle's medium
DMSO	dimethylsulfoxide
DSS	dengue shock syndrom
DENV	dengue virus
E	envelope
EBM	endothelial basal medium
ECL	enhanced chemiluminescent
ELISA	enzyme-linked immunosorbant assay
ER	endoplasma reticulum
FCS	fetal calf serum
FcR	Fc receptor
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte-colony stimulating factor
H_2DCFDA	dichlorodihydrofluorescine-diacetate
HO	heme oxygenase
HMEC	human microvascular endothelial cells
HUVEC	human umbilical vein endothelial cells

ICAM-1	intercellular cell adhesion molecule
IFNα	interferon α
IgG	immunoglobulin G
IgG	immunoglobulin M
IL-1β	interleukin-1β
IL-8	interleukin 8
IPB	immunoprecipitation buffer
JEV	japanese encephalitis virus
mab	monoclonal antibody
MAPK	mitogen-activated protein kinase
MPO	myeloperoxidase
NAC	N-acetyl-cysteine
NF-κB	nuclear factor-κB
NO	nitric oxide
NS	non-structural glycoprotein
OD	optical density
ORF	open reading frame
PBS	phosphate-buffered saline
PDI	protein disulfide isomerase
PECAM	platelet/endothelial cell adhesion molecule
PI3K	phosphatidylinositol 3 kinase
PKC	protein kinase C
PKG	cGMP-dependent protein kinase
PMA	phorbol myristate acetate
PBMC	peripheral blood mononuclear cells
PMSF	phenylmethylsulfonyl fluoride

prM	precursor membrane
PVDF	polyvinylidene difluoride
RANTES	regulated upon activation, normal T-cell expressed and secreted
RE	regulatory element
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLE	systemic lupus erythematosus
TF	transcription factor
TNF-α	tumor necrosis factor- α
TPA	12-O- tetradecanoylphorbol 13-acetate
VCAM-1	vascular cell adhesion molecule-1
WHO	World Health Organization
WNV	west nile virus
YFV	yellow fever virus

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CHAPTER 1
INTRODUCTION

1.1 Basic biology and epidemiology of dengue

1.1.1 Dengue disease

Dengue disease is probably the most important arthropod borne viral disease in terms of human morbidity and mortality. Up to one third of the world population is at risk of dengue infection. The disease is now highly endemic in more than 100 tropical countries and the number of cases has been increased dramatically during the past decades (WHO, 2009). It remains a major health problem in South-east Asia, Central America and the Pacific region, representing one of major causes of child death in several countries (Monath, 1994). Among South-east Asia countries, in the period of 2006-2008 Indonesia was reported to be the highest number of dengue with a total of 396196 cases and 3468 deaths case fatality rate (CFR) ~1%. The current situation of dengue in Indonesia is stratified by World Health Organization (WHO) as the highest epidemic category (WHO, 2008).

Dengue diseases especially dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), are serious clinical conditions that occur almost exclusively in response to the secondary infection by dengue virus (DENV) (Henchal and Putnak, 1990; Thein et al., 1997). In reality, over than 99% of the cases of viral haemorrhagic fever worldwide reports are related instead to DHF (Rothman, 2004).

Until recently, the highly domesticated *Aedes aegypt* mosquito represents the main vector for the transmission of DENV to human. However, recent observation showed that the strong ecological plasticity of *Aedes albopictus* has allowed a further spread of DENV throughout the world (Benedict et al., 2007). Moreover, the lack of proper diagnostics and inability to control mosquito populations make the disease to be prevalent and to be major public issue in the

developing countries. No preventative therapies such as vaccines or anti-viral treatments are currently available for dengue disease infections, despite its major impact on the world population (Warke et al., 2008). Geographical distribution of DENV is mostly found in the tropical and subtropical regions as shown in the Figure 1 below.

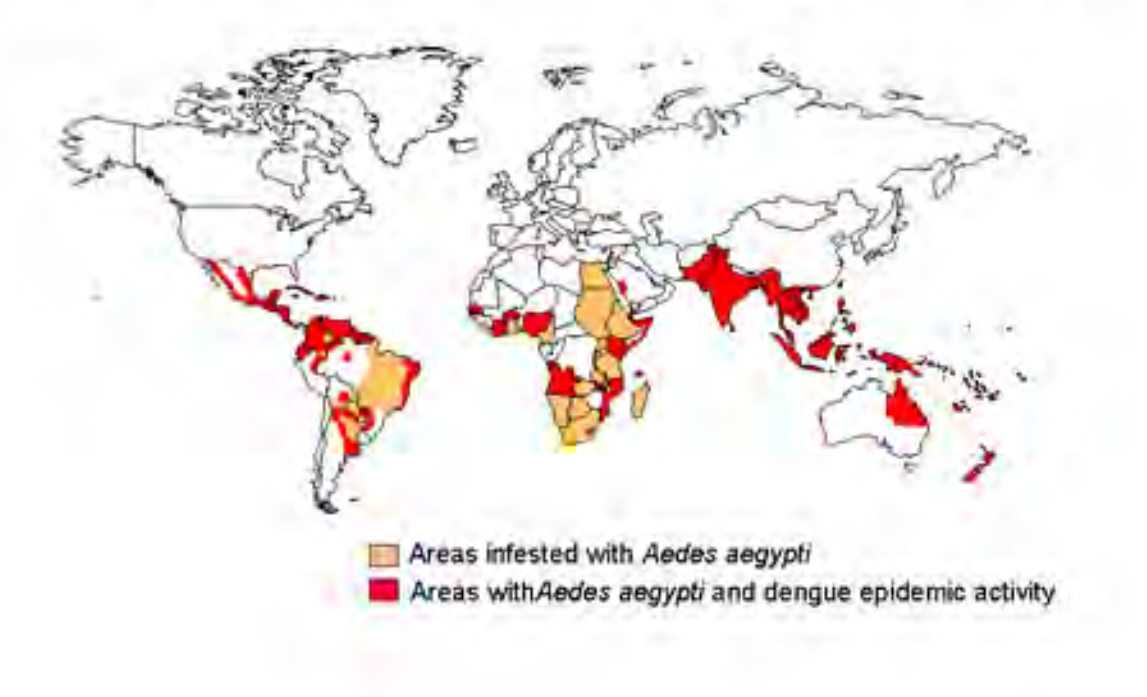

Figure 1. World distribution of dengue and *Aedes aegypti* in 2005 (CDC, 2005).

1.1.2 Clinical and pathological findings on dengue virus infection

Four different serotypes of DENV (DENV-1, DENV-2, DENV-3, and DENV-4) of the genus *Flavivirus* have been discovered. The incubation period of DENV infection varies from 3-14 days (WHO, 1997). Infections by dengue virus produce a spectrum of clinical illness ranging from a non-specific viral syndrome to severe and fatal hemorrhagic disease.

Dengue fever (DF) is a mild, self-limiting febrile illness typically associated with the following symptoms: retro-orbital pain, myalgia, arthralgia, rash, hemorrhagic manifestations, leukopenia, and headache. Most of the infected persons recover after the acute febrile period without any specific treatment (Bhamarapravati, 1989; Bhamarapravati et al., 1967; Burke et al., 1988; Gubler, 2006). There is a lower risk of death in DENV patients presenting clinical symptoms for DF.

Dengue Haemorrhagic Fever (DHF) is an acute vascular permeability syndrome accompanied by abnormalities in haemostatis. The clinical features include plasma leakage, bleeding tendency, and liver involvement (Bhamarapravati, 1989; Bhamarapravati et al., 1967; Burke et al., 1988; Henchal and Putnak, 1990). After dengue virus infection, there is a continuum from mild DF to severe DHF or DSS. It has been estimated that 4-6% of individuals with second infection develop severe DHF disease (Halstead, 2007; Mackenzie et al., 2004). In the most severe cases, clinical deterioration is characterized by severe thrombocytopenia and selective vascular leakage (Oishi et al., 2007). Furthermore, according to severity, WHO has divided DHF into 4 grades (I-IV) (WHO, 1997). Grade I and grade II are a non-shock DHF. Grade III and grade IV are cases of DHF with shock (Malavige et al., 2004). The pathogenesis, especially the mechanistic steps toward the manifestation of DHF, is not clearly understood.

Dengue shock syndrome (DSS) is associated with a very high mortality (a rate of 9.3%, increasing to 47% in instances of profound shock). Acute abdominal pain and persistent vomiting are early warning signs of impending shock. Suddenly hypotention may indicate the onset of profound shock. Prolonged shock is often accompanied by metabolite acidosis which may precipitate disseminated intravascular coagulation or enhance ongoing disseminated intravascular coagulation, which in turn could lead to massive haemorrhage. DSS may be accompanied by encephalopathy due to metabolic or electrolyte disturbance (Malavige, 2004).

1.1.3 Pathogenesis of severe dengue virus infection

It is generally believed that, as in the case for most flaviviruses infection, patients who acquire the dengue disease at the first time (primary infection) elicit lifelong protective immunity to homologous strains of DENV. Patients exposed for the second time (secondary infection) are usually susceptible to heterologous strains of DENV (Nielsen, 2009). The term secondary infection refers to the second infection by a different DENV strain of a patient who already has finished and cleared a first infection by DENV (WHO, 1997). In case of DENV, individuals are protected against reinfection with the same serotype but not against the other three serotypes that circulate globally. In fact, many epidemiological studies have demonstrated that the development of more severe DHF is associated with secondary infections with a heterotypic serotype (Burke et al., 1988; Guzman et al., 1990; Halstead et al., 1969; Sangkawibha et al., 1984; Thein et al., 1997), that led to the widely accepted hypothesis of antibody-dependent enhancement (ADE) of DENV infection (Halstead, 2003; Pang et al., 2007; Rothman and Ennis, 1999; Sullivan, 2001).

ADE theory has been a long-term thought to play a central role on the pathogenesis of severe dengue infection (Halstead, 1970). This theory is based on the observations of severe DHF manifestation in children experiencing a secondary dengue virus infection which has a different serotype (heterologous) of the previous one (Halstead and O'Rourke, 1997). During secondary infection, subneutralizing antibodies recognize DENV and form antigen-antibody complexes. This complex is recognized by cells expressing Fc receptors (FcR) such as monocytes (Mady et al., 1991). This interaction leads to enhanced uptake of virus, resulting in an increased number of cells being infected by the virus (Littaua et al., 1990, Lei et al., 2001). ADE-mediated infection has been reported in many ribonucleic acid (RNA) viruses, including flavivirus and others (Suhrbier and La Linn, 2003). However, unlike these viruses, severe dengue infections have been uniquely associated with hemorrhage. This observation suggests that the hemorrhage found in DHF patients might not be completely

explained by the ADE hypothesis. Figure 2 shows the current model of DHF pathomechanism involving specific T cells.

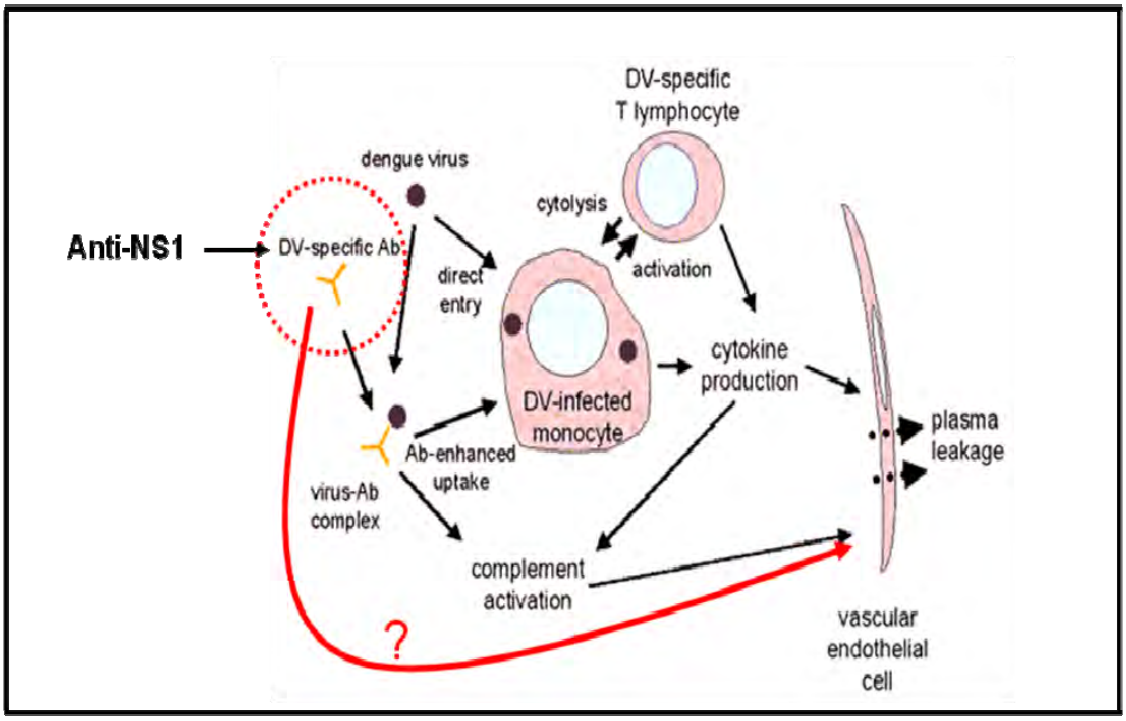

Figure 2. Immunological model of DHF pathomechanism. DENV specific memory T cells are activated following a secondary infection of the host by different DENV serotype. The activated memory T cells rapidly express cytokines (such as tumor necrosis factor-α TNF-α and interferonγ (IFN-γ). Additionally, DENV specific antibodies increase the viral burden of virus-infected cells expressing Fcγ receptors by ADE mechanism. The increased number of viral on antigen presenting cells activates memory T cells. The accumulated production of cytokines by memory and naive T cells during a secondary infection along with complement activation enhances the effect on vascular endothelial cells and lead to plasma leakage (Rothman, 2003 with some modifications).

1.1.4 Virus structure

Dengue virus belongs to the family Flaviviridae (from the Latin *flavus*, yellow), which includes yellow fever virus (YFV), Japanese encephalitis virus (JEV) and West Nile virus (WNV). DENV is an arthropod borne (Monath and Heinz, 1996) and is a small single-stranded RNA virus which comprised of four distinct serotypes (DENV 1-4). Its genome consists of a single open reading frame encoding for a large polypeptide which is cleaved by viral and host proteases in at least 10 discrete proteins. The N-terminal one quarter of the polypeptide encodes the structural proteins core (C), precursor membrane (prM/pM), envelope (E), and the remaining part contains seven nonstructural (NS) proteins, including large, highly conserved proteins NS1, NS3, and NS5 and four small hydrophobic proteins NS2A, NS2B, NS4A, and NS4B (Chambers, 1990; Henchal and Putnak, 1990; Zhang, 2003;). Figure 3 shows the gene organization of the *Flavivirus* and its resulting proteins and the location of the major targets of immune response. The DENV genome is a single-stranded sense RNA with a single open reading frame (ORF, top). The ORF is translated as a single polyprotein (middle) that cleavage by viral and host protease to yield the ten viral proteins (bottom) (Rothman, 2004).

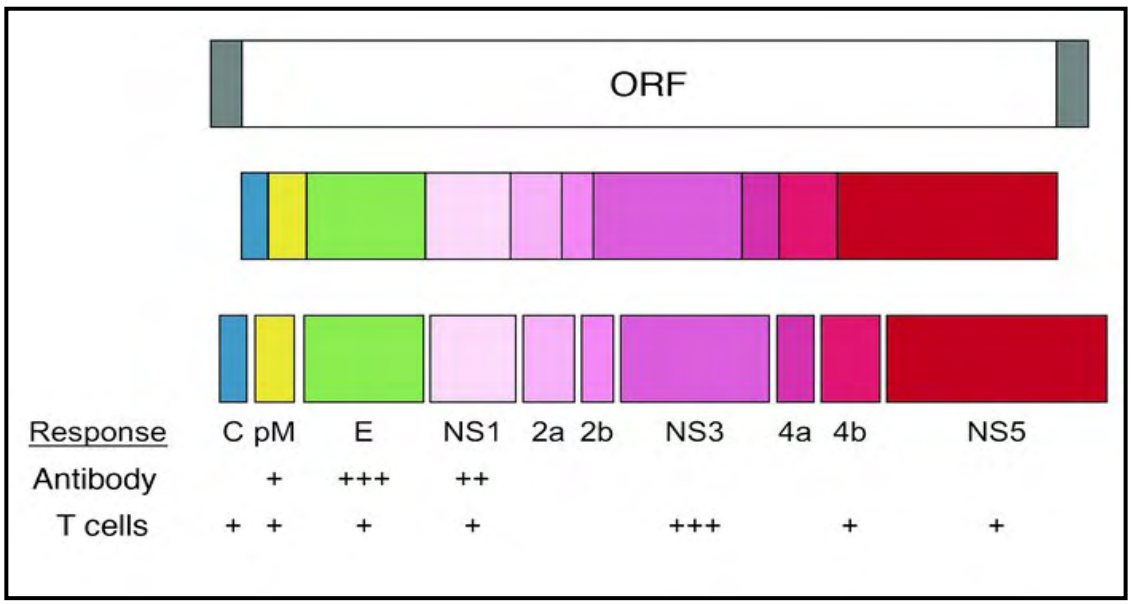

Figure 3. *Flavivirus* genome organization (Rothman, 2004)

The NS1 glycoprotein (Mr ~46 kDa) exists in a cell-associated, cell-surface, or extracellular nonvirion secreted form in infected mammalian cells (Wingkler, 1989). In contrast, the remaining NS proteins are localized on the cytoplasmic side of the endoplasma reticulum (ER) membrane (Markoff et al., 1994; Falgout and Markoff, 1995). Several reports have demonstrated that NS1 is highly immunogenic and could induce the production of complement-fixing antibody. This antibody can cause a direct complement-mediated lysis of infected target cells via interaction with the cell-surface-associated form of NS1 (Costa et al., 2007). The secretion of a viral NS1 protein that elicits protective immune response is an interesting phenomenon in *Flavivirus* biology.

Clinical study observed the presence of secreted form of NS1 in serum of patients during dengue infection (Monath and Heinz, 1996). Furthermore, several studies found that DHF patients with a secondary infection developed frequently anti-NS1 antibodies indicating that these antibodies may associate with the pathomechanism of DHF and DSS (Kuno et al., 1990; Falconar, 1997; Valdes et al., 2000). Other studies indicated that anti-NS1 antibodies may confer protection against DENV (Henchal et al., 1985; Schlessinger et al., 1993). Meanwhile the use of anti-NS1 antibodies as predictor for DHF is focused of different clinical studies (Lemes et al., 2005).

1.2 Dengue disease and endothelial cells

1.2.1 Cross-reaction of dengue antibodies with endothelial cells

Dengue haemorrhagic fever is the main cause of mortality in dengue virus infection (Valdes et al., 2000). Haemorrhagic syndrome, a feature of DHF/DSS is a hematologic abnormality resulting from multiple factors, including thrombocytopenia, coagulopathy and vasculopathy related with dysfunction of platelet and endothelial cells (Rothman et al., 1999). The clinical symptoms of DHF/DSS, which include hemorrhage, thrombocytopenia, increased vascular permeability, decreased blood pressure, and hypovolemic shock, strongly indicate a disorganization of haemostasis system in this disease.

Endothelial cells and platelets are known to play an important role in regulating vessel permeability and maintaining haemostasis (Kaiser et al., 1997). The most characteristic feature of DHF/DSS and the best indicator of disease severity is plasma leakage that results from structural damage of endothelial cells (Lei et al., 2001). Plasma leakage is caused by a diffuse increase in capillary permeability and manifests as any combination of hemoconcentration, pleural effusion, or ascites. It usually becomes evident on days 3-7 of illness, during which time dengue fever resolve (Bhamarapravati et al., 1967; Burke, 1988). Plasma leakage occurs systemically, progressing quickly, but will resolve within 1 to 2 days in patients who receive appropriate fluid resuscitation. No subsequent tissue or organ dysfunction is observed. Although perivascular edema is obvious, however, no obvious destruction of vascular endothelial cells has been reported. It was previously thought that plasma leakage was due to altered vascular permeability rather than to structural destruction of endothelial cells. The functional alteration of endothelial cells is probably caused via by-standard effects of cytokine or mediator release in dengue infection. The dengue virus can infect endothelial cells in vitro which lead to apoptosis as well as production of cytokines and chemokines such as IL-6, IL-8 and regulated upon activation normal T cell expressed and secreted (RANTES) (Avirutnan et al., 1998; Huang et al., 2000) but no infection in biopsies of patients with DHF/DSS have been demonstrated.

Disturbance of platelet as well as endothelial cell functions by antibodies may initiate the clinical manifestations of thrombocytopenia and endothelial dysfunction. Lin et al (2002) reported that anti-NS1 antibodies can cross-react with non-infected endothelial cells and induce these cells to undergo apoptosis. Recent studies showed that anti-NS1 antibodies caused thrombocytopenia in *in vitro* as well as in *in vivo* mouse model (Chang et al., 2002; Sun et al., 2007).

The endothelium acts as the primary barrier of the circulatory system could broadly affect the immune cell function and contribute to dengue pathology (Warke et al., 2003). The body releases cytokines that cause the endothelial tissue to become permeable which results in hemorrhage and plasma loss from the blood vessels. Cytokines are proteins secreted during innate and adaptive immunological responses, acting as inflammatory mediators or modulatory molecules during several haemorrhagic fevers (Marty et al., 2006). Clinical studies support a key role for cytokines in the DHF pathogenesis (Hober, 1993; Bethel, 1998; Green, 1999; Braga, 2001; Fink et al., 2006; Azeredo, 2006).

Endothelial damage and activation were observed in the acute phase of dengue virus infection (Cardier et al., 2006; Sosothikul et al., 2007). Apoptosis in microvascular endothelial cells from lung and intestine tissues was observed in fatal cases of DHF/DSS (Limonta et al., 2007). However, in the recent years a number of studies have shown that infection with DENV induces apoptosis in vitro and also in vivo (Despres et al., 1996; Marianneau et al., 1999; Couvelard et al., 1999; Huerre et al., 2001; Lei et al., 2001). Anti-NS1 antibodies generate in mice has been shown to cross-react with human fibrinogen, platelets and endothelial cells (Falconar, 1997; Falconar, 2007). The cross reactivity of dengue patient sera with endothelial cells have also been demonstrated. Endothelial cells were more reactive with DHF/DSS patient sera than DF patient sera (Lin et al., 2004).

1.2.2 Dengue virus-induced anti-endothelial cell autoantibodies

The binding reactivity of the sera of DHF/DSS with endothelial cells was detected by flow cytometric. There were higher percentages of endothelial cells reactive with DHF/DSS than those with DF patient sera. Further studies showed that platelet or endothelial cell binding activities were inhibited by pretreatment with dengue NS1. A molecular mimicry between the dengue virus and endogenous self-proteins was proposed to be one of the mechanisms for the induction of autoimmunity during dengue virus infection (Lin et al., 2003).

1.2.3 Immunopathogenetic effects of dengue virus infection on endothelial cells

Although the disruption of endothelial barrier is a hallmark of DENV infection, however, the exact mechanism is a quite few known. The endothelium is the target site for DENV infection-mediated pathology such as vascular permeability, capillary fragility (evidenced by positive tourniquet test done in patients), bleeding, and coagulopathy and hypovolemic shock during the acute phase of DHF/DSS. In vivo, the pathophysiology clinically observed is considered to be a result of both direct (viral infection) and indirect (pro-inflammatory cytokines, chemokines released by activated leukocytes) effects on endothelial cells. Apoptosis of endothelial cells caused by both virus infection and by anti-NS1 antibodies has been proposed (Andrew, 1978; Avirutnan et al., 1998; Lin et al., 2002; Huang et al., 2006).

Recently, Cheng et al. (2009) reported several candidate antigens of anti-NS1 antibodies by proteomic approach. However, the specific endothelial antigen reacting with anti-NS1 antibody is not known. Functional studies in the past demonstrated that anti-NS1 antibodies are able to cross-react with non-infected endothelial cells and trigger the intracellular signaling leading to the production of nitric oxide. Nitric oxide (NO) caused upregulation of p53 and Bax and down-

regulation of pro- and anti-apoptotic factors Bcl-2 and Bcl-xL, respectively, which leads to cytochrome-c release and caspase-3 activation (Lin et al., 2002; Lin et al., 2004). The activation of caspase-3 has been identified as a key mediator of apoptosis of mammalian cells. Furthermore, nuclear factor-κB (NF-κB) activation was found in endothelial cells after stimulation with anti-NS1 antibodies (Lin et al., 2002).

Recently, Naidu et al. (2008) described a direct association between NF-κB activation pathway and the induction of anti-apoptotic heme oxygenase-1 (HO-1). Soares et al. (1998) demonstrated that HO-1 inhibits the expression of proinflammatory genes like TNF-α or IL-1 associated with endothelial activation via a mechanism that is associated with the inhibition of NF-κB activation resulting in the modulation of adhesion molecules like E-selectin, intercellular cell adhesion molecule (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Furthermore, Foresti et al. (2003) described that NO potently up regulated HO-1 in the presence of heme and heme metabolites. Several pathological states are characterized by increased NO production and liberation of heme. This synergism may ultimately increase the defensive abilities of endothelium to counteract cell apoptosis.

1.3 Heme Oxygenase (HO)

1.3.1 Isoforms of HO

Heme oxygenase catalyzes the first and rate-limiting step of heme degradation. HO breaks up the heme tetrapyrrole ring to yield equimolar amounts of biliverdin, carbon monoxide (CO) and iron (Figure. 3). Biliverdin, in turn, is converted into bilirubin by biliverdin reductase in a non-rate-limiting enzyme reaction (Maines, 1997). Three HO isoforms have been discovered; HO-1, -2 and -3 (Tenhunen et al., 1968; Maines et al., 1986). However, the biological significance of a third isoform, HO-3, is unclear (McCoubrey et al., 1997).

HO-1 and HO-2 are products of distinct genes. Both isoforms are highly conserved throughout evolution and are found in a wide range of organisms such as bacteria, fungi, plants and mammalians. Nucleotide sequence homology among mammalians (rat, mouse, and human) is >80% or >90% for HO-1 and HO-2, respectively (Maines, 1997).

The homology between HO-1 and HO-2 genes is about 43%. The inducible HO-1 isoform (also known as 32-kDa heat-shock protein), exhibits low basal gene expression levels in most cells and tissues. High gene expression levels of HO-1 are detected in spleen and liver tissue macrophages (Kupffer cells) in which senescent erythrocytes are sequestered and destroyed. By contrast, the non-inducible HO-2 form (36 kDa) exhibits high constitutive gene expression preferentially in brain and testis (Maines, 1988; Wagener et al., 1999; Otterbein and Choi, 2000).

1.3.2 Functional significance of HO-1

HO-1 is upregulated by heme, or heme-containing compounds, and also by non-heme containing compounds that increase the cellular production of reactive oxygen species (ROS) in different cells and tissues (Immenschuh and Schröder,

2006). Due to this large array of stress stimuli that induce HO-1 gene expression, HO-1 has been considered for many years to serve cytoprotective functions against oxidative stress (Vile and Tyrell, 1994). In addition, HO-1 has been recognized to have anti-inflammatory effects (Poss and Tanegawa, 1997; Yachie et al., 1999). Different regulatory mechanisms such as modulation of proinflammatory cytokines and activation of T-cells by HO-1 have been observed (Otterbein et al., 2003; Kapturczak et al., 2004; Brusko et al., 2005).

The broad spectrum of substances that induce HO-1 suggests that various signaling pathways are involved in the regulation of HO-1 gene such as mitogen-activated protein kinases, protein kinase C, cAMP-dependent protein kinase A, or cGMP-dependent protein kinase G (Immenschuh and Ramadori, 2000).

Several studies have been done in regards to the regulation of HO-1 gene expression by different stimuli that increase the cellular ROS production (Applegate et al., 1991; Choi and Alam, 1996). It has been shown that scavengers of ROS such as N-acetyl -cysteine (NAC) inhibit the magnitude of HO-1 induction by oxidative stress (Lautier et al., 1992). These findings indicate that an increase of intracellular ROS and, thereby, the activation of redox-dependent signaling pathway play a crucial role for the regulation of HO-1 gene expression.

Although the exact mechanism of redox signaling targeted by ROS is not solved yet, changes of the cellular redox state seem to be responsible for the modification of specific regulatory protein kinases and phosphatases leading to the alteration in the regulation of gene expression (Finkel, 1998).

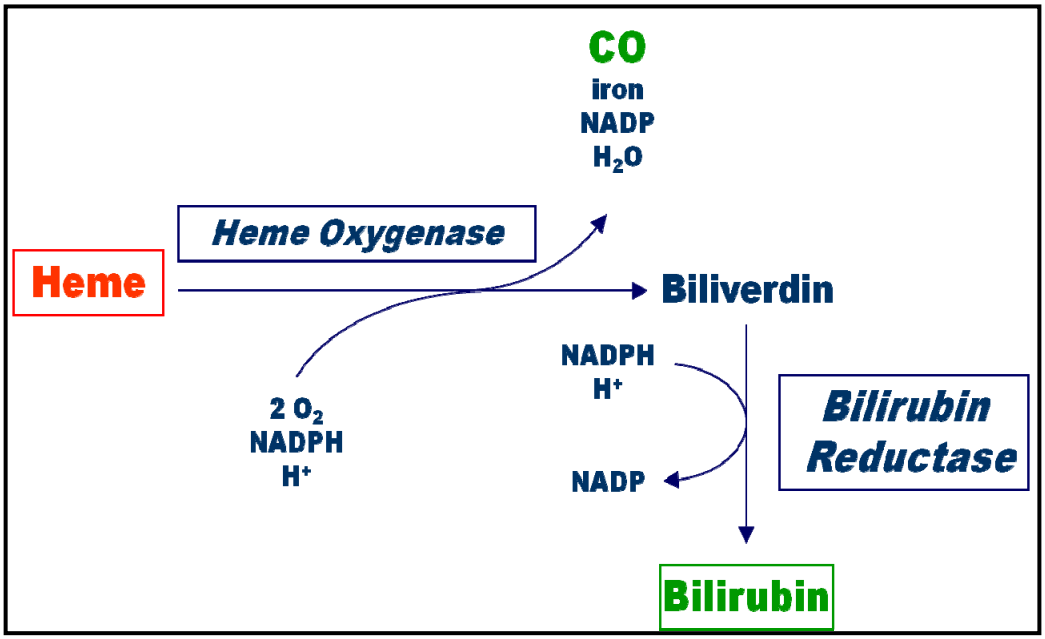

Figure 4. The enzymatic reaction catalyzed by heme oxygenase enzymes.

1.3.3 Physiological function of HO-1

Antioxidant enzyme levels are sensitive to oxidative stress. Both increased and decreased levels have been reported in different disease in which an enhancement of ROS is a cause or a consequence of the illness (Gebicki, 1997).

Major functions of HO enzyme activity comprise degradation of the prooxidant heme and production of CO and bilirubin, thereby providing protection of organs and tissue against oxidative stress (Abraham et al., 1988; Maines, 1997). More recently, accumulating evidence indicates that HO-1 is an important modulator of the inflammatory response possibly via the generation of the second messenger gas CO (Otterbein et al., 2000; 2003). An anti-inflammatory function of HO-1 has been shown in experimental models of acute complement-dependent pleurisy and heme-induced inflammation of various organs (Willis et al., 1996; Wagener et al., 1999). However, modulation of HO-1 may not only serve as a therapeutic target in inflammatory disease, but also has therapeutic implications in organ transplantation. HO-1 has been demonstrated to play a protective role in several

experimental transplantation models, in which elevated HO activity prevents the development of vascular lesions, intra-graft apoptosis, ischemia/reperfusion injury, and significantly prolongs allograft survival (Soares et al., 1998; Hancock et al., 1998; Amersi et al., 1999; Immenschuh and Ramadori, 2000).

1.3.4 Signaling pathway of HO-1

Stimulation of the HO-1 gene by most, if not all, stimuli is primarily controlled at the transcriptional level. A variety of regulatory elements (RE) and transcription factors (TF) have been demonstrated to be involved in this process (Choi and Alam 1996). The broad spectrum of substances that induce HO-1 suggests that various signaling pathways are involved in the regulation of this gene such as mitogen-activated protein kinases (MAPK), protein kinase C (PKC), cAMP-dependent protein kinase A (PKA), or cGMP-dependent protein kinase G (PKG) (Immenschuh and Ramadori, 2000).

1.4 Objectives of the study

Understanding the role of anti-NS1 antibodies binding on endothelial cells and its functional signaling consequence during dengue virus infection may help to understand the cause of vascular leakage in DHF patients which may have therapeutic benefits.

The objectives of this study were (1) to study the regulation of the anti-inflammatory gene, HO-1 on human umbilical vein endothelial cells (HUVEC) after stimulation with anti-NS1 antibodies derived from DHF patients, (2) to identify the prominent cellular signaling pathway that was activated or regulated by HO-1 gene, and (3) to characterize the target antigen on endothelial cell surface that recognized by anti-NS1 antibodies.

CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and reagents

Acrylamide/bisacrylamide 30%,	Roth, Karlsruhe, Germany
Bromophenol blue	Merck, Darmstadt, Germany
Bovine serum albumin (BSA)	Serva, Heidelberg, Germany
Cell culture lysis reagent 5x	Promega, Madison, WI, USA
Chloroform	Roth, Karlsruhe, Germany
Dimethylsulfoxid (DMSO)	Sigma-Aldrich, Munich, Germany
Dithiothreitol	Sigma-Aldrich, Munich, Germany
Dry milk	Sucofin, Zeven, Germany
Ethanol (100%)	Roth, Karlsruhe, Germany
Ethidium bromide	Dianova, Hamburg, Germany
Ethylenediamine tetraacetic acid (EDTA)	Merck, Darmstadt, Germany
Extract of yeast powder	Merck, Darmstadt, Germany
Glycerol	Sigma-Aldrich, Munich, Germany
Glycin	Sigma-Aldrich, Munich, Germany
Glycogen	Roche, Basel, Switzerland
Methanol	Roth, Karlsruhe, Germany
Penicillin/Streptomycin	Merck, Darmstadt, Germany
Phenol	Sigma-Aldrich, Munich, Germany
PMSF	Merck, Darmstadt, Germany
Ponceu S	Serva, Heidelberg, Germany
Potassium acetate	Sigma-Aldrich, Munich, Germany
Sodium acetate	Merck, Darmstadt, Germany
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, Munich, Germany
Tetra-methylethylendiamine	Serva, Heidelberg, Germany
Tris base	Sigma-Aldrich, Munich, Germany
Triton-100	Sigma Aldrich, Munich, Germany

Yeast extracts	Difco, Detroit, MI, USA
Tween 20	Sigma-Aldrich, Munich, Germany

All other standard reagents were from Sigma-Aldrich, if not indicated otherwise.

2.1.2 Pharmacological compounds

Bacitracin	Sigma-Aldrich, MO, USA
H_2DCFDA	Sigma-Aldrich, Munich, Germany
Fluorescent labeled albumin	Sigma Alrich, MO, USA
Fibronectin	Sigma-Aldrich, Munich, Germany
Heme	Sigma- Alrich, Munich, Germany
LY294002	Calbiochem, La Jolla, CA, USA
N-acetylsystein	Sedico Pharmaceutical, Cairo, Egypt
Protein G sepharose CL-4B bead	Pharmacia, Uppsala, Sweden
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich, Munich, Germany
SB203580	Calbiochem, La Jolla, CA, USA
Sulfo-NHS-Kc Biotin	Thermo Scientific, Rockford, IL, USA
TNF-α	Roche, Basel, Switzerland
TPA	Sigma-Aldrich, Munich, Germany
Wortmannin	Calbiochem, La Jolla, CA, USA

2.1.3 Markers

Rainbow TM protein molecular weight	Amersham, Freiburg, Germany
Biotin Marker	Cell Signaling, Beverly, MA, USA

2.1.4 Kits

Annexin V Kit	BD Pharmingen, San Diego, CA, USA
BCA protein assay kit	Thermo Scientific, Rockford, IL, USA.
ECL cheluminescence kit	Calbiochem, Darmstadt, Germany
Pure Melon™ gel IgG purification kit	ThermoScientific, Rockford, IL, USA.
Chemiluminescence detection system	Millipore, Billerica, MA, USA

2.1.5 Materials of cell culture

Dulbecco's Modified Eagle's medium	Gibco, Grand Island, NY, USA
Endothelial cell basal medium (EBM)	PromoCell, Heidelberg, Germany
Fetal bovine serum (FBS)	Biochrom KG, Berlin, Germany
Fetal calf serum (FCS)	Biochrom KG, Berlin, Germany
Ficoll-Paque	Amersham, Freiburg, Germany
Gentamycin	PromoCell, Heidelberg, Germany
Hepes	Gibco, Gaithersburg, MD, USA
RPMI 1640	Gibco, Gaithersburg, MD, USA
Sodium-pyruvate	Gibco, Gaithersburg, MD, USA
Trypsin-EDTA (1X) (w/o Ca & Mg)	Gibco, Grand Island, NY, USA
Tissue culture dish 6 cm	Falcon, Heidelberg, Germany
Tissue culture flash	Falcon, Heidelberg, Germany
Tissue culture 6-well plate (surface area 9.6 cm2)	Greiner, Frickenhausen, Germany
Transwell	Costar, Cole Parmer, IL, USA

2.1.6 Cell cultures

HUVEC human endothelial cells	ATCC, Walkerville, MD, USA
Eahy 926 ECV304 human endothelial cells	ECCC, Braunschweig, Germany
U 937 monocytic cells line	ATCC, Manassas, VA, USA

2.1.7 Antigen and antibodies

Primary antibodies:

Mab anti-NS1 (clone DN 2)	Abcam, Cambridge, UK
Mab againts HO-1	Stressgen, Victoria, BC, Canada
GAPDH	HyTest, Turku, Finland
PDI-antibody clone 77	Abcam, La Jolla, CA, USA
RL 90 mab against PDI	Novus Biological, CA, USA

Mabs against PECAM-1 (clone GI18), CD177 (7D8), and CD31 were produced and characterized in our laboratory.
Purified IgG was isolated from secondary dengue infected patient sera and healthy donor sera.

Secondary antibodies

Goat anti-rabbit IgG-HRP	Acris Antibodies, Hiddenhausen, Germany
Goat anti-mouse IgG-HRP	Acris Antibodies, Hiddenhausen, Germany
Goat anti-human IgG-HRP	DPC Biermann, Bad Nauheim, Germany
Rabbit anti-mouse IgG-HRP	Acris Antibodies, Hiddenhausen, Germany

Goat anti-mouse FITC	Invitrogen, Carlsbad, CA, USA
Streptavidin horseradish peroxidase	Amersham Life Science, Arlington, IL, USA

Antigen

NS1 antigen	Prospec-Tanytechno Gene, Rehovot, Israel

2.1.8 Other materials

3 mm Whatman paper	Schleicher and Schuell, Dassel, Germany
Lumi-LightPLUS western blotting substrate	Roche, Mannheim, Germany
Polyvinylidene fluoride membranes (PVDF)	Millipore, Bedford, MA, USA

2.1.9 Instruments

AMAXA electroporation system	Amaxa, Koln, Germany
Blotting-semidry Whatman	Biometra, Göttingen, Germany
Centrifuge: RC5C	Sorvall, Wiesloch, Germany
Densitometry	Alpha Innotech, San Leandro, CA, USA
ELISA reader	Bio-rad, Munich, Germany
FACS Calibur	BD Pharmingen, San Diego, CA, USA
Fluorometry	Beckmann, Munich, Germany
Fluorescence microplate reader	Bio-Tek, Bad Friedrichshall, Germany
Fluorchem FC2 gel documentation system	Bio-Tek, Bad Friedrichshall, Germany
Hettich Rotixa/RP	Hettich, Tuttlingen, Germany

Hettich Mikro 22R	Hettich, Tuttlingen, Germany
Electrophoresis apparatus	Bio-Rad, Munich, Germany
Incubator CO2	Heraeus, Hanau, Germany
Laminair HB2448	Heraeus, Hanau, Germany
Microscope Axiovert 10	Zeiss, Oberkochen, Germany
Spectrophotometer	Beckmann, Munich, Germany
Nanodrop spectrophotometer	Biocompare, San Francisco, CA, USA

2.2 Methods

2.2.1 Sample collection

Serum samples were obtained from the Cipto Mangunkusumo Hospital, Jakarta Indonesia. The serum samples were collected from confirmed cases of dengue virus infected patients (n=16) during dengue outbreak in 2007. Serum samples were selected from patients who were classified as having secondary dengue virus infection according to the WHO criteria (WHO, 1997). All serological characterizations to identify dengue virus infection and to discriminate between primary and secondary infection were done at the Cipto Mangunkusumo Hospital, Jakarta. WHO evaluated diagnostic test (Panbio Dengue IgM Capture ELISA and Panbio Dengue Duo Casette) was used. Serum samples from healthy donors were used as control (n=5).

2.2.2 IgG purification

IgG was isolated from serum samples using Immuno Pure Melon™ gel IgG purification kit. 500 µl Melon gel was added to the column and centrifuge as recommended by the manufacturer. After washing with 300 µl purification buffer, 500 µl of diluted serum in gel purification buffer (1:10) was added to the gel and mixed end-over-end for 5 minutes. The gel then centrifuged and purified IgG was then collected in a collection tube. IgG concentration was determined by a nanodrop spectrophotometer.

2.2.3 Determination of anti-NS1 antibodies by solid phase ELISA

Antibody against NS1 antigen in patient sera was analyzed by the solid phase ELISA, according to Vasquez et al. (1997). Microtiter wells were coated with 50 µl of recombinant NS1 antigen (5 µg/ml) in 0.1 M bicarbonate buffer, pH 9.5.

After blocking with 1% BSA in phosphate-buffered saline (PBS), diluted serum sample (1:50 in blocking buffer) was added and incubated for 1 h at 37^{o}C. Wells were washed 5 times with 200 µl PBS 0.1% Tween 20 phosphate-buffered saline tween (PBST), 100 µl of horse radish peroxidase labeled goat anti-human IgG was added for 1 h at 37^{o}C. Reaction was measured in ELISA reader. Sample was considered positive if the OD_{492} > 0.3.

2.2.4 Cell culture preparation

HUVEC were cultured in fresh endothelial basal medium (EBM) supplemented with hydrocortisone (1 mg/ml), gentamycin sulphate (50 mg/ml), amphotericin-B (1 µl/ml), and 2% fetal calf serum (FCS). Human monocytic cells line U937 were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin. Human EA.hy 926 endothelial cells line was maintained in DMEM supplemented with 10% FCS and 100 IU/ml penicillin and 100 µg/ml streptomycin. Cell cultures were maintained until confluence at 37^{o}C in a controlled environment of 100% humidity and 5% $CO_{2.}$

2.2.5 Cell culture stimulation

Aliquots of HUVEC in six-well flat bottom plates containing 2 ml EBM were stimulated with different concentrations of mab anti-NS1 (2-20 µg/ml) and different time periods (1-48 h). After washing with 0.9% NaCl, cells were lysed with 200 µl lysis buffer containing 50 mM tris, 150 mM NaCl, 1% triton x-100 and 2 mM phenylmethylsulfonyl fluoride (PMSF). The endothelial cell lysate was then centrifuged for 5 min at 13.000 g at 4^{o}C and the protein concentration of the supernatant was determined by bicinchoninic acid (BCA) protein assay kit. Endothelial proteins were used for western blotting (see 2.2.6).

For the analysis of sera from dengue virus infected patients, purified IgG from dengue virus infected patient (10 µg/ml) was added either to HUVEC or U937 cells for 18 h. Heme (10 µg/ml in DMSO) was used as a positive control. In some experiment, HUVEC were treated with mab anti-NS1 (10 µg/ml) and DHF IgG (10 µg/ml) in the presence of NS1 antigen (10 µg/ml).

In some experimental setting, HUVEC were treated with phosphatidylinositol 3-kinase (pI3K) inhibitors (wortmannin, LY294002 and p38 inhibitor, SB203580 in a concentration of 10 µM for 30 min prior to incubation with mab anti-NS1 (10 µg/ml) and DHF IgG (10 µg/ml) for 18 h.

In some experimental setting, HUVEC were growth as mentioned above until confluence. After confluence HUVEC were stimulated with 4 µl 2 mM bacitracin and mab anti-RL-90 (10 µg/ml) for 18 h and lysed.

2.2.6 Western blotting analysis

Total endothelial protein (5 µg) was incubated for 5 min at 95^{o}C using non reduced loading buffer. Total endothelial protein was separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto polyvinylidene difloride (PVDF) membranes. After blocking with Tris-buffer saline containing 5% skim milk, 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20 for 1 h at room temperature, membranes were incubated with antibody against HO-1 (1:2,000 dilution), detected with peroxidase labelled goat anti-rabbit IgG (dilution 1:10,000) The enhanced chemiluminescent (ECL) chemiluminescent kit detection system was applied for detections; as recommended by the manufacturer.

In the control experiment strips were stained with antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; dilution 1:10,000). The signals were visualized with the Fluorchem FC2 gel documentation system.

2.2.7 Immunoprecipitation

Human EA.hy 926 endothelial cells were maintained in DMEM supplemented with 10% FCS, 100 IU/ml penicillin, and 100 µg/ml streptomycin. After confluence, 2 x 10^8 cells were washed three times with PBS and incubated with 1 ml Sulfo-NHS_LC Biotin (1 mg/ml) for 30 min on ice. Labeled cells were resuspended in 200 µl lysis buffer containing 50 mM tris, 150 mM NaCl, 1% triton x-100, and 2 mM phenylmethylsulfonyl fluoride (PMSF).

After centrifugation at 10,000 g for 10 min, cell lysates were precleared for 30 min with 50 µl of 20% protein G-Sepharose CL-4B beads in the presence of 33.3 µl normal human serum for 30 min. Aliquots of 50 µl precleared cell lysates were incubated with 5 µg/ml control mouse IgG (mab 7D8; 5 µg/ml), mab anti-NS1 (DN 2; 5 µg/ml), anti PDI RL-90; 5 µg/ml, anti-PECAM-1 (clone Gi18, 5 µg/ml), or 50 µl human serum overnight at 4^{o}C.

Immunocomplexes were washed five times with immunoprecipitation buffer (IPB; 10 mM Tris HCl at pH 7.4). Bound proteins were released by boiling in SDS buffer for 5 minutes at 95^{o}C. After centrifugation at 10,000 g for 2 min, samples were analyzed by SDS-PAGE and blotted on PVDF membrane as described above. Membrane was incubated with 8.3 µl streptavidin horseradish peroxidase secondary antibody (1:8,000 dilution) for 30 min at room temperature. After washing, precipitated protein was detected by using ECL chemiluminescence kit as recommended by the manufacturer.

In some experimental setting a preclearing procedure prior to immunoprecipitation was performed. The cell lysates for immunoprecipitation were prepared as described above. After centrifugation at 10,000 g for 10 min, cell lysates were precleared for 30 min with 50 µl of 20% protein G-Sepharose CL-4B beads in the presence of 33.3 µl normal human serum for 30 min. Aliquots of 50 µl precleared cell lysates were incubated with 10 µl DHF IgG (5 µg/ml). Preclearing with DHF Ig was repeated 3 times. After preclearing, the cell lysates were incubated with mab anti-PDI and anti-CD31 (as control) at 4^{o}C

overnight. Immunocomplexes were washed five times with washing buffer (IPB; 10 mM Tris HCl at pH 7.4). Bound proteins were released by boiling in SDS buffer for 5 minutes at 95^{o}C. After centrifugation at 10.000 g for 2 min, samples were analyzed by SDS-PAGE and blotted on PVDF membrane as described above. Membrane was incubated with 8.3 µl streptavidin horseradish peroxidase secondary antibodies (1:8,000) for 30 min at room temperature. After washing, precipitated protein was detected by using an enhanced ECL chemiluminescence kit as recommended by the manufacturer.

2.2.8 Flow cytometry analysis

2.2.8.1 Analysis of cells apoptosis

HUVEC were treated with mab anti-NS1 (10 µg/ml) and DHF (10 µg/ml) for 18 h. After washing with PBS, cells were resuspended in binding buffer (10 mM Hepes, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4). Aliquots of 2 x 10^4 cells were incubated with 5 µl fluorescein labeled annexin V in 100 µl binding buffer at room temperature for 15 min in the dark. Labelled cell were analyzed by flow cytometry.

2.2.8.2 Analysis of ROS production

For the measurement of ROS in HUVEC, the green fluorescence dye (5,6-carboxy-2',7'-dichlorodihydrofluoresceine diacetate (carboxy-H_2DCFDA) in ethanol was used as recommended by the manufacturer. Aliquots of HUVEC in six-well flat bottom plates containing 2 ml EBM were stimulated with 10 µg/ml of mab anti-NS1 and incubated for 18 h. Subsequently, 3 µl of 10 µM carboxy-H_2DCFDA was added for 20 min. As positive control, 10 µM tetradecanoylphorbol 13-acetate (TPA) was used. The cells were washed 3 times with PBS (pH 7.4 at 37°C). The presence of fluorescent dye in cells was

detected with flow cytometry. In addition, the inhibition of ROS production with NAC was measured by flow cytometry. In brief, aliquots of HUVEC in six-well flat bottom plates containing 2 ml EBM were treated with different NAC concentrations (10-30 mM/ml) for 30 min. After washings with 0.9% NaCl, cells were further incubated with fresh serum free EBM and then stimulated with mab anti-NS1 (10 µg/ml) and patient IgG (10 µg/ml) for 18 h.

2.2.8.3 Analysis of antibody binding on endothelial cells

In some experiments, HUVEC cells were untreated or treated with 2 µg/ml TNF-α for 1 h. Cells were washed using EBM serum free medium and incubated with mab anti-NS1(10 µg/ml) and DHF IgG (10 µg/ml) for 18 h. After stimulation cells were washed using cold PBS and incubated with fluorescein conjugated secondary antibodies and analyzed by flow cytometry.

2.2.9 Analysis of endothelial permeability

For the measurement of endothelial permeability, HUVEC were grown on gelatin-coated Costar transwell and were treated with mab anti-NS1 (10 µg/ml) and patient IgG (10 µg/ml) for 18 h. Thereafter, fluorescent labeled albumin (40 ng/ml) was added to the luminal chamber. After a period of times, samples were collected from the bottom of chambers and analyzed by fluorometry.

2.2.9 Quantification analysis

Signals from Western blots were evaluated by videodensitometry scanning and quantification with Imagequant software. The relative densities of bands were expressed as fold-induction normalized to GAPDH from at least three independent experiments.

2.2.10 Statistical analysis

Statistical difference was analyzed by Student's *t* test and presented as mean values ± S.E. from at least three independent experiments. A value of $p \leq 0.05$ was considered as was statistically significant.

CHAPTER 3
RESULTS

3.1 Anti-NS1 antibodies in dengue virus infected patients

The presence of anti-NS1 antibodies in the serum of patients with secondary infection from both DF and DHF patients during acute infection was analyzed using a solid phase ELISA. Sample was considered positive if the OD_{492} value > 0.3.

Anti-NS1 antibodies were detected in eight (50%) serum samples from DF/DHF patients (Table 1). Anti-NS1 antibodies were detected only in patients with secondary type of infection, while all serum samples from healthy donors were negative (Table 2). The result also demonstrated that the presence of anti-NS1 antibodies of DHF patients is greater than DF patients. Anti-NS1 antibodies were detected in six serum samples of DHF patients with secondary infection, while anti-NS1 antibodies of DF patients with secondary infection were detected only in two serum samples.

Table 1. Determination of anti-NS1 antibodies of DF and DHF patients by solid phase ELISA. Sample was considered positive if the OD_{492} > 0.3.

Sample Code	Dengue Virus Infection Category	Anti-NS1 antibodies (OD_{492} value)
D1	DHF stadium I	0.738
D2	DHF stadium I	0.979
D3	DHF stadium I	0.191
D4	DHF stadium I	0.979
D5	DHF stadium I	0.228
D6	DF	0.639
D7	DF	0.294
D8	DHF stadium I	1.284
D9	DHF stadium I	0.260
D10	DHF stadium I	0.297
D11	DHF stadium I	0.218
D12	DHF stadium II	0.856
D13	DHF stadium I	1.373
D14	DHF stadium I	0.228
D15	DF	0.268
D16	DHF stadium I	0.764

Table 2. Determination of anti-NS1 antibodies of healthy donors by solid phase ELISA**.** Sample was considered positive if the OD_{492} > 0.3.

Sample Code	Dengue Virus Infection Category	Anti-NS1 antibody (OD_{492} value)
N1.	Healthy donor	0.128
N2.	Healthy donor	0.179
N3.	Healthy donor	0.056
N4.	Healthy donor	0.079
N5.	Healthy donor	0.104

3.2 The influence of anti-NS1 antibodies on the regulation of HO-1

To investigate the mechanism of how HO-1 expression is regulated on endothelial cells, the expression of HO-1 in HUVEC that were treated with mab anti-NS1, IgG from DHF patient with positive NS1 (DHF IgG) by immunoblotting were examined, IgG from healthy donor (normal IgG) was run as control. As shown in Figure 5A, treatment HUVEC with mab anti-NS1 and DHF IgG markedly-increased HO-1 protein expression in a dose-dependent manner with a maximum of 10 µg/ml. In addition, anti-NS1 antibody-induced HO-1 protein levels in time-dependent with a maximum level of expression after 18 h (Figure 5B). These results indicate that anti-NS1 antibodies in dengue virus infected patients are capable to upregulate the anti-apoptotic HO-1 protein expression on endothelial cells.

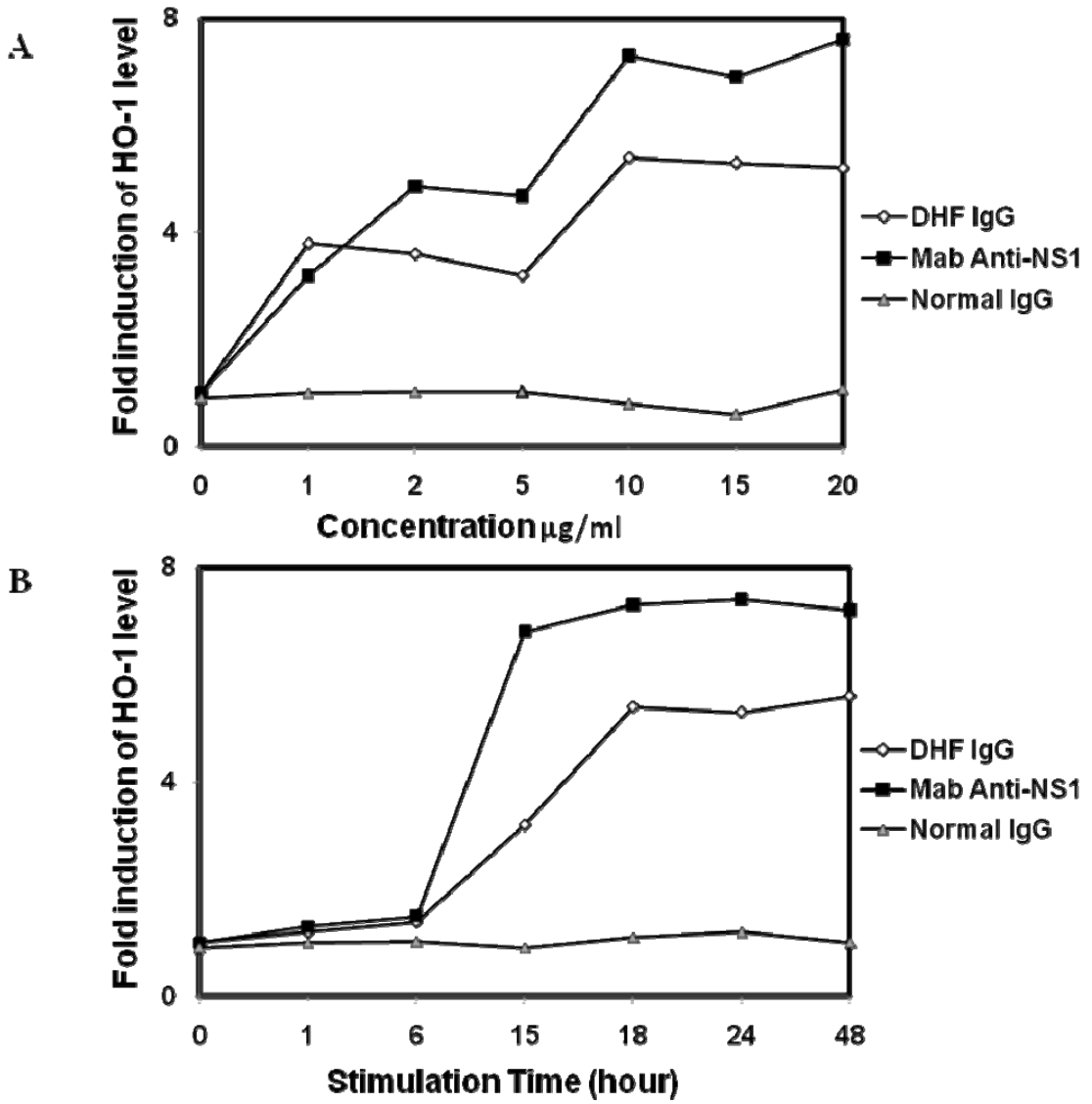

Figure 5. Influence of anti-NS1 antibodies on HO-1 upregulation in HUVEC. HUVEC were treated with mab anti-NS1, DHF IgG, and normal IgG in different antibody concentrations (A) and different stimulation times (B).

For comparison, the effect of these stimuli in endothelial was also determined on IgG from DHF patient with negative NS1 that run parallel with IgG from DHF patient with positive NS1, and mab anti-NS1. Heme and normal IgG were used as positive and negative control, respectively. No upregulation was observed when endothelial cells were treated with purified IgG from sera DHF patients without anti-NS1 antibodies. In contrast, significance upregulation was detected with purified IgG containing anti-NS1 antibodies. Similar result was obtained with mab anti-NS1 (Figure 6).

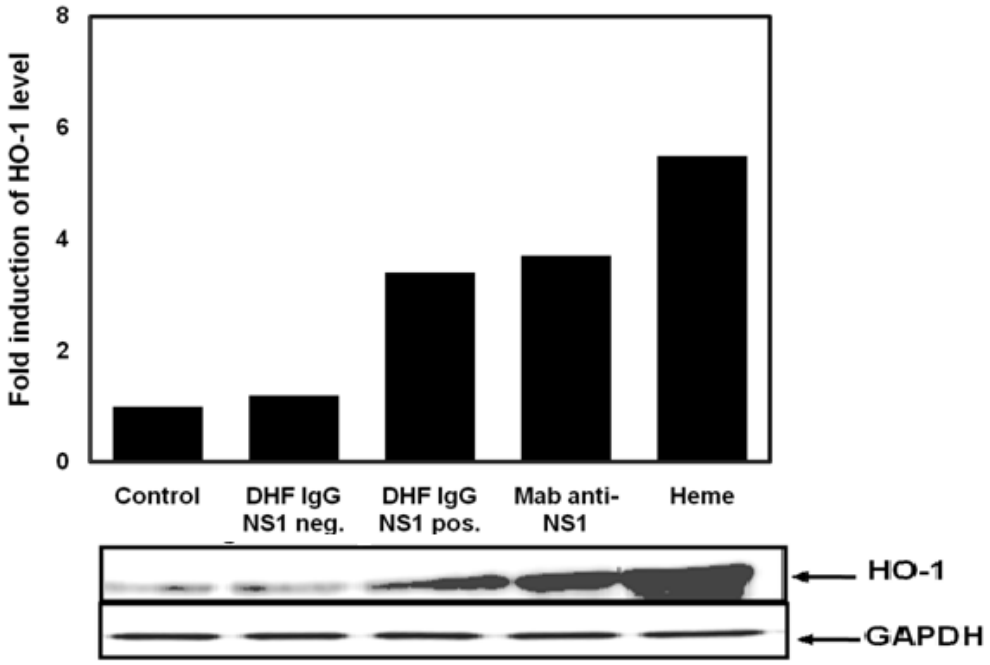

Figure 6. **Anti-NS1 antibodies induce HO-1 upregulation in HUVEC**. No upregulation was observed when cells treated with purified IgG from serum of DHF patient without anti-NS1 antibodies. In contrast, significance upregulation was detected with purified IgG containing anti-NS1 antibody. Similar result was obtained with mab anti-NS1. The relative band densities were expressed as fold-induction, normalized to GAPDH band from three independent experiments.

To verify the specificity of HO-1 upregulation in endothelial cells, human monocytic cells line U937 were treated with IgG from DHF patient with negative NS1, IgG from DHF patient with positive NS1, and mab anti-NS1. Positive and negative controls were heme and normal IgG, respectively. No upregulation of HO-1 by mab anti-NS1 or DHF IgG was observed in these cells (Figure 7).

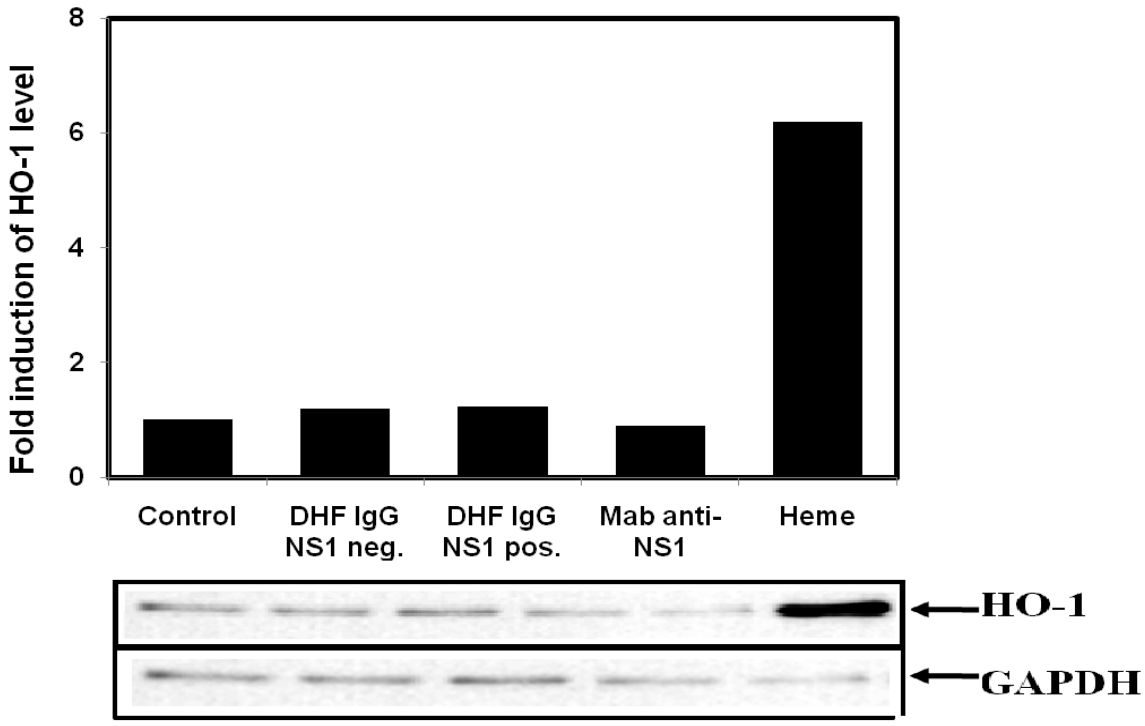

Figure 7. Anti-NS1 antibodies did not induce HO-1 upregulation in U937 cells. There was no upregulation of HO1 when U937 cells were treated with purified IgG from serum of healthy donor, DHF IgG NS1 negative, DHF IgG NS1 positive, mab anti-NS1 antibodies. The relative band densities were expressed as fold-induction, normalized to GAPDH band from three independent experiments.

3.3 Inhibition of anti-NS1 antibody-mediated HO-1 induction by NS1 antigen

To investigate whether HO-1 upregulation induced by anti-NS1 antibodies alone or by antigen-antibody complex, NS1 antigen was used as an inhibitor. Upregulation of HO-1 was detected when endothelial cells were stimulated with both NS1 antigen and mab anti-NS1. In contrast, NS1 antigen blocked the anti-NS1 antibodies binding on endothelial cells and abolished the upregulation of HO-1 activity, no upregulation of HO-1 was detected when endothelial cells were stimulated with both NS1 antigen and anti-NS1 antibody, simultaneously (Figure 8).

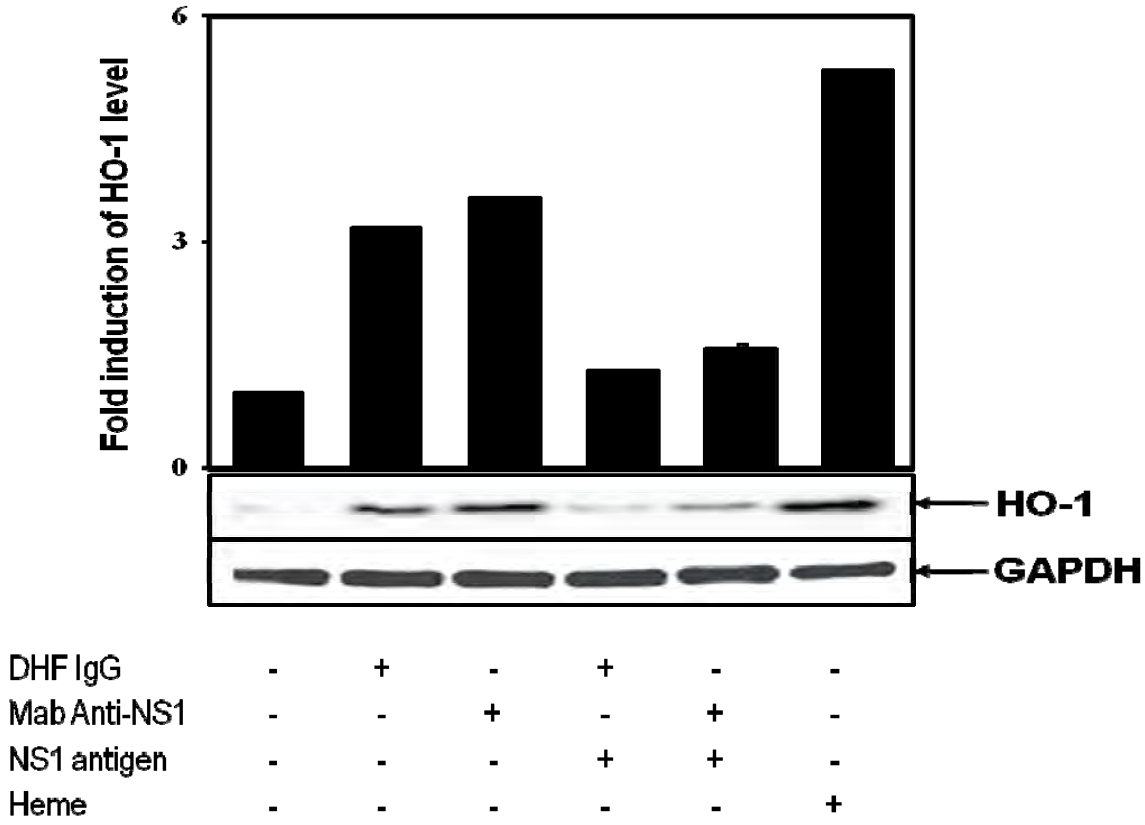

Figure 8. Inhibition of anti-NS1 antibody-mediated HO-1 induction by NS1 antigen. HUVEC were treated with DHF IgG (10 µg/ml) and mab anti-NS1 (10 µg/ml) in the absence or presence of purified NS1 antigen (10 µg/ml) for 18 h. After cell lysed, HO-1 expression was analyzed as described in figure above. The relative band densities were expressed as fold-induction, normalized to GAPDH band from three independent experiments.

3.4 Anti-NS1 antibodies activate HO-1 via pI3K pathway

The pI3K signaling pathway has recently been demonstrated to be involved in the induction of HO-1 gene expression (Martin et al., 2004). To evaluate the regulatory role of this pathway for the anti-NS1 antibody-dependent induction of HO-1 expression various pharmacological inhibitors were tested. Upregulation of HO-1 expression by anti-NS1 antibodies was markedly reduced by pretreatment with the pI3K inhibitors, LY294002, and wortmannin (Figure 9).

By contrast, pretreatment with the p38 inhibitor SB203580 did not affect anti-NS1 antibody-dependent induction of HO-1. These data suggest that the pI3K signaling pathway plays a major regulatory role for the induction of HO-1 by anti-NS1 antibody. HO-1 induction is a crucial mechanism of resistance against oxidative stress, and understanding the signaling pathways involved in HO-1 induction will help develop new strategies for the prevention and treatment of diseases associated with oxidative stress. The data suggest that the pI3K signaling pathway plays a major regulatory role for the induction of HO-1 by anti-NS1.

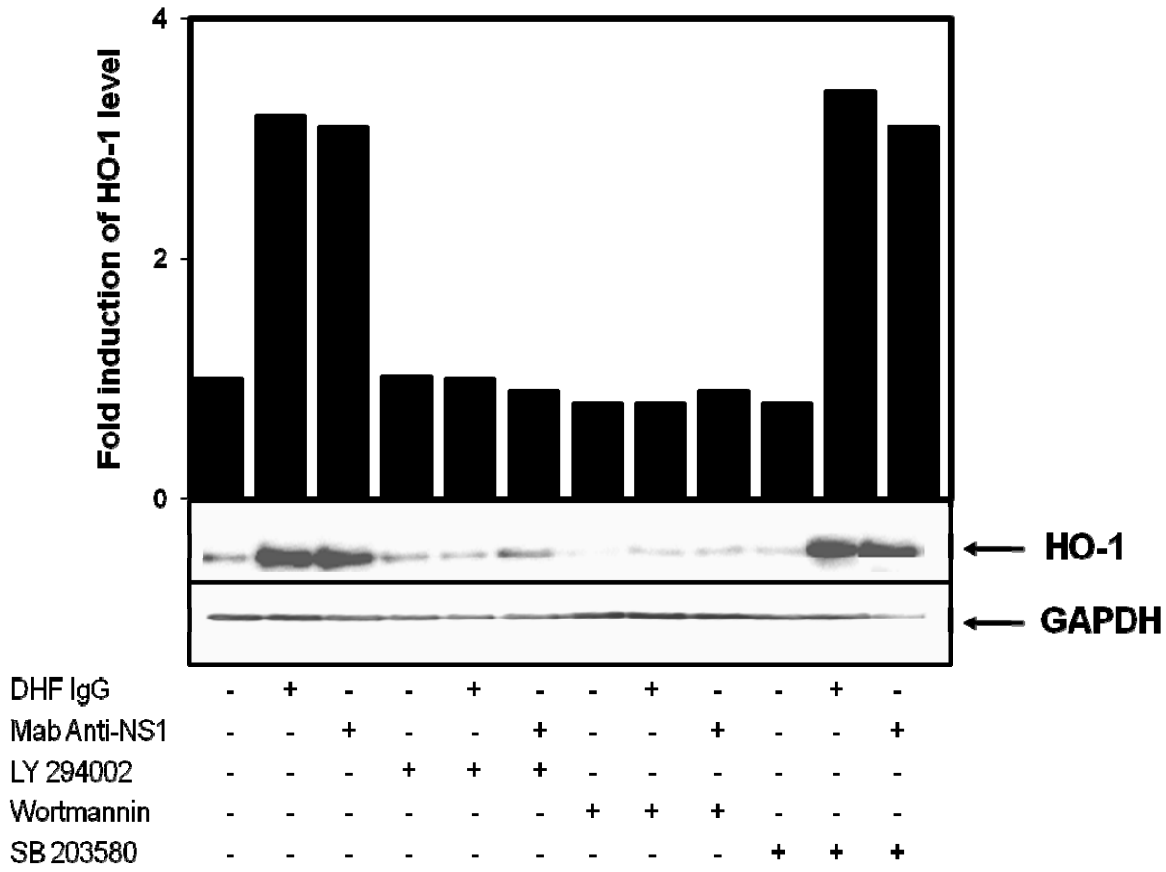

Figure 9. Anti-NS1 antibodies activate HO-1 on endothelial cells via pI3K pathway. HUVEC were incubated with different pI3K inhibitors LY294002 (10 µM/ml), wortmannin (10 µM/ml) and p38 inhibitor SB203580 (10 µM/ml) for 30 min prior to incubation with mab anti-NS1 and DHF IgG for 18 h. Cells were lysed and HO-1 expression was analyzed by immunoblotting as described. The relative band densities were expressed as fold-induction, normalized to GAPDH band from three independent experiments.

3.5 Anti-NS1 antibodies increase accumulation of cellular ROS

In order to investigate the signaling pathway on anti-NS1 antibody-induced apoptosis, ROS production was monitored in HUVEC. The histograms and the percentages of ROS production are shown in Figure 10. Treatment with anti-NS1 antibodies and DHF IgG caused a prominent increased of ROS expression as demonstrated by both the percentages of positive cells and the mean fluorescence intensity from flow cytometry. In the control experiment, no positive staining was observed with normal IgG. Pretreatment HUVEC with NAC decreased ROS expression in these cells. NAC is cysteine analog commonly used to treat acetaminophen overdose (Kelly, 1998), NAC can protect against ROS through the restoration of intracellular glutathione (Juurlink and Paterson, 1998; Ratan et al., 1994).

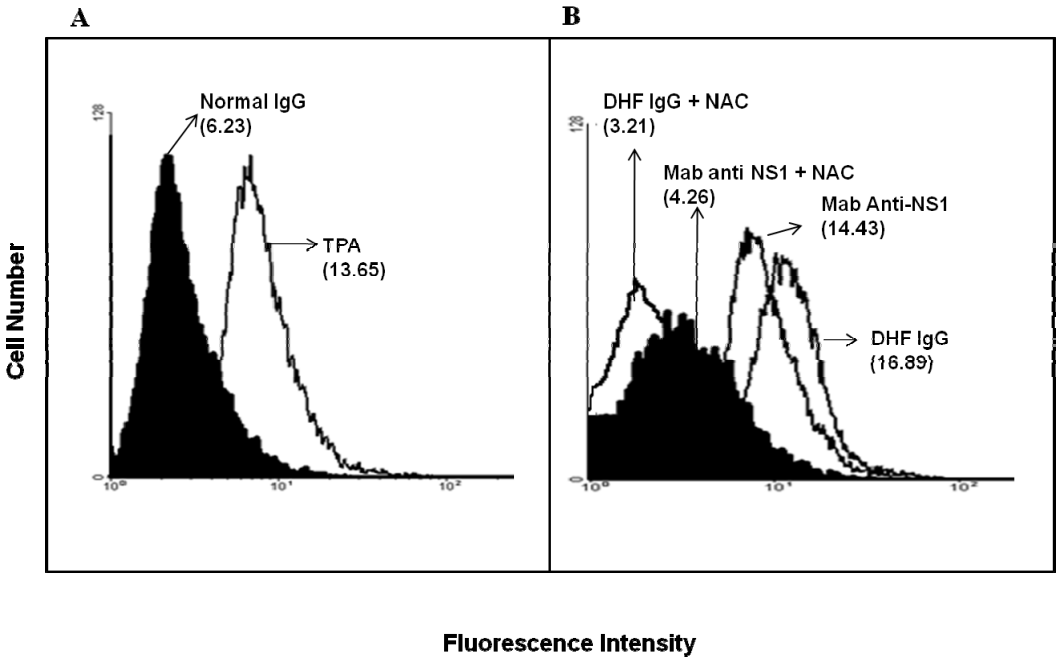

Figure 10. Anti-NS1 antibodies induce ROS production on endothelial cells. HUVEC were stimulated with DHF IgG (10 µg/ml) and mab anti-NS1 (10 µg/ml) for 18 h in the presence or absence of NAC (30 mM), TPA was run as positive control. Thereafter, membrane-permeable fluorescence dye carboxy-H_2DCFDA was added and incubated for 20 min.

To determine whether ROS as potential secondary messengers would be involved in HO-1 upregulation in HUVEC, the effect of antioxidant NAC on anti-NS1 antibodies induction of HO-1 was examined. Pretreatment with NAC decreased anti-NS1 antibody-dependent HO-1 upregulation in a dose-dependent manner (Figure 11). This result suggests the involvement of ROS on the induction of HO-1 by anti-NS1 antibodies.

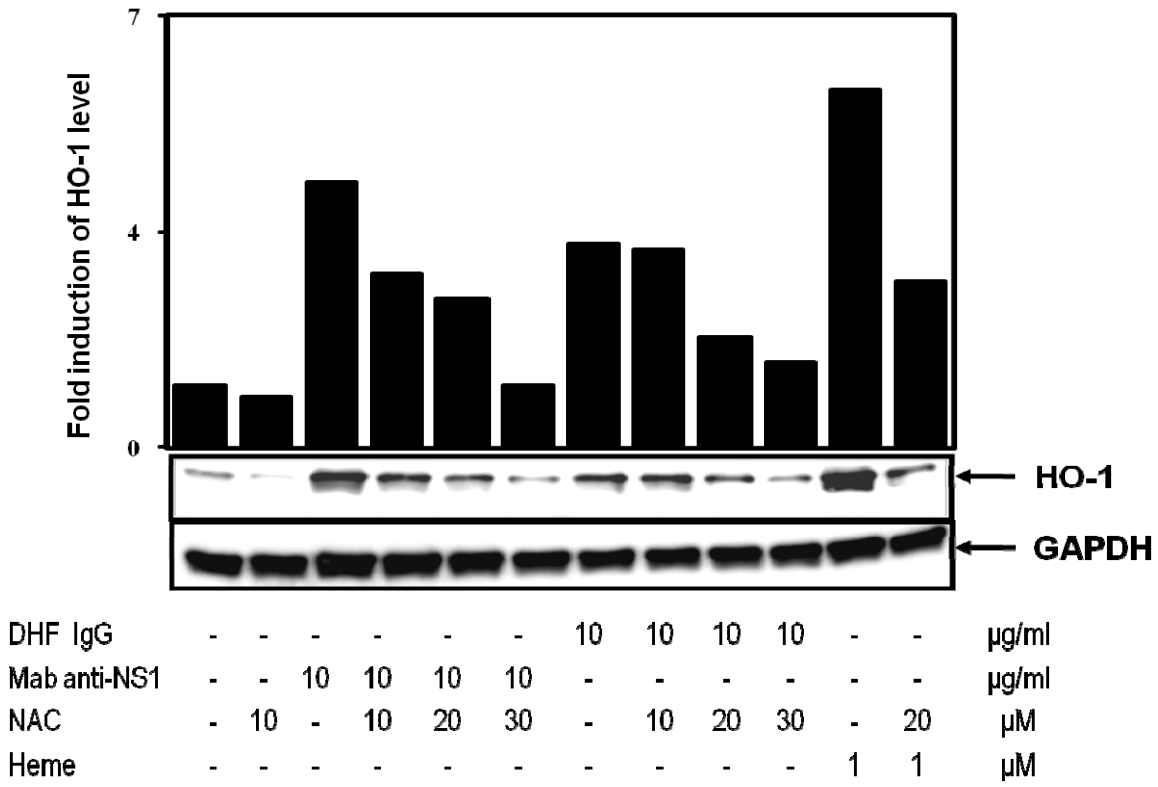

Figure 11. Effect of NAC on HO-1 upregulation induced by anti-NS1 antibodies. HUVEC were treated with NAC at concentrations of 10, 20, and 30 mM for 30 min prior to incubation with mab anti-NS1 and DHF IgG. Cells were lysed and analyzed by immunoblotting as described. The relative band densities were expressed as fold-induction, normalized to GAPDH band from three independent experiments.

3.6 Anti-NS1 antibodies induce apoptosis of endothelial cells

The ability of anti-NS1 antibodies to induced endothelial cells apoptosis should be tested. HUVEC were treated with mab anti-NS1, DHF IgG. Mab anti-CD177 and normal IgG were run as controls. Cells apoptosis was measured using flow cytometry, the histograms and the percentages of apoptotic cells are shown in Figure 12. Cells apoptosis was inducible by mab anti-NS1 and DHF IgG. In the control, normal IgG and mab anti-CD177 did not induce cell apoptosis.

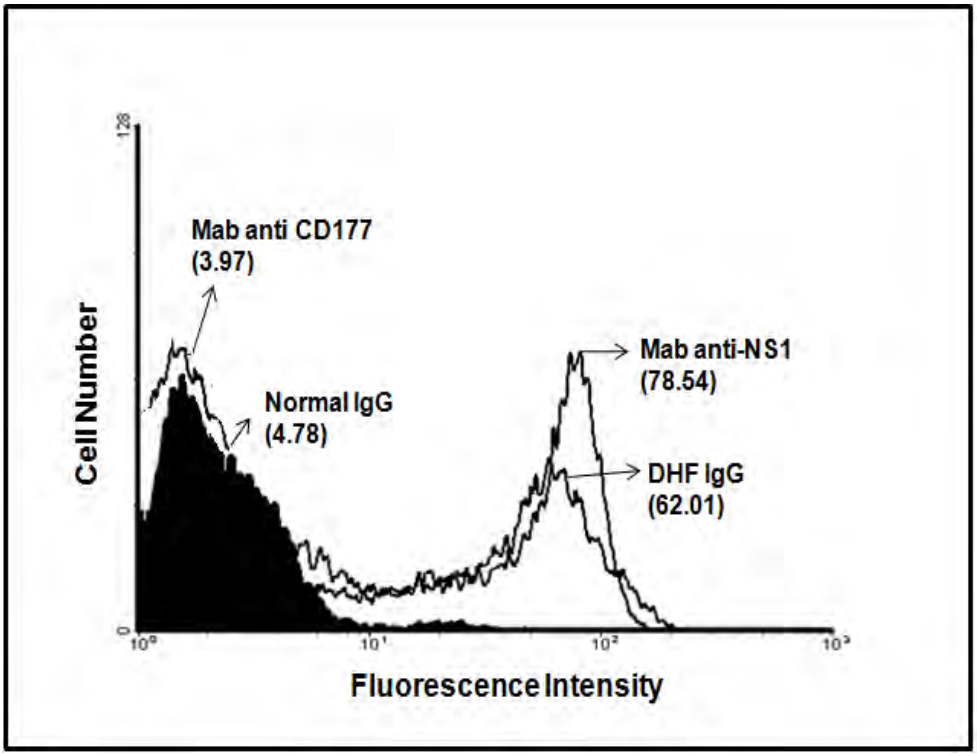

Figure 12. Anti-NS1 antibodies induce apoptosis of endothelial cells. HUVEC were treated with mab anti-NS1 (2 µg/ml), and DHF IgG (2 µg/ml). As negative controls, mab anti-CD177 (5 µg/ml) and normal IgG (5 µg/ml) were used. After incubation for 18 h cells were analyzed by flow cytometry.

3.7 Anti-NS1 antibodies binding onto endothelial cells

To characterize the binding site of anti-NS1 antibodies, flow cytometry analysis with resting and activated endothelial cells was performed. The histograms and the percentages of binding cells are shown in Figure 13. DHF IgG reacted with primary HUVEC as well as with endothelial cell line EaHy. These reactions increased after stimulating these cells with TNF-α.

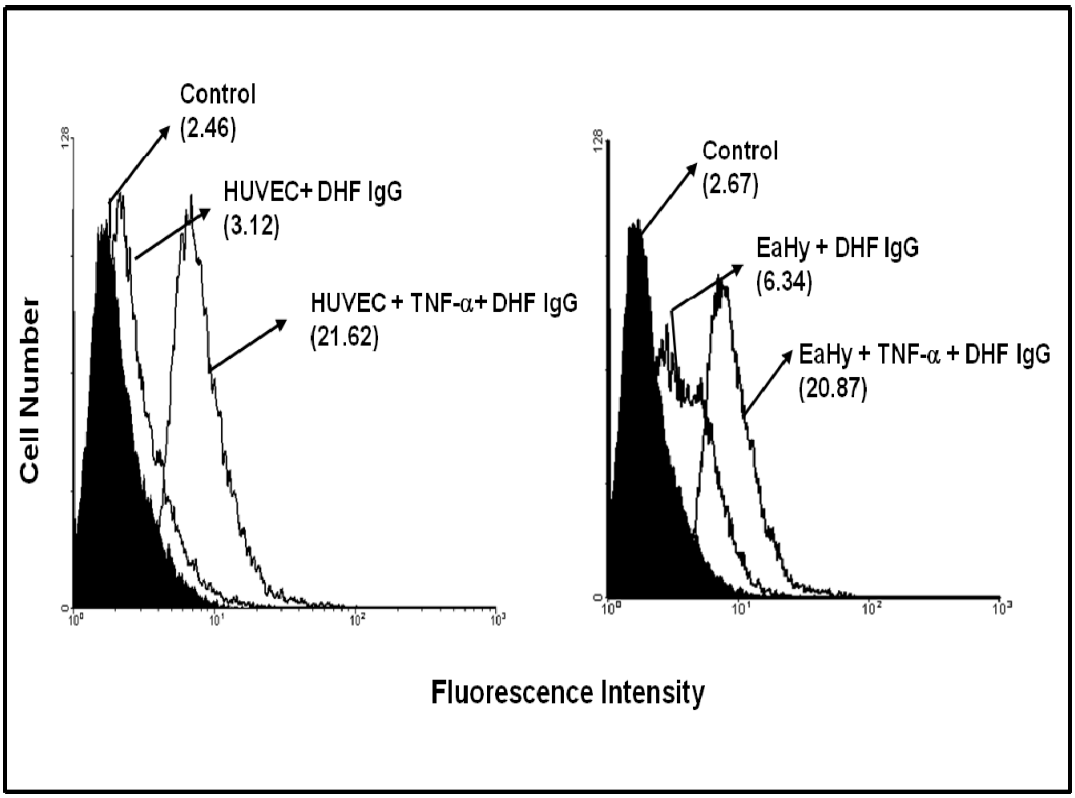

Figure 13. Flow cytometry analysis of anti-NS1 antibodies binding onto endothelial cells. HUVEC and Eahy cells were treated with DHF IgG (10 µg/ml) and mab anti-NS1 (10 µg/ml) before and after stimulation with TNFα (2 µg/ml). After washing bound IgG was detected using fluorescence labeled secondary antibody by flow cytometry. Isotype control was run in parallel.

3.8 Anti-NS1 antibodies of DHF patients react with PDI antigen on endothelial cells

To investigate the binding of anti-NS1 antibodies to protein disulfide isomerase (PDI) on endothelial cells, immunoprecipitation of biotinylated Eahy cells with mab anti-NS1, anti-PDI, and DHF IgG was performed. Anti-NS1 antibodies bound to membrane protein at molecular weights 62-72 kDa, corresponding to the molecular weight of PDI (Figure 14). Similar band was also found by immunoprecipitation with anti-PDI. To confirm the identity of PDI preclearing experiments were performed.

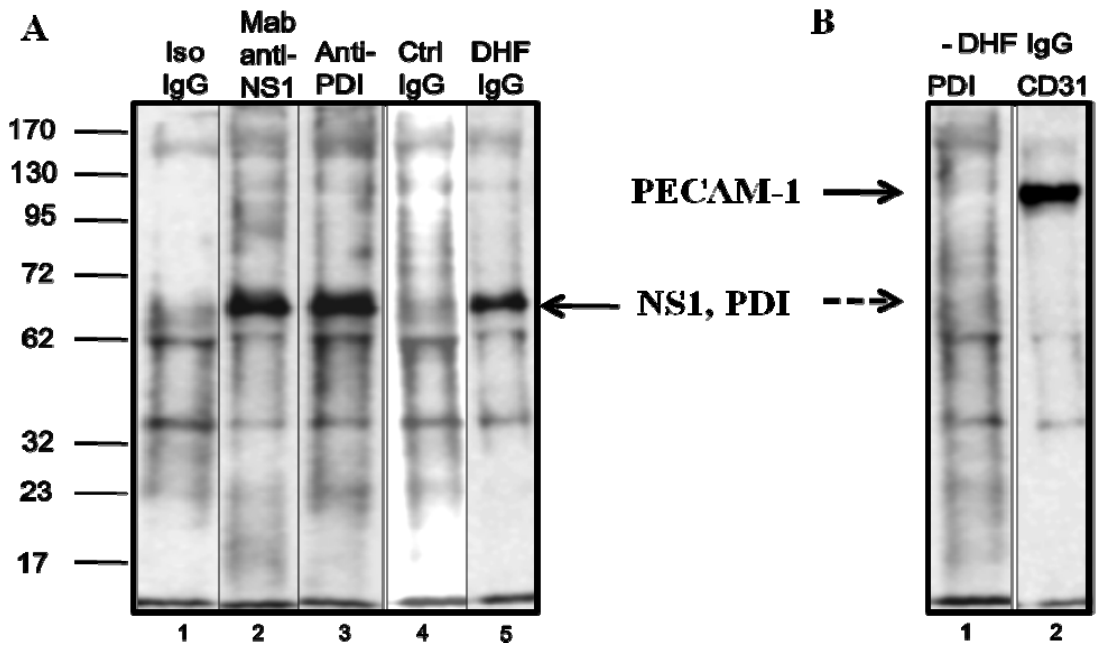

Figure 14. Immunoprecipitation analysis of anti-NS1 antibodies with endothelial cells. A) Eahy cells were labelled with biotin, lysed and precipitated with mab anti-NS1 (5 µg/ml), anti-PDI (5 µg/ml), normal IgG (5 µg/ml), and DHF IgG (5 µg/ml). Immunoprecipitates were separated on 7.5% SDS-PGE under reducing conditions. After blotting, antigens recognized by antibodies were visualized by streptavidin chemiluminescence system. B) Biotin labelled Eahy cell lysates were precipitated extensively (three times) with DHF IgG (5 µg/ml). Precleared cell lysates were then precipitated with anti-PDI or anti-CD31 as control.

After preclearing with IgG from DHF patients, cells lysates were precipitated with anti-PDI or mab anti-CD31. Whereas specific band for CD31 was detected, no PD1 protein could be precipitated by anti-PDI. This result demonstrates that antibody in DHF patients react with PDI on endothelial cells.

3.9 Inhibition of PDI abolishes HO-1 upregulation mediated by anti-NS1 antibodies

To further investigate the binding mechanism between anti-NS1 antibodies and PDI, bacitracin was applied as PDI inhibitor (Swiatkowska et al., 2000). Pretreatment of HUVEC cells with bacitracin and RL-90 caused inhibition of anti-NS1 antibody-induced HO-1 upregulation on protein level as shown in Figures 15 and 16, respectively.

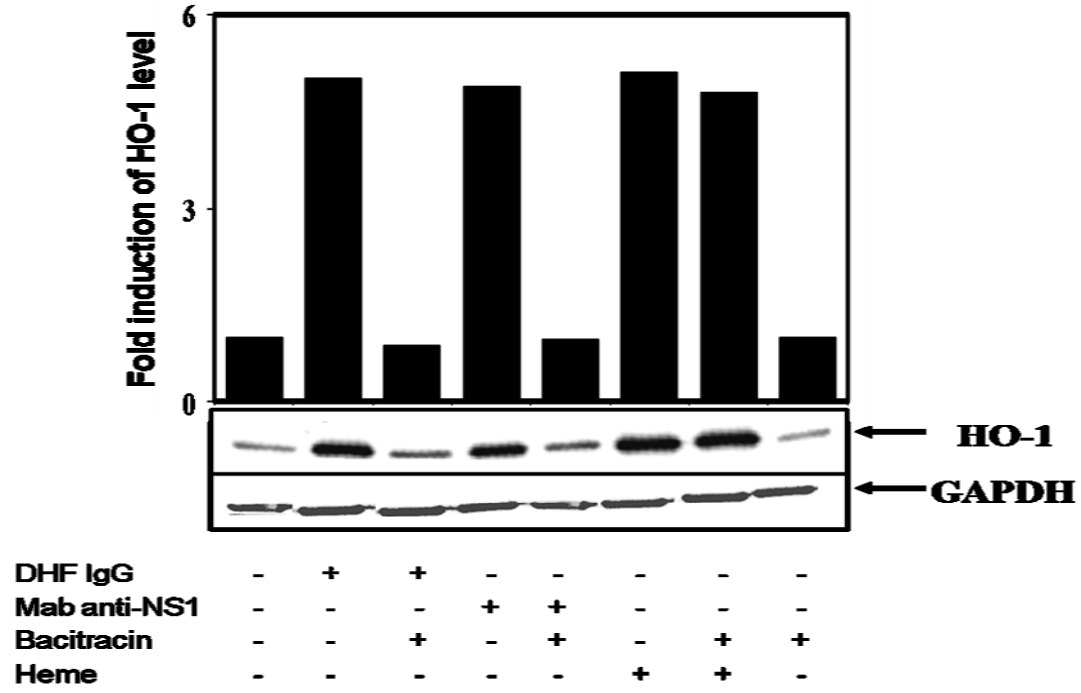

Figure 15. Inhibition of PDI with bacitracin abolishes HO-1 upregulation mediated by anti-NS1 antibodies. HUVEC were incubated for 30 min in the absence or presence of 4 µl bacitracin (2 mM). After washing cells were treated with mab anti-NS1 (10 µg/ml), DHF IgG (10 µg/ml) or heme (1 µM) as control for 18 h. Cells were lysed and HO-1 expression was analyzed by immunoblotting as described. The relative band densities were expressed as fold-induction, normalized to GAPDH band from three independent experiments.

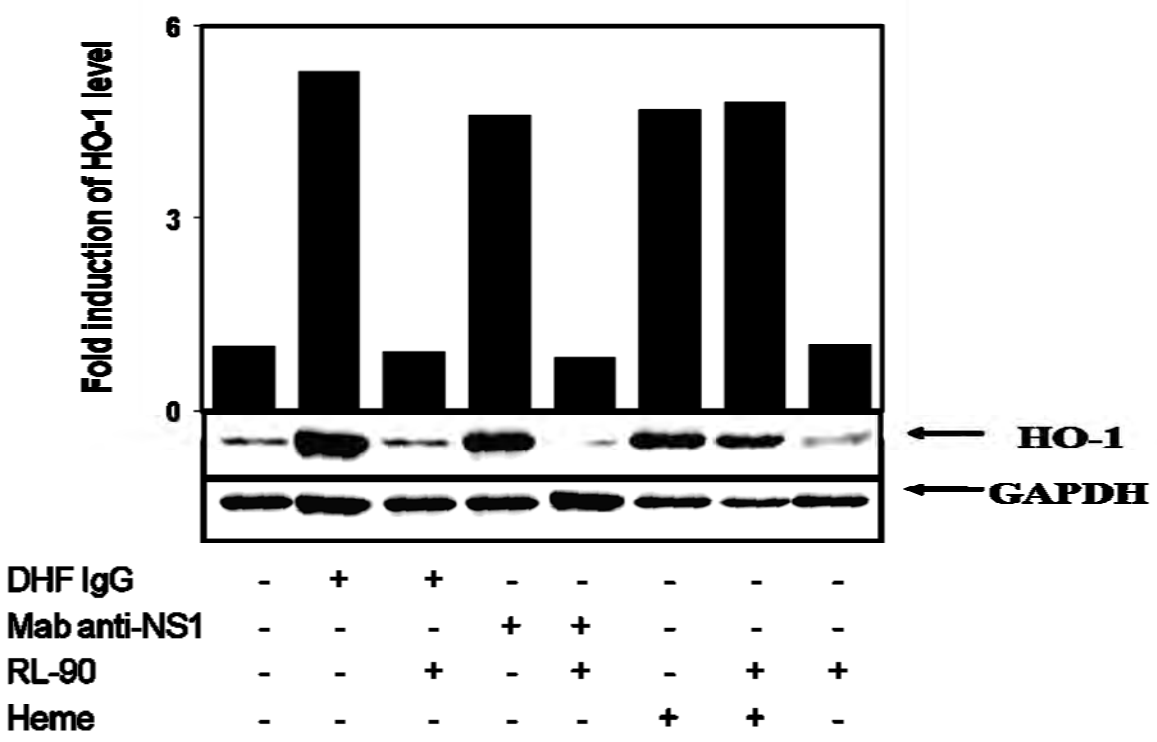

Figure 16. Inhibition of PDI with RL-90 abolishes HO-1 upregulation mediated by anti-NS1 antibodies. HUVEC were incubated for 30 min in the absence or presence of 10 µg/ml mab RL-90 against PDI. After washing cells were treated with mab anti-NS1 (10 µg/ml), DHF IgG (10 µg/ml) and heme (1 µM) as control for 18 h. Cells were lysed and HO-1 expression was analyzed by immunoblotting as described. The relative band densities were expressed as fold-induction, normalized to GAPDH band from three independent experiments.

3.10 Permeability disturbance of endothelial cells by anti-NS1 antibodies

To investigate whether anti-NS1 antibodies increasing endothelial permeability, labelled markers (albumin-FITC) through tightly confluent HUVEC monolayers were measured. Stimulation of HUVEC with mab anti-NS1 or IgG from DHF patients IgG increased transendothelial migration of albumin FITC in comparison to HUVEC treated with isotype control 7D8 (mab anti-CD177) or normal human IgG (Figure 16).

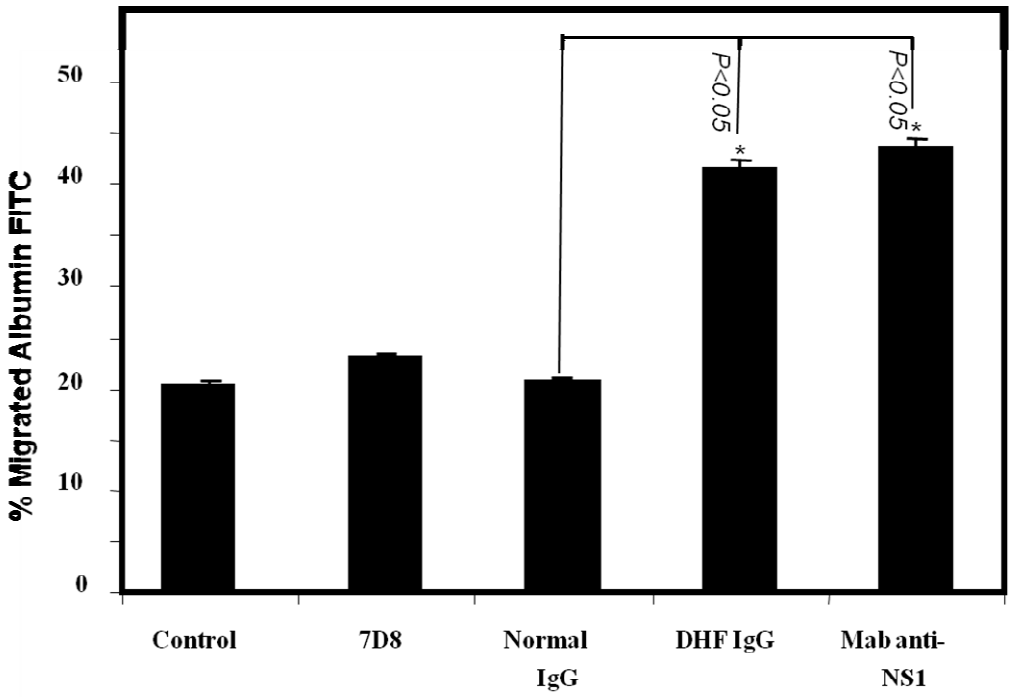

Figure 17. Analysis of endothelial permeability with anti-NS1 antibodies. HUVEC were grown for 2 days on collagen-coated Transwell filters to confluence, then incubated with PBS buffer (control), isotype control (mab 7D8; 10 µg/ml), normal IgG (10 µg/ml), DHF IgG (10 µg/ml) and mab anti-NS1 (10 µg/ml) for 18 h. Fluorescence labeled albumin (Albumin-FITC; 40 ng/ml) were then added in to the upper chamber. Transwell were measured by fluorescence reader and expressed as percentage of the total albumin-FITC. Data represent means ± S.E from at least three independent experiments. Student's *t* tests: **P*<0.05 vs normal IgG.

CHAPTER 4
DISCUSSION

4.1 Anti-NS1 antibodies cause accumulation of cellular ROS, apoptosis, and permeability disturbance on endothelial cells

Dengue haemorrhagic fever is the main cause of mortality in dengue virus infection (Valdes et al., 2000). Haemorrhagic syndrome, a feature of DHF is a hematologic abnormality resulting from multiple factors, including thrombocytopenia, coagulopathy and vasculopathy related with destruction/dysfunction of platelet and endothelial cells (Rothman et al., 1999). Although the exact pathomechanism is not very well defined, available data strongly suggest that in the most cases of DHF immune mediated mechanism play also an important role in the destruction of platelets and disturbance of endothelial function (Lin et al., 2006; Lei et al., 2008).

It is well known, that antibodies against DENV can augment secondary DENV infection through the phenomenon called antibody-dependent enhancement (ADE) (Morens et al., 1994; Anderson et al., 1997). At certain concentration, sub-neutralizing antibodies against DENV form antigen/antibody complexes, which are recognized by monocytes via Fc receptors (Mady et al., 1991), leading to enhanced virus uptake, resulting in an increased number of virus infected cells (Littaua et al., 1990, Lei et al., 2001). These antibodies are IgG subclass and recognized DENV structural proteins such as E and prM peptides (Henchal, et al, 1985).

Several evidences indicated a mechanism of molecular mimicry in which antibodies against non-structural protein NS1 of DENV (anti-NS1 antibodies) can also cross react with platelet and endothelial cells, and thereby may induce platelet destruction and endothelial disturbance in DHF patients (Falconar et al., 1997, Lin et al., 2004). Interestingly, Lin et al (2004) showed a strong cross-reaction between sera from DHF/DSS with endothelial cells, but not with sera

from DF patients. In line with these observations, we found by the use of solid phase ELISA that anti-NS1 antibodies derived during acute phase of DHF reacted strongly with NS1 antigen as comparison to sera from DF patients.

Recent study demonstrated that anti-NS1 antibodies recognize an immunodominant RGD- and ELK/KLE motifs of NS1 molecule, which is present on human clotting factors (fibrinogen, factor VII, IX, X) as well as on cell adhesion molecules, particularly integrin such as αIIbβ3, αvβ3 (Chang et al., 2002; Falconar, 2007). However, direct binding of anti-NS1 antibodies to these adhesive molecules have not been well documented (Wiwanitkit, 2006).

Recently Cheng et al. (2008) found that anti-NS1 antibodies react with several proteins on Human microvascular endothelial cells (HMEC-1) endothelial cell line including ATP synthase beta chain, PDI, vimentin, and heat shock protein 60. To identify the target antigen on endothelial cells recognized by anti-NS1 antibodies, we performed immunoprecipitation with surface labelled HUVEC, and found that anti-NS1 antibodies from DHF patients reacted with membrane protein of 62-72 kDa corresponding to the apparent molecular weight of PDI. By the use of pre-clearing experiment approach we could definitely identified PDI as the target antigen of anti-NS1 antibodies. This is in accordance with the recent study reported by Cheng et al. (2009). The authors demonstrated that anti-NS1 antibodies recognized PDI on platelet surface causing inhibition of platelet aggregation induced by ADP. Further analysis showed that anti-NS1 antibodies bound to amino acid residues 311-330 of DENV NS1, which shares sequence homology with the thioredoxin domain of PDI.

Interestingly, PDI has been shown to play a role on the regulation of integrin activation (Essex et al., 2006). Swiatkowska et al. (2008) showed that modulation of the thiol isomerase activity of PDI by divalent manganese cation leads to PDI/αvβ3 integrin complex formation resulting in integrin-transition; from resting to the ligand-competent state. This mechanism may explain the

phenomenon of integrin co-precipitation by anti-NS1 antibodies under certain *in vitro* experimental conditions.

After the identification of PDI as target antigen of anti-NS1 antibodies, there was any question about the functional consequence of this antibody binding for endothelial cell function(s). The results above were found that incubation of HUVEC with purified anti-NS1 from DHF IgG resulted in significance increased production of cellular ROS which could be specifically inhibited by the anti-oxidant drug, NAC.

It is possible that also endothelial cells contribute to ROS production during a dengue infection (Gil et al., 2004). It is well known that ROS can initiate and regulate the transcription and activation of large series of mediators in cells which culminate in common mechanism of cell damage including apoptosis and necrosis (Gil et al., 2004). ROS attack polyunsaturated fatty acid and initiative lipid per-oxidation which can ultimately lead to a loss or alteration of cell membrane function (Rothman and Ennis, 1999; Kurane and Takasaki, 2001). In fatal cases of DHF and DSS, cell apoptosis process of endothelial cells from lung and intestine tissue was observed (Limonta et al., 2007).

In accordance to the previous observations described by Lin and co-workers (Lin et al., 2003), in this study was found that anti-NS1 antibodies can induce endothelial cells to undergo apoptosis. These findings suggest that ROS-modulated endothelial cells apoptosis may disturb endothelial barrier and contribute thereby to the pathogenesis of vascular leakage in DHF patients. Indeed, we observed that treatment of endothelial cells with anti-NS1 antibodies caused increased penetration of fluorescence labelled albumin indicating leakage of barrier function of these cells which may result in spontaneous haemorrhage and plasma loss from the blood vessels. However, it has been suggested that the increased vascular permeability observed in DHF is caused by a malfunction rather than a structural destruction of endothelial cells (Rothman and Ennis, 1999; Kurane and Takasaki, 2001).

Generation of ROS has been detected when endothelial cells were stimulated by cytokines (Matsubara et al., 1986), a process which commonly occurs during dengue infection (Anderson et al., 1997). The cytokine secretion of dengue infected cells may result in activation of non-infected endothelial cells (Anderson et al., 1997; Halstead, 2007; Basu and Chaturvedi, 2008). High levels of TNF-α, IL-6 and IL-8 were measured in sera of patients with DHF/DSS (Hober et al., 1993; Avirutnan et al., 1998; Raghupathy et al., 1998). This study showed that treatment of endothelial cells with TNFα increased the expression of PDI on the cell surface. The up-regulation of PDI surface expression could facilitate the binding of anti-NS1 antibodies to endothelial cells, and in turns accelerate ROS production; a process which may decline the fate of DHF spectacularly.

In line with this observation was found that inhibition of ROS production with the antioxidant NAC reduced basal HO-1 expression in these cells. NAC is cysteine analog commonly used to treat acetaminophen overdose (Kelly, 1998), NAC can protect against reactive oxygen species through the restoration of intracellular glutathione (Ratan et al., 1994; Juurlink and Paterson, 1998).

4.2 Anti-NS1 antibodies regulate the anti-apoptotic HO-1 on endothelial cells via activation of pI3K

On the other hand, several reports indicate that HO-1 has a cytoprotective role by its ability to break down the pro-oxidant heme to the powerful anti-oxidants products biliverdin and bilirubin (Yi and Hazel, 2005). This effect has been demonstrated under both *in vitro* (Vile and Tyrell, 1994; Abraham et al., 1995) and *in vivo* conditions (Nath et al., 1992; Otterbein et al., 1995).

HO-1, an inducible heme-degrading enzyme, exerts a potent anti-inflammatory effect through the production of carbon monoxide and bilirubin. Expression of HO-1 is up-regulated by multiple stress stimuli and the enzymatic products of this reaction has not only antioxidant cytoprotective, but also anti-inflammatory functions (Kyriakis et al., 2001; Orozco et al., 2007; Pamplona et al., 2007; Chora et al., 2007; Chung et al., 2008).

Major functions of HO-1 comprise the degradation of the pro-oxidant heme and the production of bilirubin, which provide protection of tissue and organs against oxidative stress (Abraham et al., 1988; Maines et al., 1997). More recently, HO-1 turns to be an important modulator of the inflammatory response possibly via the generation of second messenger gas CO (Otterbein et al., 2002; 2003). Accumulation data indicate that modulation of HO-1 may not only serve as therapeutic target for heme-induced inflammation diseases (Willis et al., 1996; Wagener et al., 2001), but also has therapeutic implications in organ transplantation. Several studies demonstrated that the induction of HO-1 activity prevents the development of vascular lesions, intra-graft apoptosis, and significantly prolongs allograft survival (Soares et al., 1998; Hancock et al., 1998; Immenschuh and Ramadori et al., 2000).

Recently Iwasaki et al. (2010) demonstrated that the ligation of HLA class I antigen on endothelial cells by low concentration of HLA class I antibodies protects endothelial cell against complement destruction by induction of HO-1 gene in a PI3K/Akt dependent manner.

The present study found that purified anti-NS1 antibodies from DHF patients caused specific induction of HO-1 in endothelial cells which can be inhibited by soluble recombinant NS1 antigen underlying the importance of this mechanism in endothelial cells. In addition, we could demonstrate that pI3K signaling pathway is also involved in the HO-1 regulation mediated by anti-NS1 antibody. Treatment of HUVEC cells with specific pI3K inhibitors (LY294002 and Wortmanin) blocked significantly anti-NS1 antibodies-mediated HO-1 expression. In addition, stimulation of endothelial cells with anti-NS1 antibodies caused an elevation of Akt-phosphorylation, which was specifically inhibited by pI3K inhibitors.

It is known that HO-1 is inducible during the oxidative stress caused for example by different substances which are able to modify intracellular glutathione levels (Applegate and Philip, 1991). In line with this observation, this study found that inhibition of ROS production with the antioxidant NAC resulted in dose-dependent reduction of basal HO-1 expression.

NAC is a cysteine analog commonly used to treat acetaminophen overdose (Kelly, 1998). Studies in the past documented that NAC protects cells against ROS through the restoration of intracellular glutathione (Ratan et al., 1994; Juurlink & Paterson, 1998). Treatment with NAC results in a rapid recovery of reduced glutathione level in brain and reduction of oxidative stress following in traumatic brain injury (Juurlink and Paterson, 1998; Xiong et al., 1999).

In this study it was found that Anti-NS1 antibodies increased the generation of intracellular ROS and up-regulated the expression of HO-1, and suppressed by the pretreatment with NAC. It is likely that NAC acts as a scavenger for some specific anti-NS1 antibody-induced oxidative reaction that triggers HO-1 induction.

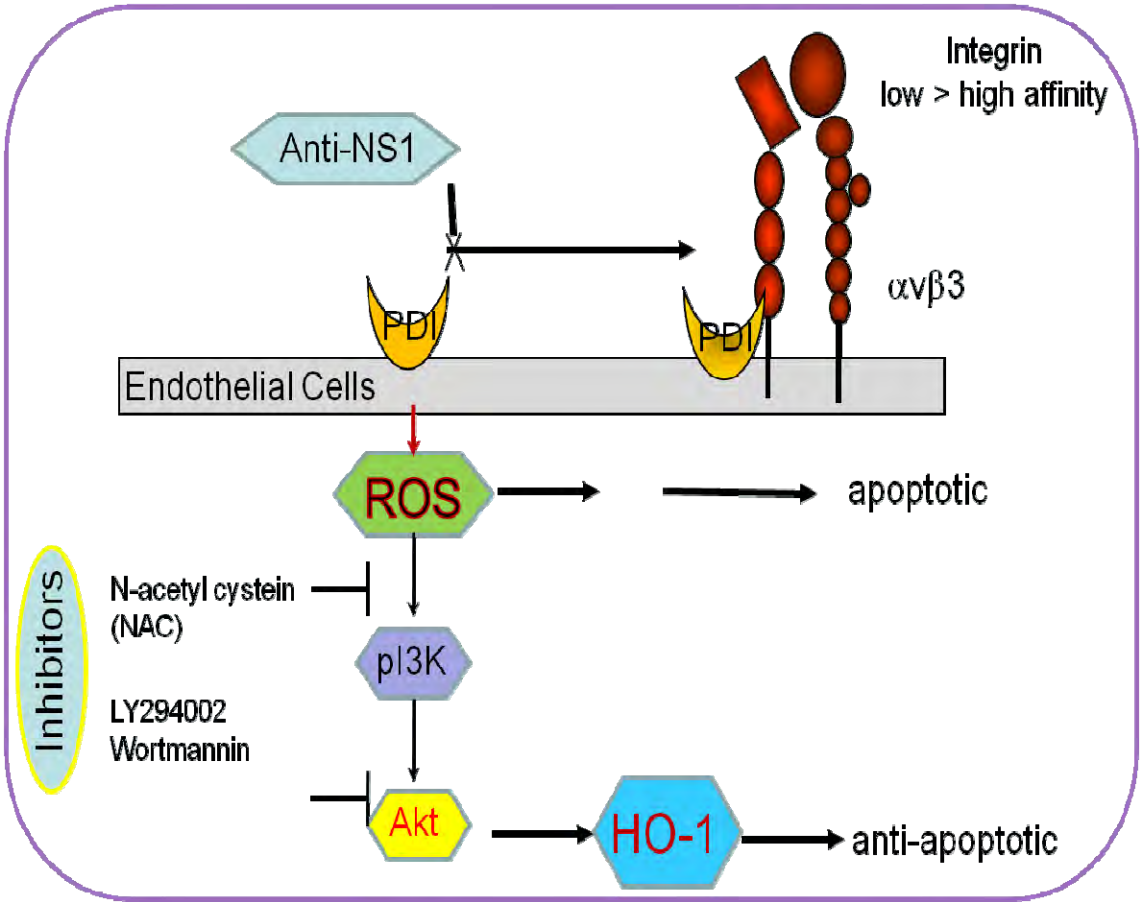

Figure 18. Possible mechanism of endothelial leakage induced by anti-NS1 antibodies. In one site, binding of anti-NS1 antibodies to endothelial cell surface via PDI causes ROS production leading to apoptosis and integrin activation ($\alpha v\beta 3$) which may result in disturbance of cellular haemostasis. Another side, this interaction induces consequently down stream signalling via pI3K signalling pathway which resulted in the upregulation of the anti-apoptotic HO-1. Thus, the balance between ROS secretion and the production of HO-1 (anti-apoptotic) induced by anti-NS1 antibodies is important for the regulation of vascular leakage and for the prevention of irreversible endothelial damage in DHF patients.

In overall, this information provides new insight into the molecular mechanism of antibody mediated endothelial disturbance in DHF. This knowledge may help us to define a new therapeutic strategy for the patients with DHF.

CHAPTER 6
SUMMARY

Dengue haemorrhagic fever (DHF) is characterized by thrombocytopenia, increased vascular permeability and haemorrhage. The antibody against NS1 of the dengue virus seems to play a role in the pathogenesis of dengue virus disease due to its cross-reaction with endothelial cells. Recently, it has been demonstrated that anti-NS1 antibodies induced endothelial cells to undergo cell apoptosis. However, the exact mechanism underlying this antibody-mediated cell apoptosis is not well-known.

In this study, the influence of anti-NS1 antibodies of DHF patients on the regulation of HO-1 in HUVEC was investigated. Sera derived from DHF patients with or without anti-NS1 antibodies were analyzed. Incubation of HUVEC with purified anti-NS1 antibodies from dengue virus infected patients caused HO-1 upregulation in a time and dose manner which was attenuated by the NS1 antigen. This upregulation was not observed with IgG from patients without NS1 antibodies. Furthermore, reduction of HO-1 was observed, when HUVEC were pretreated with pI3K pathway inhibitors (LY294002 and wortmannin) prior to stimulation with anti-NS1 antibodies. In contrast, no inhibition was detectable with p38 MAPK inhibitors (SB203580). These results indicate that the pI3K signaling pathway plays a major regulatory role for the induction of HO-1 by anti-NS1.

In addition, stimulation of HUVEC with anti-NS1 antibodies induced the production of ROS, induced endothelial cell apoptosis, and increased cell permeability. These mechanisms probably contribute to the pathomechanism of vascular leakage and haemorrhage, which is often clinically observed in dengue fever patients.

Protein disulfide isomerase (PDI) was found expressed on the endothelial surface as a target antigen for anti-NS1 antibodies by the use of immunoprecipitation approaches and flow cytometry analysis.

Taken together, this study indicates that anti-NS1 antibodies bind to endothelial cells via PDI as a surface target protein. This interaction leads to production of ROS which can induce cell apoptosis and disturbance of endothelial cell permeability. On the other hand, ROS initiates the induction of the anti-apoptotic gene HO-1 via the pI3K signalling pathway. Thus, the balance between ROS (apoptotic) and HO-1 (anti-apoptotic) production induced by anti-NS1 antibodies may have significance as the cause of vascular leakage in some DHF patients.

CHAPTER 6
ZUSAMMENFASSUNG

Das Dengue Hämorrhagische Fieber (DHF) geht mit Thrombozytopenie, erhöhter Gefäßpermeabilität und Blutungen einher. Der Antikörper gegen das Nicht-Struktur-Protein NS1 des Dengue Virus scheint in der Pathogenese der Erkrankung eine Rolle aufgrund seiner Kreuzreaktion mit Endothelzellen zu spielen. Kürzlich wurde gezeigt, dass anti-NS1 in Endothelzellen Apoptose veranlasst. Der genaue Mechanismus hierfür ist jedoch nicht bekannt.

In dieser Studie untersuchten wir den Einfluss von NS1-Antikörpern von DHF-Patienten auf die Regulierung von HO-1 in HUVEC in vitro. Die Inkubation von HUVEC mit gereinigtem anti-NS1-IgG verursachte einen dosisabhängigen Anstieg von HO-1, der mit IgG von Patienten ohne NS1-Antikörper nicht beobachtet wurde. Dagegen wurde eine Abnahme von HO-1 beobachtet, wenn die PI3K-Signalkaskade-Inhibitoren LY294002 oder Wortmannin vor der Behandlung mit anti-NS1 verabreicht wurden. Im Gegensatz dazu war ein solcher Effekt nicht mit dem p38 MAPK-Inhibitor SB203580 feststellbar. Diese Ergebnisse zeigen die Beteiligung von PI3K an der Signalkaskade des anti-NS1-vermittelten HO-1-Anstiegs.

Zusätzlich veranlasste die Behandlung von HUVEC mit dem anti-NS1-Antikörper die Produktion von ROS, die Zunahme von Apoptosen und eine erhöhte Permeabilität der Endothelzellen. Diese Mechanismen tragen wahrscheinlich zum Pathomechanismus der stark erhöhten Gefäßpermeabilität bei, die klinisch bei Dengue Fieber-Patienten öfter zu beobachtenist.

Als Gegenmechanismus verursacht ROS aber über die Induktion von HO-1 via PI3K/Akt-Signalkaskade (siehe oben) auch eine anti-apoptotische Wirkung. So kann das Gleichgewicht zwischen der direkten Wirkung von ROS (Apoptoseinduktion) und anti-apoptotischer Wirkung des anti-NS1-Antikörpers

über HO-1 wichtig für die Ausprägung der Gefäßpermeabilitätserhöhung bei DHF-Patienten sein.

Weiterhin konnte in dieser Arbeit als Zielantigen der anti-NS1-Antikörper auf Endothelzellen die Protein-Disulfidisomerase (PDI) nachgewiesen werden.

CHAPTER 7
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