Virology 418 (2011) 74–84

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Induced autophagy reduces virus output in dengue infected monocytic cells

Mingkwan Panyasrivanit [a], Michael P. Greenwood [b], David Murphy [b], Ciro Isidoro [c], Prasert Auewarakul [d], Duncan R. Smith [a,*]

[a] Molecular Pathology Laboratory, Institute of Molecular Biosciences, Mahidol University, Thailand
[b] Laboratories of Integrative Neurosciences and Endocrinology (LINE), University of Bristol, Bristol, UK
[c] Laboratorio di Patologia Molecolare, Dipartimento di Scienze Mediche, Università del Piemonte Orientale 'A. Avogadro', Via Solaroli 17, 28100 Novara, Italy
[d] Department of Microbiology, Siriraj Hospital, Mahidol University, Bangkok, Thailand

ARTICLE INFO

Article history:
Received 8 June 2011
Returned to author for revision 3 July 2011
Accepted 13 July 2011
Available online 2 August 2011

Keywords:
ADE
Autophagy
Dengue
ER
Monocytic cells

ABSTRACT

While several studies have shown a role for autophagy in the replication of dengue virus (DENV), these studies have been performed in directly infected cells. However, in severe cases of DENV infection the critical cell in the disease is believed to be monocytes which are poorly infected directly, but are highly susceptible to antibody enhanced infection. This study sought to determine the involvement of autophagy in the DENV infection of monocytic cells, using U937 cells as a model system. While the induction of autophagy was seen in response to DENV-2 infection, biochemical induction of autophagy resulted in a significant decrease in virus output. Down regulation of autophagy resulted in only a very slight increase in intracellular virus levels. In monocytic cells autophagy is not a significant part of the DENV replication mechanism, and there are distinct cell type specific differences in the DENV–autophagy interaction.

Introduction

Dengue viruses (DENVs) are transmitted to humans by *Aedes* mosquitoes and can cause an acute febrile illness with retro-orbital pain, myalgia, arthralgia and hemorrhagic manifestation called dengue fever (DF). In some infections the individual may develop massive bleeding, thrombocytopenia, evidence of plasma leakage such as pleural effusion, ascites and a rise of hematocrit called dengue hemorrhagic fever (DHF). If the plasma leakage leads to hypovolemic shock the syndrome is called dengue shock syndrome (DSS) which has a high mortality rate (Gubler, 1998; Kurane, 2007). At present specific treatments or a preventive vaccine for dengue infection is not available, although appropriate supportive treatment significantly reduces the mortality rate. However, the social and economic cost from dengue infections is onerous due to the high incidence of infection around the world (Mathers et al., 2007).

DENVs are enveloped positive single-stranded RNA viruses, consisting of four heterologous serotypes termed DENV serotypes 1, 2, 3 and 4 (DENV-1 to 4). The viral genome is translated as a single polyprotein which is co- and post-translationally processed into 3 structural (envelope (E), pre-membrane (prM) and capsid (C)) proteins and 7 non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins (Henchal and Putnak, 1990; Perera and Kuhn, 2008). The virus enters the host cells by receptor-mediated endocytosis (Krishnan et al., 2007; Suksanpaisan et al., 2009; van der Schaar et al., 2007) where low-pH conditions in the endosome induce uncoating of the viral genome followed by translation and replication of the virus (Modis et al., 2004; Mukhopadhyay et al., 2005).

The reason why some cases of DENV infection, but not all, develop severe symptoms (DHF and DSS) has been widely studied, and both virus and host factors have been proposed to contribute to the pathogenesis of severe dengue (Murgue et al., 2000; Rico-Hesse, 2007; Vaughn et al., 2000; Wang et al., 2006). While previous reports have implicated a role for specific genotypes or lineages in determining severity (Murgue et al., 2000; Vaughn et al., 2000; Wang et al., 2006), numerous studies have implicated second infections with a heterologous DENV as being a major cause of a more severe disease presentation, through the process termed antibody dependent enhancement (ADE) of infection (Halstead et al., 2010; Halstead and O'Rourke, 1977a; Halstead et al., 1980). While lifelong immunity is generated against subsequent infections with a homotypic virus, only transient protection is provided against heterotypic infections (Guzman et al., 2000; Sangkawibha et al., 1984) and it is believed that the presence of pre-existing sub-neutralizing antibodies from a previous heterotypic infection facilitate entry of the virus to Fc receptor-bearing cells such as monocytes, leading to increased virus uptake and replication in these cells (Goncalvez et al., 2007; Kliks et al., 1988). This model is strongly supported by a retrospective seroepidemiologic study in Cuba of the 1981 DENV-2

* Corresponding author at: Molecular Pathology Laboratory, Institute of Molecular Biosciences, Mahidol University, 25/25 Phuttamonthon 4 Road, Salaya, Nakhon Pathom, 73170, Thailand. Fax: +66 2441 1013.
E-mail address: duncan_r_smith@hotmail.com (D.R. Smith).

0042-6822/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2011.07.010

epidemic, which showed that 98% of patients with symptomatic dengue infection were the consequence of secondary infections, while 97% of patients without illness (but with a positive serology test) were primary infection (Guzman et al., 1990).

Several recent studies have shown that DENV infection activates the cellular autophagy pathway (Heaton and Randall, 2010; Khakpoor et al., 2009; Lee et al., 2008; Panyasrivanit et al., 2009). Autophagy is a conserved lysosomal degradation pathway responsible for degradation of long life proteins and organelles to maintain the homeostasis of macromolecules in eukaryotic cells (Eskelinen and Saftig, 2009; Levine and Klionsky, 2004; Meijer and Codogno, 2006). Autophagy begins with sequestration of cytoplasmic materials inside a double membrane vesicle called an autophagosome (Dunn, 1990a). This structure can either directly fuse with lysosomes to form autophagolysosomes, or with endosomes to form amphisomes (Gordon and Seglen, 1988) prior to fusion with lysosomes (Dunn, 1990b). Fusion of autophagic vesicles with lysosomes leads to degradation of the cytoplasmic materials (Mizushima, 2007). Two ubiquitin-like conjugations of autophagy (Atg) proteins are essential for autophagosome formation. Firstly the covalent linkage between Atg5 and Atg12 is crucial for elongation of the pre-autophagosomal membrane and formation of the autophagosome (Mizushima et al., 1998) and secondly the conjugation of microtubule-associated protein 1 light chain 3 (LC3; Atg8) with phosphatidylethanolamine converts the cytosolic form of LC3 (LC3-I) to the membrane bound LC3 (LC3-II) form which integrates into the autophagosomal membrane (Ferraro and Cecconi, 2007; Levine and Klionsky, 2004; Mizushima, 2007; Yang and Klionsky, 2010). LC3 is commonly used as a marker to monitor autophagy where the amount of LC3-II reflects the existence of autophagosomes (Mizushima, 2004, 2007).

While studies on the interaction of DENV and the autophagic machinery to date all propose that autophagy is activated during DENV infection and that autophagy is required for efficient DENV replication (Heaton and Randall, 2010; Khakpoor et al., 2009; Lee et al., 2008; Panyasrivanit et al., 2009) the mechanics of the interaction remain controversial. While we have previously proposed that autophagic vacuoles can act as sites of replication for DENV (Khakpoor et al., 2009; Panyasrivanit et al., 2009), other authors have recently suggested that the induction of autophagy helps virus replication through alterations in lipid metabolism and increase in β-oxidation and generation of ATP (Heaton et al., 2010; Heaton and Randall, 2010). However all of these studies have been undertaken primarily on liver cell lines (Heaton and Randall, 2010; Khakpoor et al., 2009; Lee et al., 2008; Panyasrivanit et al., 2009) and while the liver and hepatocytes in particular are generally seen as a viable target for DENV (reviewed in Smith and Khakpoor, 2009), monocytes are of greater significance, particularly in the severe forms of dengue (Halstead et al., 2010; Halstead and O'Rourke, 1977b; Kurane and Ennis, 1992; Rothman and Ennis, 1999).

In this study we sought to characterize the interaction between DENV and autophagy in monocytic cells, using the cell line U937 as a model. These cells are poorly infected directly but are highly permissive under ADE infection conditions, and are thus believed to be a suitable model for secondary infection of monocytes (O'Sullivan and Killen, 1994). While our results support the induction of autophagy by DENV infection under ADE conditions, we found that, in marked contrast to previous studies on directly infected cells (Heaton and Randall, 2010; Khakpoor et al., 2009; Lee et al., 2008; Panyasrivanit et al., 2009) induction of autophagy reduced viral yield while inhibition of autophagy only slightly increased levels of the virus. These results are somewhat similar to a defense interaction between DENV and autophagy (but with significant differences) as opposed to the subversive interaction seen in liver cells (Heaton and Randall, 2010; Khakpoor et al., 2009; Lee et al., 2008; Panyasrivanit et al., 2009), and indicate that the DENV/autophagy interaction has a significant cell type specific component.

Results

Induction of autophagy in U937 cells

This study aimed to investigate the relationship between autophagy and DENV-2 in monocytic cells, and used the human monocytic cell line U937 as a model (O'Sullivan and Killen, 1994). To confirm that the autophagy pathway was inducible in these cells, cells were subjected to two well characterized autophagy induction conditions, namely starvation and treatment with rapamycin (Noda and Ohsumi, 1998). The induction of autophagy was observed by co-immunofluorescence using antibodies against LC3 and a marker of endosomal and lysosomal membranes, LAMP1, with co-localization of these two markers indicating maturation of autophagic vacuoles during autophagy. The cellular localization of these markers was examined in control cells cultured under normal conditions, in cells incubated in Earle's Balanced Salt Solution for 1 h (starvation condition) and in cells cultured in the presence of 100 nM rapamycin for 1 h. A significantly higher degree of co-localization between LC3 and LAMP1 was observed in both starved (mean Pearson correlation coefficient 0.54, 95% confidence interval (CI) 0.51–0.57; $P<0.001$) and rapamycin (mean Pearson correlation coefficient 0.4, 95% CI 0.37–0.43; $P<0.001$) treated cells compared to control cells (mean Pearson correlation coefficient −0.01, 95% CI −0.05–0.03; Fig. 1(a)).

Induction of autophagy in response to DENV-2 infection in U937 cells

To determine whether autophagy was induced in response to DENV infection, U937 cells were mock-infected or infected in the presence of dilutions of the monoclonal antibody HB-114 (Henchal et al., 1982) previously shown to result in at least 80% infection of cells (20 p.f.u. per cell/1:200 final antibody dilution; Klomporn et al., 2011). Cells were harvested at 1 to 3 days post infection (d.p.i) for protein extraction and samples were analyzed for LC3 expression by Western blotting. The autophagic membrane associated form of LC3 (LC3-II) in DENV-2 infected U937 cells increased on day 1, day 2 and day 3 post infection in comparison to mock-infected cells (Fig. 1(b)), suggesting induction of autophagy in DENV-2 infection. To show that induction of autophagy results from DENV-2 infection, not physical interaction of the viruses and cells, Western blot of LC3 was performed in U937 cells infected with DENV-2 in the absence of enhancing antibody (no ADE). Result showed no increase of LC3-II level in non-ADE-DENV-2 infection as compared to mock-infection (Fig. 1(c)). Localization of LC3 and LAMP1 was examined in mock-infected and (ADE) DENV-2 infected U937 cells. A significant increase in co-localization was observed in (ADE) DENV-2 infected U937 cells (mean Pearson correlation coefficient 0.27, 95% CI 0.22–0.32; $P=0.021$) compared to mock-infected cells (mean Pearson correlation coefficient 0.13, 95% CI 0.04–0.22; Fig. 1(d)). Collectively these results indicate that autophagy was induced in response to DENV-2 ADE-mediated infection of U937 cells.

Effect of autophagy induction and inhibition of autophagosome-lysosome fusion on DENV-2 production

To further investigate the interaction between autophagy and DENV, two autophagy modulators were used namely rapamycin, an autophagy inducer (Noda and Ohsumi, 1998) and L-Asparagine (L-Asn), an inhibitor of autophagosome–lysosome fusion (Hoyvik et al., 1991). First, the effects of these two autophagy modulators in U937 cells were confirmed. U937 cells were treated with either 100 nM rapamycin or 30 mM L-Asn for 24 h and total proteins were extracted and subjected to Western blot analysis of LC3. An increase of LC3-II was observed following rapamycin treatment compared to control indicating the induction of autophagy, consistent with the LC3 and LAMP1 co-localization analysis. LC3-II expression also increased following L-Asn

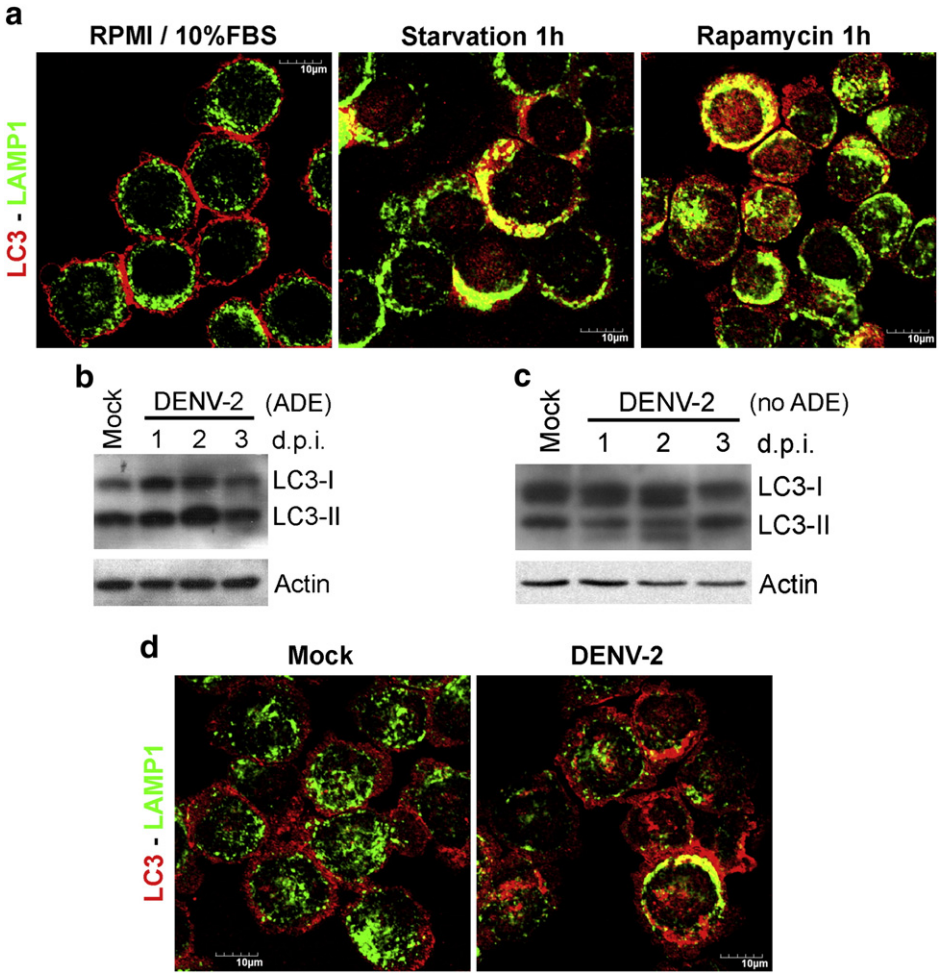

Fig. 1. Induction of autophagy in U937 cells in response to DENV-2 infection. (a) Immunofluorescence of LC3 (red) and LAMP1 (green) in U937 cells cultured in RPMI/10%FBS, 1× EBSS (starvation) or 100 nM rapamycin/RPMI/10%FBS for 1 h. The fluorescent signals were observed under a confocal microscope. Representative merged, non contrast adjusted images are shown. (b) and (c) Western blot of LC3 and actin from U937 cells mock-infected or ADE-infected with DENV-2 at 20 p.f.u. per cell (b) or DENV-2 infected with 20 p.f.u. per cell without antibody (c) for 1–3 days p.i. (d) Immunofluorescence of LC3 (red) and LAMP1 (green) in U937 cells mock-infected or ADE-infected with DENV-2 at 20 p.f.u. per cell for 3 days. The fluorescent signals were observed under a confocal microscope. Representative merged, non contrast adjusted images are shown.

treatment due to an accumulation of LC3-II following inhibition of autophagosome–lysosome fusion (Fig. 2(a)).

To confirm that rapamycin and L-Asn were not toxic to U937 cells prior to determining the effects on virus output, Annexin V/propidium iodide (PI) staining was performed to determine the amount of cell death in U937 cells treated with rapamycin and L-Asn at days 1 to 3 post treatment. Cells treated with 2.5% DMSO for 24 h were used as a positive control. The percentage cell death was calculated from the amounts of PI positive and Annexin V/PI positive cells. No toxicity of these chemicals was observed in U937cells (Fig. 2(b)).

To determine the effect of rapamycin and L-Asn on DENV-2 production in U937 cells, the cells were infected with DENV-2 in the presence of HB-114 as previously and in the presence or absence of these two modulators. The culture supernatants and cells were collected to determine extracellular and intracellular virus productions at 1 to 3 d.p.i. Results showed a significant reduction of both extracellular ($P = 0.001$ at 2 and 3 d.p.i.) and intracellular ($P < 0.05$ at 1, 2 and 3 d.p.i.) virus production in the presence of rapamycin compared to DENV-2 infection in the absence of autophagy modulators (Figs. 2(c) and (d)). Therefore, increased autophagy may have deleterious effects on DENV-2 production in U937 cells.

DENV-2 infection in the presence of L-Asn resulted in a marginal decrease of extracellular virus and similar level of intracellular virus titer in comparison to DENV-2 infection in the absence of autophagy modulators (Figs. 2(c) and (d)). This would suggest that autophagosome–lysosome fusion has only a small effect on DENV-2 production in U937 cells.

Effect of Vps34^{dn} mediated autophagy inhibition on DENV-2 production

To further investigate the interaction between autophagy and DENV in U937 cells we sought to down regulate autophagy to determine the effect on virus production. The commonly used biochemical autophagy inhibitor, 3-methyladenine, was significantly toxic to U937 cells at 5–10 mM (data not shown) which is the recommended concentration to inhibit autophagy (Klionsky et al., 2007; Seglen and Gordon, 1982). Previous studies have shown that over-expression of a dominant negative mutant of Vps34 (Vps34^{dn}), an autophagy regulatory molecule (Nobukuni et al., 2007; Petiot et al., 2000), inhibits autophagy (Castino et al., 2008; Castino et al., 2010; Trincheri et al., 2008). We therefore constructed a lentiviral expression vector expressing Vps34^{dn} which was transduced into HEK293T/17 and U937 cells, and the inhibition effect of over-expression of Vps34^{dn} on autophagy was investigated by Western blot analysis of LC3. The results showed a reduction of LC3-II form in HEK293T/17 cells expressing Vps34^{dn}

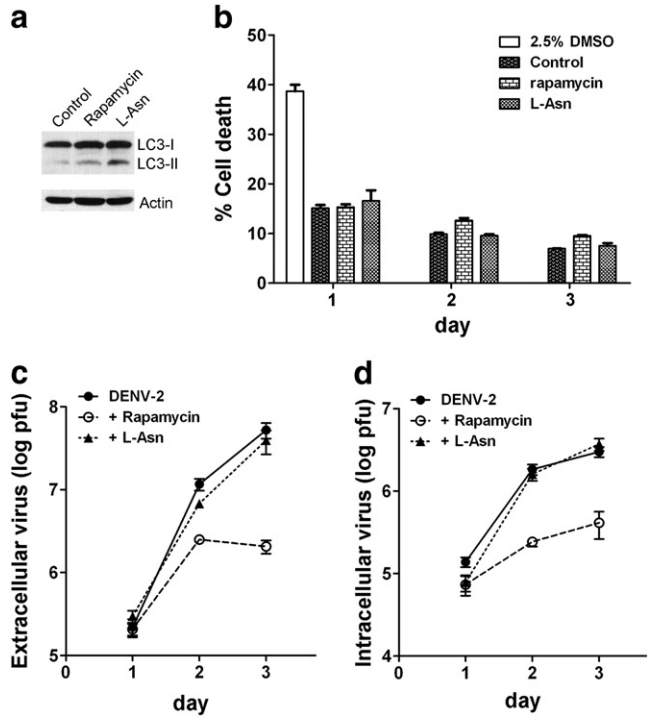

Fig. 2. Effect of autophagy induction and inhibition of autophagic lysosomal fusion on DENV-2 production. (a) Western blot of LC3 and actin in U937 cells cultured in normal complete medium or in the presence of 100 nM rapamycin or 30 mM L-Asn for 24 h. (b) Determination of cell death after treatment of U937 cells with autophagy modulators rapamycin and L-Asn by Annexin V/PI staining. U937 cells were cultured in normal complete medium or in presence of 100 nM rapamycin or 30 mM of L-Asn. The cells were harvested and stained with Annexin V/PI on days 1, 2 and 3 p.i. The signals were observed by flow cytometry. The percentage cell death was calculated from the number of PI- and both Annexin V and PI-positive cells. (c) Extracellular and (d) intracellular virus production from U937 cells ADE-infected with DENV-2 in the presence or absence of 100 nM rapamycin or 30 mM L-Asn on days 1, 2 and 3 p.i. The titer was determined by standard plaque assay. The experiment was undertaken independently in triplicate with duplicate assay of virus titers by standard plaque assay. Virus yield is shown in log scale with error bars (±SEM).

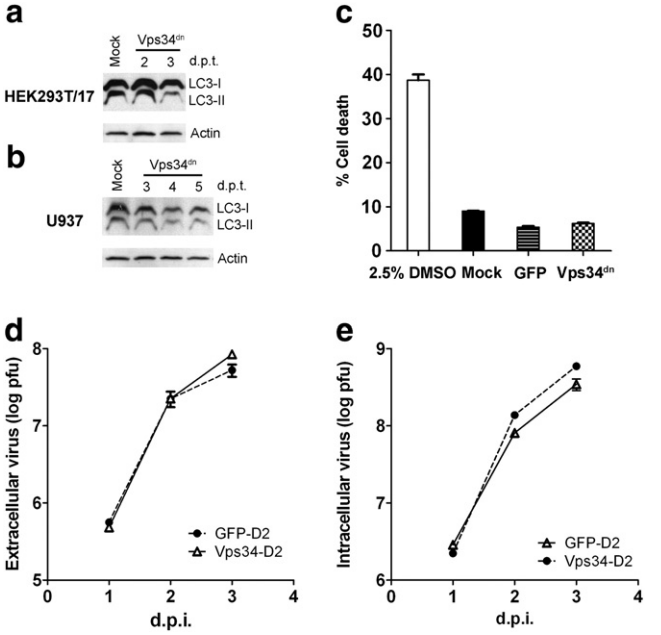

Fig. 3. Effect of Vps34^{dn} mediated autophagy inhibition on DENV-2 production. (a) Western blot of LC3 and actin in HEK293T/17 cells mock-transduced or transduced with a lentivirus expressing Vps34^{dn} for 2 and 3 days post transduction (d.p.t.). (b) Western blot of LC3 and actin in U937 cells mock-transduced or transduced with lentivirus expressing Vps34^{dn} for 3 to 5 d.p.t. (c) Determination of a degree of cell death in transduction of lentivirus in U937 cells by Annexin V/PI staining. U937 cells mock-transduced or transduced with lentivirus expressing GFP or Vps34^{dn} were stained with Annexin V and PI at 5 d.p.t. The signals were observed by flow cytometry. The percentage cell death was calculated from the number of PI- and both Annexin V and PI-positive cells. (d) Extracellular and (e) intracellular virus production from U937 cells mock-transduced or transduced with lentivirus expressing GFP or Vps34^{dn} for 4 days and then ADE-infected with DENV-2 for 1 to 3 days. The titer was determined by standard plaque assay. The experiment was performed in three independent replicates and duplicates for plaque assay. Virus yield is shown in log scale with error bars (±SEM).

on day 3 post transduction as compared to mock (Fig. 3(a)). Lower LC3-II expression was also observed in U937 cells expressing Vps34^{dn} on days 3 to 5 post transduction as compared to mock infected cells (Fig. 3(b)).

To show that the lentiviral transductions did not affect cell viability, Annexin V/PI staining was performed to determine the amount of cell death in U937 cells mock-transduced or transduced with lentivirus expressing Vps34^{dn} or a lentivirus expression vector expressing GFP as a control. The results showed no significant cell death in the cells transduced with these lentiviruses (Fig. 3(c)).

To determine the effect of autophagy inhibition on DENV-2 infection of U937 cells, cells were transduced with the lentivirus expressing Vps34^{dn} or a lentivirus expressing GFP as a control, and on day 4 post transduction, cells were infected with DENV-2 at 20 p.f.u. per cell in the presence of monoclonal antibody HB-114 to induce ADE of infection. Extracellular and intracellular virus production was examined by standard plaque assay at days 1, 2 and 3 post infection. There was no significant difference in extracellular virus production in U937 cells transduced with lentivirus expressing Vps34^{dn} on 1 and 2 d.p.i. compared to GFP-transduced cells, whereas a marginal, but statistically significant increase in virus production was observed on 3 d.p.i. ($P=0.031$) (Fig. 3(d)). Similarly, a small but statistically significant increase in intracellular virus was observed in U937 transduced with lentivirus expressing Vps34^{dn} compared to GFP-transduced cells on days 2 ($P=0.001$) and 3 ($P=0.007$) post infection (Fig. 3(e)).

Effect of autophagy modulation on DENV proteins

The level of DENV E and NS1 protein expression was examined in DENV-2 infection in response to autophagy modulation (Fig. 4). The results showed a reduction of DENV E and NS1 proteins when autophagy was induced by treatment of rapamycin, while no alteration of dengue E and NS1 expression was observed in L-Asn treatment compared to DENV-2 infection (Fig. 4(a)). Inhibition of autophagy by over-expression of Vps34^{dn} resulted in an increase of dengue E and NS1 level in comparison to Mock- and GFP-transduced cells (Fig. 4(b)).

The DENV replication complex does not co-localize with autophagy markers

Immunofluorescent co-localization studies were performed to determine the site of DENV-2 replication in U937 cells. An extremely low level of co-localization of autophagic marker LC3 and dsRNA was observed in DENV-2 infected U937 cells (mean Pearson correlation coefficient 0.13, 95% CI 0.11–0.15; Fig. 5(a)). A significant increase in co-localization was observed when autophagy was induced by treatment of rapamycin (mean Pearson correlation coefficient 0.29, 95% CI 0.26–0.32; $P<0.001$) while no significant alteration was observed in upon inhibition of autophagosome–lysosome fusion by treatment with L-Asn (mean Pearson correlation coefficient 0.11, 95% CI 0.09–0.13) (Fig. 5(a)).

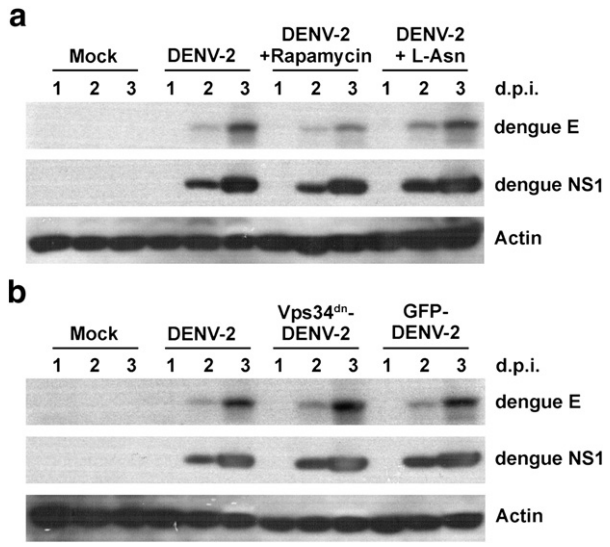

Fig.4. Expression of DENV viral proteins under autophagy modulation. (a) Western blot of dengue E and NS1 proteins in U937 cells mock-infected or ADE-infected with DENV-2 in the presence or absence of either 100 nM rapamycin or 30 mM L-Asn at 1 to 3 d.p.i. Expression of actin was used as an internal control. (b) Western blot of dengue E and NS1 proteins in U937 cells mock-transduced or transduced with lentivirus expressing GFP or VPS34^{dn} and then mock-infected or ADE-infected with DENV-2 for 1, 2 and 3 days. Expression of actin was used as an internal control.

The co-localization of a marker of mature autophagolysosomes and lysosomes (cathepsin D) was also examined in relation to dsRNA. Consistent with the LC3 and dsRNA analysis, no localization was seen between cathepsin D and dsRNA in DENV infected cells (mean Pearson correlation coefficient −0.03, 95% CI −0.05 to −0.01), or in U937 cells infected with DENV in the presence of L-Asn (mean Pearson correlation coefficient −0.06, 95% CI −0.08 to −0.04). A slight degree of co-localization (mean Pearson correlation coefficient 0.1, 95% CI 0.05–0.15; $P<0.001$) was observed between dsRNA and cathepsin D in the presence of rapamycin (Fig. 5(b)).

ER, stress and autophagy

A number of studies have proposed that DENV replication occurs in association with the ER (Perera and Kuhn, 2008). We therefore examined the co-localization of dsRNA and a predominantly ER resident marker, calnexin. A high degree of co-localization between dsRNA and calnexin was observed (mean Pearson correlation coefficient 0.46, 95% CI 0.45–0.47; $P<0.001$ (Fig. 6(a)) implying that the ER is the major site of DENV-2 replication in U937 cells.

We have recently shown that DENV-2 infection induces ER stress and activation of the unfolded protein response (UPR) pathway in U937 cells (Klomporn et al., 2011). As several studies have shown that induction of the UPR can induce autophagy (Bernales et al., 2006; Bernales et al., 2007), it is possible that the increase in autophagy seen here results from ER stress and UPR activation. To determine if autophagy is induced in U937 cells in response to ER stress, cells were treated with various concentrations of the sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin, a commonly used ER stress inducer (Bachar et al., 2009; Bertolotti et al., 2000; Won et al., 2009). Splicing of the XBP-1 transcript, a hallmark of events of UPR activation (Yoshida et al., 2001), was shown to occur in response to thapsigargin treatment of U937 cells (Fig. 6(b)). The induction of autophagy by thapsigargin treatment was investigated by Western blot analysis of LC3. The results showed a clear induction of autophagy in the presence of thapsigargin, as evidenced by a significant dose dependent increase in levels of LC3-II (Fig. 6(c)).

The effect of thapsigargin on DENV-2 production was investigated. The toxicity of thapsigargin to U937 cells was evaluated prior to determining the effects on virus output. Annexin V/PI staining was performed to determine the amount of cell death in the presence of 5 nM thapsigargin. The result showed a marked increase in cell death as a consequence of thapsigargin treatment on days 2 and 3 post treatment which may result from prolong ER stress-induced apoptosis (Li et al., 2006; Nakagawa et al., 2000) (Fig. 6(d)). U937 cells were then infected with DENV-2 in the presence of 5 nM thapsigargin. Extracellular and intracellular virus productions were determined by standard plaque assay. The results showed significant reduction of both extracellular and intracellular virus on 1, 2 and 3 d.p.i. ($P<0.05$) (Figs. 6(e) and (f)). Lastly, to confirm that autophagy induction by rapamycin did not in itself induce ER stress, U937 cells were treated with 100 nM rapamycin for three days and analyzed for the induction of XBP-1 splicing in parallel with negative (no treatment) and positive (5 nM thapsigargin for 24 h) controls. Results showed that rapamycin treatment did not induce XBP-1 splicing (Fig. 6(g)).

Discussion

Autophagy has been shown to play a role in the host cell/virus interaction for a number of different viruses. In what is apparently the simplest interaction, autophagy is activated as a defense mechanism to eliminate the invading pathogen. However, because of this innate antiviral process, many viruses have evolved to either down regulate autophagy through the expression of specific viral gene products, or to subvert the autophagic process for their own replication strategy (reviewed in Espert et al., 2007; Kirkegaard et al., 2004). Studies from several groups have determined that DENV activates autophagy upon infection, and uses the autophagic process as part of its replication strategy (Heaton and Randall, 2010; Khakpoor et al., 2009; Lee et al., 2008; Panyasrivanit et al., 2009). While we have proposed that this occurs through DENV utilizing autophagic structures as sites for replication (Khakpoor et al., 2009; Panyasrivanit et al., 2009), others have suggested that DENV induced autophagy results in alterations in lipid metabolism which facilitates DENV replication (Heaton and Randall, 2010). The two models are far from mutually exclusive, and both agree that the induction of autophagy is an important component of the DENV replication strategy.

To date, the studies investigating the relationship between autophagy and DENV have been undertaken almost exclusively on liver cells (Heaton and Randall, 2010; Khakpoor et al., 2009; Lee et al., 2008; Panyasrivanit et al., 2009). While hepatocytes are almost certainly a true *in vivo* target of DENV (Suksanpaisan et al., 2007), a significant amount of evidence has suggested that the more severe forms of DENV infection are associated with a second DENV infection, from a heterologous serotype to the first infection. The increased severity is proposed to result from monocytic cells becoming susceptible to infection through the intermediary of non-neutralizing antibodies from the first infection allowing internalization of virus/antibody complexes to monocytic cells through the Fc receptor (Halstead et al., 2010; Halstead and O'Rourke, 1977a; Halstead et al., 1980).

Surprisingly, while DENV-2 induced autophagy in monocytic cells, the relationship between autophagy and DENV in these cells was not subversive as described in liver cells, with DENV utilizing the autophagic process as part of its replication strategy (Heaton and Randall, 2010; Khakpoor et al., 2009; Lee et al., 2008; Panyasrivanit et al., 2009). Instead, activation of autophagy significantly reduced virus output while inhibition of autophagy resulted in only marginal increases in virus production. This would suggest that under normal infection conditions there is a limited interaction between autophagy and DENV. As such, while the interaction between DENV and autophagy in monocytic cells is not one of defense, which is characterized by low or null virus output (Lee and Iwasaki, 2008), neither is it one of avoidance or subversion.

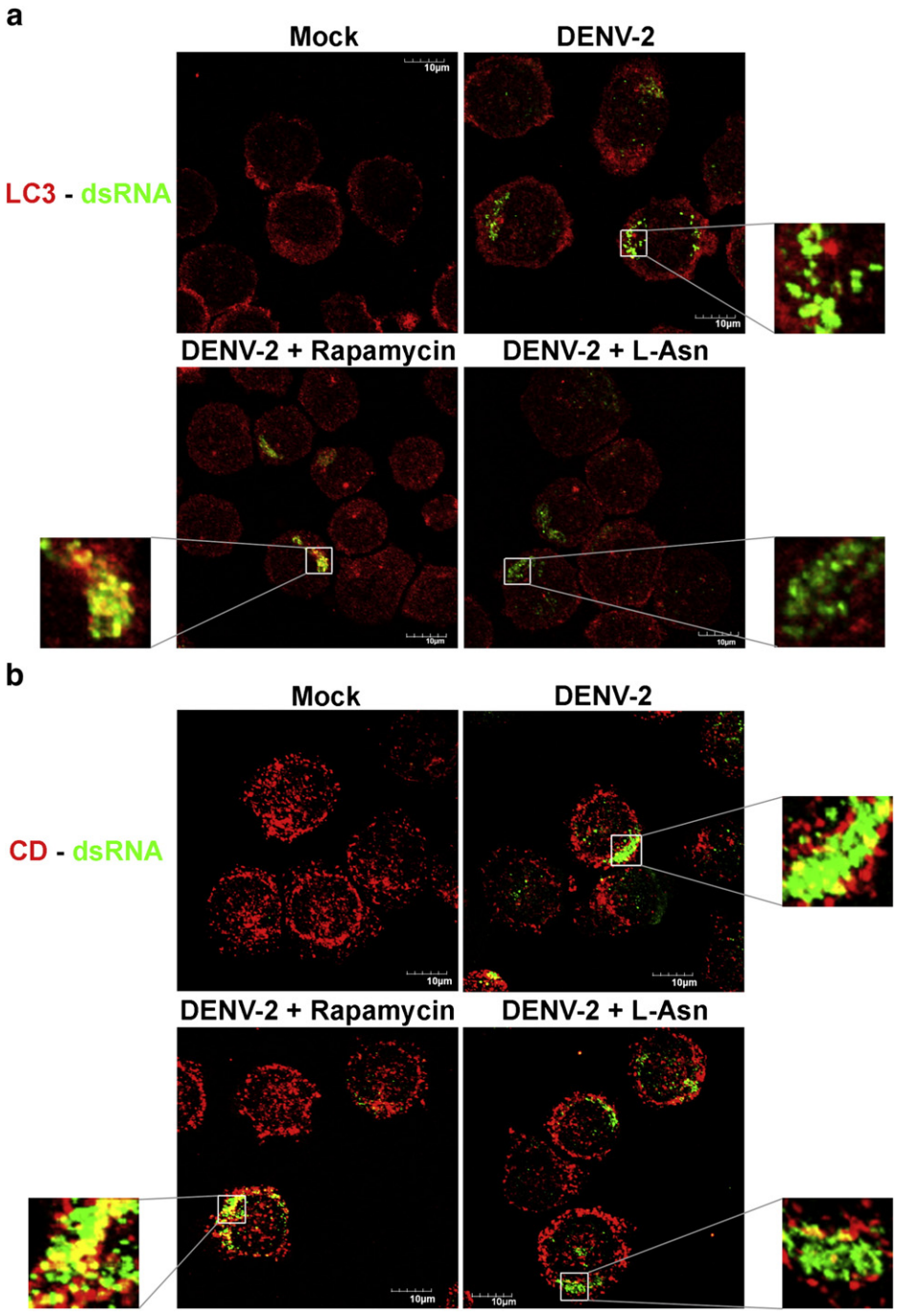

Fig. 5. Localization of DENV-2 replication complex. Immunofluorescence of (a) LC3 (red) and dsRNA (green) or (b) cathepsin D (CD) (red) and dsRNA (green) was performed in U937 cells mock-infected or ADE-infected with DENV-2 in the presence or absence of 100 nM rapamycin or 30 mM L-Asn for 3 days. The fluorescent signals were observed under a confocal microscope. Representative merged, non contrast adjusted images are shown.

Under normal infection conditions, no co-localization was observed between a marker of the DENV replication complex (dsRNA) and markers of autophagy (LC3 or cathepsin D), while significant co-localization was noted between dsRNA and the predominantly ER resident marker calnexin. However, when autophagy was strongly induced with rapamycin, some co-localization was observed between dsRNA and markers of autophagy. While the origin of the autophagic membranes is of some dispute, it is generally accepted that the ER is a major source of autophagic membranes (Tooze and Yoshimori, 2010; Yla-Anttila et al., 2009) and therefore it is likely that the increased co-localization seen between autophagic markers and dsRNA seen in rapamycin treated cells reflects the utilization of ER membranes, together with associated DENV replication complexes in the formation of autophagic vacuoles. In this way, the reduction of DENV production seen in rapamycin treated cells could result from a loss of ER resident replication sites when the membranes are co-opted for autophagic vesicles.

The extremely marginal increase in DENV production seen upon down regulation of autophagy would also be consistent with this model, where under normal infection conditions there is only a small contribution of ER membranes (and associated DENV replication potential) to autophagy. This model is similarly consistent with the results seen on Western analysis where rapamycin results in a reduction of DENV E and NS1 proteins, while down regulation of

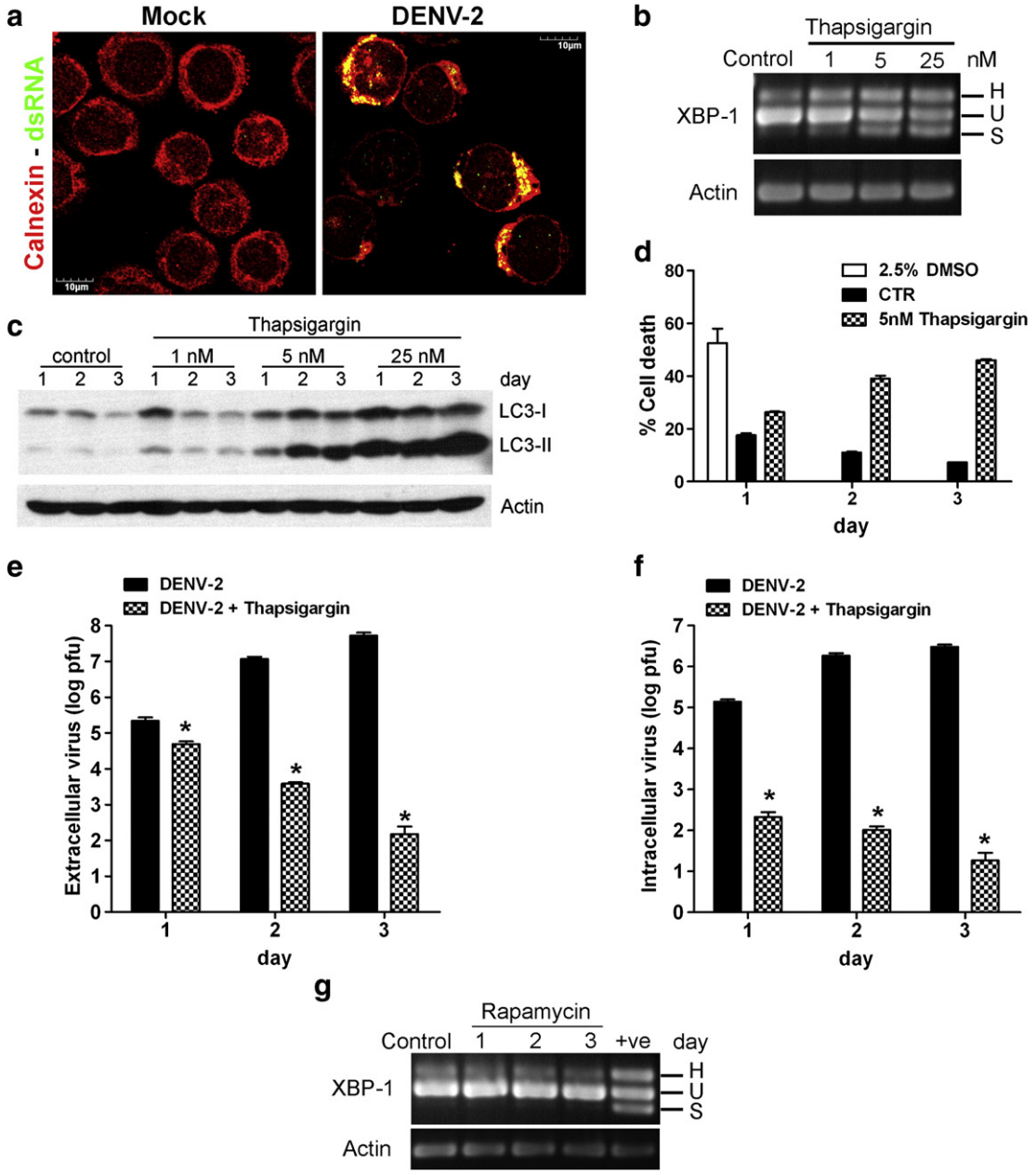

Fig. 6. ER stress induced autophagy in DENV-2 infected U937 cells. (a) Immunofluorescence of calnexin (red) and dsRNA (green) was performed in U937 cells mock-infected or ADE-infected with DENV-2 for 3 days. The fluorescent signals were observed under a confocal microscope. Representative merged, non contrast adjusted images are shown. (b) Splicing of XBP-1 transcripts was examined in U937 cells cultured in RPMI/10%FBS (control) or in the presence of 1, 5 and 25 nM of thapsigargin for 24 h by RT-PCR. H: heteroduplex; U: unspliced; S: sliced form of XBP-1. (c) Western blot of LC3 and actin in U937 cells cultured in normal complete medium (control) or in the presence of 1, 5 and 25 nM of Thapsigargin for 1 to 3 days. (d) Determination of cell death in U937 cells treated with 5 nM thapsigargin by Annexin V/PI staining. The signals were observed by flow cytometry on days 1 to 3 post treatment. Percentage of cell death was calculated from the number of PI- and both Annexin V and PI-positive cells. (e) Extracellular and (f) intracellular virus production from U937 cells ADE-infected with DENV-2 in the presence or absence of 5 nM thapsigargin on days 1, 2 and 3 post infection. The titer was determined by standard plaque assay. The experiment was undertaken independently in triplicate with duplicate assay of virus titers by standard plaque assay. Virus yield is shown in log scale with error bars (±SEM). * indicates statistically significant compared to control ($P<0.05$). (g) U937 cells were either not treated (Control) or treated with either 100 nM rapamycin for 1, 2 or 3 days (rapamycin 1 to 3) or with 5 nM thapsigargin for 24 h as a positive control (+ve) and analyzed for the splicing of the XBP-1 transcript by RT-PCR. H: heteroduplex; U: unspliced; S: sliced form of XBP-1.

autophagy results in a slight increase in the levels of these proteins. Again, the ER membranes being co-opted under increased autophagy would serve to reduce the amount of protein translation occurring in the ER.

While the data suggests a minimal interaction between autophagy and DENV production under normal conditions, the induction of autophagy was observed in response to infection.

We have recently shown that infection of monocytic cells by DENV results in the activation of multiple ER stress pathways, which ultimately result in apoptosis of the cells (Klomporn et al., 2011). Several studies have suggested a direct link between ER stress and autophagy (Bernales et al., 2006; Bernales et al., 2007), and it is likely then that the induction of autophagy seen in response to DENV infection occurs as a result of activation of ER stress and activation of the unfolded protein response, rather than a direct DENV mediated activation of autophagy. Indeed, treatment of U937 cells with thapsigargin showed the induction of ER stress as well as the induction of autophagy. Under these conditions, DENV output was

reduced in a similar manner to the reduction seen with rapamycin. While we note that long term treatment with thapsigargin induced cell death, a significant reduction in virus production was observed prior to significant levels of cell death.

In this way it can be hypothesized that in U937 cells biochemical activation of autophagy (either directly through rapamycin, or indirectly through induction of the UPR) reduces virus output as a consequence of recruitment of ER membranes to autophagic vacuoles (Bernales et al., 2006; Bernales et al., 2007) thus reducing indirectly the number of available sites of replication for DENV.

Inhibition of autophagy did result in a small, but statistically significant increase in both intracellular and extracellular viruses. This would suggest that under normal conditions there is some (albeit small) interaction between DENV and autophagy. Apart from its functions to maintain homeostasis in the cell, autophagy also has a role in the immune system where it mediates endogenous major histocompatibility complex (MHC) class II antigen processing (Deretic, 2006; Nimmerjahn et al., 2003; Paludan et al., 2005). Since cells of a monocyte/macrophage lineage are antigen presenting cells, autophagic degradation of DENV-2 in these cells may also involve antigen processing. In this case, the proportion of DENV produced that would normally be lost during the process of autophagy mediated antigen presentation is no longer lost, and is detected on analysis of virus levels.

Given that DENV infected monocytic cells undergo apoptosis within a few days, it is difficult to see that the marginally increased cellular levels of virus would have profound effects on the cell. However, a possible reduction in antigen presentation as a consequence of down regulation of autophagy could have serious consequences. Thus treatment approaches that seek to down regulate autophagy, based upon the subversion reaction observed in other cell types (Heaton and Randall, 2010; Khakpoor et al., 2009; Lee et al., 2008; Panyasrivanit et al., 2009) could have serious long term immune consequences by muting the efficacy of the immune response.

Overall, the interaction between autophagy and DENV in monocytic cells described here does not fit any of the classical models of virus/host cell autophagy interaction (Espert et al., 2007; Kirkegaard et al., 2004), but does show that the interaction between DENV and autophagy is mediated in a cell type specific manner.

Materials and methods

Cells and viruses

U937 cells (human monocytic cell line, ATCC CRL-1593.2) were cultured in RPMI 1640 medium (RPMI; Gibco™ Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco™ Invitrogen) and 100 unit/ml of penicillin–streptomycin (PAA Laboratories GmbH). LLC-MK2 cells (rhesus monkey kidney cell line, ATCC CCL-7) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco™ Invitrogen) supplemented with 5% FBS and 100 unit/ml of penicillin–streptomycin. HEK293T/17 cells (human embryonic kidney cell line, ATCC CRL-11268) were cultured in DMEM supplemented with 10% FBS and 100 unit/ml of penicillin–streptomycin. All cells were incubated at 37 °C in a humidified incubator with 5% CO_2. C6/36 cells (the *Aedes albopictus* cell line, ATCC CRL-1660) were cultured in minimum essential medium (MEM; Gibco™ Invitrogen) supplemented with 10% FBS and the same antibiotics as the cell lines above at 28 °C.

Dengue virus serotype 2 (DENV-2) strain 16681 was propagated in C6/36 cells. The medium containing virus was collected at the day providing a maximum virus titer (Sakoonwatanyoo et al., 2006) and subsequently centrifuged to remove cell debris. The virus was stored at −80 °C until use. The virus titer was determined by standard plaque assay as previously described (Sithisarn et al., 2003) on LLC-MK2 cells.

Cell starvation and chemical treatments

U937 cells were centrifuged at 400×g for 5 min to remove culture medium. The cell pellets were washed once with PBS (for starvation) and then resuspended with 1× EBSS for starvation condition, complete culture medium containing 100 nM rapamycin (Sigma Chemical Company), 30 mM L-Asparagine (L-Asn; Sigma) or 1, 5 and 25 nM Thapsigargin (Sigma). The cells were then incubated under standard conditions until harvesting.

Infection of U937 cells

DENV-2 infection of U937 cells was undertaken exactly as described elsewhere (Klomporn et al., 2011). Briefly, a pre-calculated 20 p.f.u. per cell of DENV-2 and a 1:200 dilution of a pan specific mouse monoclonal anti-dengue E protein antibody (HB114) produced from mouse hybridoma cell line D3-2H2-9-21 (ATCC HB114, isotype IgG2a) were incubated for 1 h at 4 °C in RPMI medium. U937 cells were centrifuged at 400×g for 5 min to remove culture medium and resuspended with the antibody–virus mixture. The cells were then incubated at 37 °C, 5% CO_2 for 2 h with constant agitation. After 2 h, complete medium was added to give a final cell density of 3×10^5 cells/ml. For infection in the presence of autophagy modulators, U937 cells were pre-treated for 1 h with 100 nM rapamycin, 30 mM L-Asn or 5 nM thapsigargin (Sigma) in RPMI/10%FBS and infection was performed as described above. The cells were incubated under standard conditions until harvesting of the cells or culture medium. For non-ADE-DENV-2 infection, U937 cells were directly incubated with DENV-2 at 20 p.f.u. per cell at 37 °C, 5% CO_2 for 2 h with constant agitation and then complete medium was added to give a final cell density of 3×10^5 cells/ml.

Intracellular and extracellular virus titration by standard plaque assay

Extracellular and intracellular virus titers were determined essentially as described elsewhere (Thepparit and Smith, 2004). Extracellular virus titers were determined directly from cell supernatants. To determine the intracellular virus titer, cells in each sample were harvested and centrifuged at 400×g for 5 min to remove culture medium. The cells were washed with RPMI and resuspended in BA-1 medium (1× medium199/Earle's balanced salts (HyClone), 0.05 M Tris–HCl pH 7.6, 1% BSA fraction V (PAA), 0.075% $NaHCO_3$, 100 units/ml penicillin–streptomycin). The cell suspensions were mixed and intracellular virus was released from the cells by one freeze–thaw cycle and sonicating at 4 °C for 5 min. The extracellular virus and intracellular virus titers were determined by standard plaque assay on LLC-MK2 cells as previously described (Sithisarn et al., 2003). The virus titer values were derived from three-independent experiments assayed in duplicate.

Indirect immunofluorescence

50,000–100,000 U937 cells from each experimental condition were collected and centrifuged at 400×g for 5 min to remove culture medium. The cells were washed once with RPMI and spun down onto glass cover slips using StatSpin® CytoFuge 2 (Iris Sample Processing). The cells were fixed in ice-cold absolute methanol for 20 min and washed twice with PBS. The subsequent steps were undertaken exactly as previously described (Panyasrivanit et al., 2009). Primary antibodies used were a rabbit polyclonal anti-MAP-LC3 antibody (sc-28266, Santa Cruz Biotechnology), a mouse monoclonal anti-CD107a antibody (LAMP1, 555798, BD Bioscience), a mouse monoclonal anti-dsRNA antibody (J2-0702, Scicons, Hungary), a rabbit polyclonal anti-calnexin antibody (ab10286, Abcam) and a rabbit polyclonal anti-cathepsin D antibody (Ab-2, IM 16, Calbiochem). All primary antibodies were used at a concentration of 1:50 with the exception of the anti-dsRNA antibody which

was used at a dilution of 1:100. Secondary antibodies used were a rhodamine red™-X-conjugated goat anti-rabbit IgG antibody (111-295-144, Jackson ImmunoResearch Laboratories) (1:50), a FITC-conjugated goat anti-mouse IgG antibody (02-18-06, KPL) (1:20), and an Alexa™ 647-conjugated donkey anti-rabbit IgG antibody (A31573, Molecular Probes) (1:100). The cells were viewed under an Olympus FluoView 1000 confocal microscope as described elsewhere (Panyasrivanit et al., 2009).

The degree of co-localization was analyzed in non-contrast adjusted pictures using ImageJ software with PSC co-localization plug-in as described elsewhere (Panyasrivanit et al., 2009). A degree of co-localization was determined as Pearson correlation coefficients, which represent the linear relationship of the signal intensity from the green and red channels. At least 20 cells were analyzed for each condition. Statistical significance ($P<0.05$) between data sets was determined by independent sample t tests using SPSS software (SPSS Inc., Chicago, IL).

Protein extraction and Western blot assay

Cells were harvested and centrifuged to remove culture medium. The cell pellets were washed with cold RPMI and resuspended in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS). The cell lysates were incubated on ice for 30 min with periodic mixing and sonicated twice at 4 °C for 5 min. The cell lysates were then centrifuged at 10,000×g for 10 min and supernatants were collected. Proteins were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked in blocking solution (5% skimmed milk/0.05% tween20/TBS) for 2 h at room temperature or overnight at 4 °C and incubated with primary antibodies: a rabbit polyclonal anti-LC3B antibody (ab48394, Abcam) (1:2000 dilution), a goat polyclonal anti-actin (I-19) antibody (sc-1616, Santa Cruz Biotechnology) (1:500 dilution), a mouse monoclonal anti-dengue NS1 antibody (Puttikhunt et al., 2003) (1:200 dilution) or a mouse monoclonal anti-dengue virus type 1–4 antibody (MA1-27093, Pierce) (1:1000 dilution) diluted in blocking solution at room temperature for at least 3 h (for LC3) or 4 °C overnight (for all other antibodies) followed by incubation with an appropriate secondary antibody conjugated with HRP at room temperature for 1 h. The signal was visualized using Pierce® ECL Western blotting substrate (Thermo Fisher Scientific).

Annexin V and Propidium iodide staining

2×10^5 cells were harvested from each condition at the indicated time points. The cells were washed and resuspended in 1× ApoAlert™ Annexin V binding buffer (630202, Clontech). 1 μl of ApoAlert™ Annexin V conjugated FITC (630201, Clontech) and 10 μl of 50 μg/ml Propidium iodide (PI; Sigma) were added to the cells and samples were incubated in darkness at room temperature for 15 min. The signals were detected using a FACS Calibur flow cytometer. The positive control in all experiments was U937 cells treated with 2.5% DMSO in RPMI/10%FBS for 24 h. The percentage cell death was calculated from the amounts of PI-positive and Annexin V/PI-positive cells. All experiments were undertaken as three independent replicates.

Construction of lentiviruses

A cDNA clone encoding a kinase deficient (dominant negative) mutant of rat Vps34 was the kind gift of Dr. HW Davidson (University of Cambridge) (Row et al., 2001). The full length cDNA of Vps34 which included two point mutations (Asp^{743}-Ala, Asn^{748}-Ile) was excised from plasmid pcDNA3puro and expressed in place of GFP in the lentiviral vector pRRL.SIN.CPPT.CMV.GFP.WPRE (engineered from Addgene Plasmid 12252). The lentiviral transfer vectors pRRL.SIN.CPPT.CMV.GFP.WPRE and pRRL.SIN.CPPT.CMV.Vps34.WPRE were propagated in Stbl3 competent cells (Invitrogen) to reduce homologous recombination. All plasmid constructs were purified by two rounds of CsCl ultracentrifugation. Viruses were generated by transient transfection of the transfer vector together with 3 separate packaging plasmids (pMDLg/pRRE, pRSV-Rev, PMD2.G (Addgene)) into HEK293T/17 cells by the calcium phosphate method. After 8 h in the presence of the transfection precipitate the culture medium was changed and replaced with fresh media. Culture supernatant containing lentiviruses were collected 48 h after transfection, cell debris was removed by centrifugation, and the supernatant was filtered. Virus stocks were stored at −80 °C until used.

Lentivirus transduction and DENV-2 infection in U937 cells over-expressing $Vps34^{dn}$

4×10^5 HEK293T/17 cells were seeded in 12-well tissue culture plates for 24 h prior to viral transduction. For U937 cells, 5×10^5 cells were centrifuged at 420×g for 5 min to remove culture medium. The cells then were incubated with 400 μl crude lentivirus expressing GFP or $Vps34^{dn}$ in the presence of 8 μg/ml polybrene (hexadimethrine bromide; H9268, Sigma) for 1 h at 37 °C 5% CO_2. Control experiments were performed with 400 μl DMEM/10%FBS. After an hour of incubation, RPMI/10%FBS was added to a final volume of 1 ml. The cells were incubated under standard conditions and fresh medium was added daily. A high transduction efficiency (>70%) of the GFP lentivirus was demonstrated in U937 cells by fluorescent microscopy and flow cytometry (data not shown).

To perform DENV-2 ADE-infection in U937 cells transduced with lentiviruses, transduced U937 cells were harvested at day 4 post transduction and infected DENV-2 at 20 p.f.u. per cell in the presence of dilutions of anti-dengue E protein monoclonal antibody HB114 as described elsewhere (Klomporn et al., 2011). Extracellular and intracellular virus production was determined as previously described. Experiments were performed as three independent replicates with duplicate assay of virus titers by standard plaque assay.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed as previously described elsewhere (Klomporn et al., 2011). Briefly, U937 cells were harvested by centrifugation and the cells were washed once with cold RPMI. RNA extraction was performed using TRI Reagent® (Molecular Research Center, Inc.). 2 μg of total RNA was reverse transcribed to cDNA by using ImpromII™ reverse transcriptase (Promega) and oligo (dT). The cDNA was amplified by PCR using specific primer for XBP-1 (Yoshida et al., 2001): sense primer 5′-CCTTGTAGTTGAGAACCAGG-3′, antisense primer 5′-GGGGCTTGGTATATATGTGG-3′ and actin: sense primer 5′-GAAGATGACCCCAGATCATGT-3′, antisense primer 5′-ATCTCTTGCTCGAAGTCCAG-3′ (Lithanatudom et al., 2010). PCR conditions were denaturation at 94 °C for 10 s (XBP-1) and 20 s (actin), annealing at 55 °C for 20 s (XBP-1) and 60 °C for 15 s (actin) and extension at 72 °C for 30 s (XBP-1) and 20 s (actin). PCR-amplified fragment size for XBP-1 are 416 (spliced form) and 442 bp (unspliced form), and for actin is 330 bp. PCR products were separated by electrophoresis on 2% agarose gel. Bands were visualized by ethidium bromide staining.

Acknowledgments

This work was supported by grants from the Thailand Research Fund and Mahidol University. M.P. is supported by a Thai Royal Golden Jubilee Research Scholarship.

References

Bachar, E., Ariav, Y., Ketzinel-Gilad, M., Cerasi, E., Kaiser, N., Leibowitz, G., 2009. Glucose amplifies fatty acid-induced endoplasmic reticulum stress in pancreatic beta-cells via activation of mTORC1. PLoS One 4, e4954.

Bernales, S., McDonald, K.L., Walter, P., 2006. Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response. PLoS Biol. 4, e423.

Bernales, S., Schuck, S., Walter, P., 2007. ER-phagy: selective autophagy of the endoplasmic reticulum. Autophagy 3, 285–287.

Bertolotti, A., Zhang, Y., Hendershot, L.M., Harding, H.P., Ron, D., 2000. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nat. Cell Biol. 2, 326–332.

Castino, R., Thepparit, C., Bellio, N., Murphy, D., Isidoro, C., 2008. Akt induces apoptosis in neuroblastoma cells expressing a C98X vasopressin mutant following autophagy suppression. J. Neuroendocrinol. 20, 1165–1175.

Castino, R., Bellio, N., Follo, C., Murphy, D., Isidoro, C., 2010. Inhibition of PI3k class III-dependent autophagy prevents apoptosis and necrosis by oxidative stress in dopaminergic neuroblastoma cells. Toxicol. Sci. 117, 152–162.

Deretic, V., 2006. Autophagy as an immune defense mechanism. Curr. Opin. Immunol. 18, 375–382.

Dunn Jr., W.A., 1990a. Studies on the mechanisms of autophagy: formation of the autophagic vacuole. J. Cell Biol. 110, 1923–1933.

Dunn Jr., W.A., 1990b. Studies on the mechanisms of autophagy: maturation of the autophagic vacuole. J. Cell Biol. 110, 1935–1945.

Eskelinen, E.L., Saftig, P., 2009. Autophagy: a lysosomal degradation pathway with a central role in health and disease. Biochim. Biophys. Acta 1793, 664–673.

Espert, L., Codogno, P., Biard-Piechaczyk, M., 2007. Involvement of autophagy in viral infections: antiviral function and subversion by viruses. J. Mol. Med. 85, 811–823.

Ferraro, E., Cecconi, F., 2007. Autophagic and apoptotic response to stress signals in mammalian cells. Arch. Biochem. Biophys. 462, 210–219.

Goncalvez, A.P., Engle, R.E., St Claire, M., Purcell, R.H., Lai, C.J., 2007. Monoclonal antibody-mediated enhancement of dengue virus infection in vitro and in vivo and strategies for prevention. Proc. Natl. Acad. Sci. U. S. A. 104, 9422–9427.

Gordon, P.B., Seglen, P.O., 1988. Prelysosomal convergence of autophagic and endocytic pathways. Biochem. Biophys. Res. Commun. 151, 40–47.

Gubler, D.J., 1998. Dengue and dengue hemorrhagic fever. Clin. Microbiol. Rev. 11, 480–496.

Guzman, M.G., Kouri, G.P., Bravo, J., Soler, M., Vazquez, S., Morier, L., 1990. Dengue hemorrhagic fever in Cuba, 1981: a retrospective seroepidemiologic study. Am. J. Trop. Med. Hyg. 42, 179–184.

Guzman, M.G., Kouri, G., Valdes, L., Bravo, J., Alvarez, M., Vazques, S., Delgado, I., Halstead, S.B., 2000. Epidemiologic studies on dengue in Santiago de Cuba, 1997. Am J Epidemiol 152, 793–799 discussion 804.

Halstead, S.B., O'Rourke, E.J., 1977a. Antibody-enhanced dengue virus infection in primate leukocytes. Nature 265, 739–741.

Halstead, S.B., O'Rourke, E.J., 1977b. Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. J. Exp. Med. 146, 201–217.

Halstead, S.B., Porterfield, J.S., O'Rourke, E.J., 1980. Enhancement of dengue virus infection in monocytes by flavivirus antisera. Am. J. Trop. Med. Hyg. 29, 638–642.

Halstead, S.B., Mahalingam, S., Marovich, M.A., Ubol, S., Mosser, D.M., 2010. Intrinsic antibody-dependent enhancement of microbial infection in macrophages: disease regulation by immune complexes. Lancet Infect. Dis. 10, 712–722.

Heaton, N.S., Randall, G., 2010. Dengue virus-induced autophagy regulates lipid metabolism. Cell Host Microbe 8, 422–432.

Heaton, N.S., Perera, R., Berger, K.L., Khadka, S., Lacount, D.J., Kuhn, R.J., Randall, G., 2010. Dengue virus nonstructural protein 3 redistributes fatty acid synthase to sites of viral replication and increases cellular fatty acid synthesis. Proc. Natl. Acad. Sci. U. S. A. 107, 17345–17350.

Henchal, E.A., Putnak, J.R., 1990. The dengue viruses. Clin. Microbiol. Rev. 3, 376–396.

Henchal, E.A., Gentry, M.K., McCown, J.M., Brandt, W.E., 1982. Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. Am. J. Trop. Med. Hyg. 31, 830–836.

Hoyvik, H., Gordon, P.B., Berg, T.O., Stromhaug, P.E., Seglen, P.O., 1991. Inhibition of autophagic-lysosomal delivery and autophagic lactolysis by asparagine. J. Cell Biol. 113, 1305–1312.

Khakpoor, A., Panyasrivanit, M., Wikan, N., Smith, D.R., 2009. A role for autophagolysosomes in dengue virus 3 production in HepG2 cells. J. Gen. Virol. 90, 1093–1103.

Kirkegaard, K., Taylor, M.P., Jackson, W.T., 2004. Cellular autophagy: surrender, avoidance and subversion by microorganisms. Nat. Rev. Microbiol. 2, 301–314.

Kliks, S.C., Nimmanitya, S., Nisalak, A., Burke, D.S., 1988. Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. Am. J. Trop. Med. Hyg. 38, 411–419.

Klionsky, D.J., Cuervo, A.M., Seglen, P.O., 2007. Methods for monitoring autophagy from yeast to human. Autophagy 3, 181–206.

Klomporn, P., Panyasrivanit, M., Wikan, N., Smith, D.R., 2011. Dengue infection of monocytic cells activates ER stress pathways, but apoptosis is induced through both extrinsic and intrinsic pathways. Virology 409, 189–197.

Krishnan, M.N., Sukumaran, B., Pal, U., Agaisse, H., Murray, J.L., Hodge, T.W., Fikrig, E., 2007. Rab 5 is required for the cellular entry of dengue and West Nile viruses. J. Virol. 81, 4881–4885.

Kurane, I., 2007. Dengue hemorrhagic fever with special emphasis on immunopathogenesis. Comp. Immunol. Microbiol. Infect. Dis. 30, 329–340.

Kurane, I., Ennis, F.E., 1992. Immunity and immunopathology in dengue virus infections. Semin. Immunol. 4, 121–127.

Lee, H.K., Iwasaki, A., 2008. Autophagy and antiviral immunity. Curr. Opin. Immunol. 20, 23–29.

Lee, Y.R., Lei, H.Y., Liu, M.T., Wang, J.R., Chen, S.H., Jiang-Shieh, Y.F., Lin, Y.S., Yeh, T.M., Liu, C.C., Liu, H.S., 2008. Autophagic machinery activated by dengue virus enhances virus replication. Virology 374, 240–248.

Levine, B., Klionsky, D.J., 2004. Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev. Cell 6, 463–477.

Li, J., Lee, B., Lee, A.S., 2006. Endoplasmic reticulum stress-induced apoptosis: multiple pathways and activation of p53-up-regulated modulator of apoptosis (PUMA) and NOXA by p53. J. Biol. Chem. 281, 7260–7270.

Lithanatudom, P., Leecharoenkiat, A., Wannatung, T., Svasti, S., Fucharoen, S., Smith, D. R., 2010. A mechanism of ineffective erythropoiesis in beta-thalassemia/Hb E disease. Haematologica 95, 716–723.

Mathers, C.D., Ezzati, M., Lopez, A.D., 2007. Measuring the burden of neglected tropical diseases: the global burden of disease framework. PLoS Negl. Trop. Dis. 1, e114.

Meijer, A.J., Codogno, P., 2006. Signalling and autophagy regulation in health, aging and disease. Mol. Aspects Med. 27, 411–425.

Mizushima, N., 2004. Methods for monitoring autophagy. Int. J. Biochem. Cell Biol. 36, 2491–2502.

Mizushima, N., 2007. Autophagy: process and function. Genes Dev. 21, 2861–2873.

Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M.D., Klionsky, D.J., Ohsumi, M., Ohsumi, Y., 1998. A protein conjugation system essential for autophagy. Nature 395, 395–398.

Modis, Y., Ogata, S., Clements, D., Harrison, S.C., 2004. Structure of the dengue virus envelope protein after membrane fusion. Nature 427, 313–319.

Mukhopadhyay, S., Kuhn, R.J., Rossmann, M.G., 2005. A structural perspective of the flavivirus life cycle. Nat. Rev. Microbiol. 3, 13–22.

Murgue, B., Roche, C., Chungue, E., Deparis, X., 2000. Prospective study of the duration and magnitude of viraemia in children hospitalised during the 1996–1997 dengue-2 outbreak in French Polynesia. J. Med. Virol. 60, 432–438.

Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B.A., Yuan, J., 2000. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature 403, 98–103.

Nimmerjahn, F., Milosevic, S., Behrends, U., Jaffee, E.M., Pardoll, D.M., Bornkamm, G. W., Mautner, J., 2003. Major histocompatibility complex class II-restricted presentation of a cytosolic antigen by autophagy. Eur. J. Immunol. 33, 1250–1259.

Nobukuni, T., Kozma, S.C., Thomas, G., 2007. hvps34, an ancient player, enters a growing game: mTOR Complex1/S6K1 signaling. Curr. Opin. Cell Biol. 19, 135–141.

Noda, T., Ohsumi, Y., 1998. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. J. Biol. Chem. 273, 3963–3966.

O'Sullivan, M.A., Killen, H.M., 1994. The differentiation state of monocytic cells affects their susceptibility to infection and the effects of infection by dengue virus. J. Gen. Virol. 75 (Pt 9), 2387–2392.

Paludan, C., Schmid, D., Landthaler, M., Vockerodt, M., Kube, D., Tuschl, T., Munz, C., 2005. Endogenous MHC class II processing of a viral nuclear antigen after autophagy. Science 307, 593–596.

Panyasrivanit, M., Khakpoor, A., Wikan, N., Smith, D.R., 2009. Co-localization of constituents of the dengue virus translation and replication machinery with amphisomes. J. Gen. Virol. 90, 448–456.

Perera, R., Kuhn, R.J., 2008. Structural proteomics of dengue virus. Curr. Opin. Microbiol. 11, 369–377.

Petiot, A., Ogier-Denis, E., Blommaart, E.F., Meijer, A.J., Codogno, P., 2000. Distinct classes of phosphatidylinositol 3′-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. J. Biol. Chem. 275, 992–998.

Puttikhunt, C., Kasinrerk, W., Srisa-ad, S., Duangchinda, T., Silakate, W., Moonsom, S., Sittisombut, N., Malasit, P., 2003. Production of anti-dengue NS1 monoclonal antibodies by DNA immunization. J. Virol. Meth. 109, 55–61.

Rico-Hesse, R., 2007. Dengue virus evolution and virulence models. Clin. Infect. Dis. 44, 1462–1466.

Rothman, A.L., Ennis, F.A., 1999. Immunopathogenesis of dengue hemorrhagic fever. Virology 257, 1–6.

Row, P.E., Reaves, B.J., Domin, J., Luzio, J.P., Davidson, H.W., 2001. Overexpression of a rat kinase-deficient phosphoinositide 3-kinase, Vps34p, inhibits cathepsin D maturation. Biochem. J. 353, 655–661.

Sakoonwatanyoo, P., Boonsanay, V., Smith, D.R., 2006. Growth and production of the dengue virus in C6/36 cells and identification of a laminin-binding protein as a candidate serotype 3 and 4 receptor protein. Intervirology 49, 161–172.

Sangkawibha, N., Rojanasuphot, S., Ahandrik, S., Viriyapongse, S., Jatanasen, S., Salitul, V., Phanthumachinda, B., Halstead, S.B., 1984. Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. Am. J. Epidemiol. 120, 653–669.

Seglen, P.O., Gordon, P.B., 1982. 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. Proc. Natl. Acad. Sci. U. S. A. 79, 1889–1892.

Sithisarn, P., Suksanpaisan, L., Thepparit, C., Smith, D.R., 2003. Behavior of the dengue virus in solution. J. Med. Virol. 71, 532–539.

Smith, D.R., Khakpoor, A., 2009. Involvement of the liver in dengue infections. Dengue Bull. 33, 75–86.

Suksanpaisan, L., Cabrera-Hernandez, A., Smith, D.R., 2007. Infection of human primary hepatocytes with dengue virus serotype 2. J. Med. Virol. 79, 300–307.

Suksanpaisan, L., Susantad, T., Smith, D.R., 2009. Characterization of dengue virus entry into HepG2 cells. J. Biomed. Sci. 16, 17.

Thepparit, C., Smith, D.R., 2004. Serotype-specific entry of dengue virus into liver cells: identification of the 37-kilodalton/67-kilodalton high-affinity laminin receptor as a dengue virus serotype 1 receptor. J. Virol. 78, 12647–12656.

Tooze, S.A., Yoshimori, T., 2010. The origin of the autophagosomal membrane. Nat. Cell Biol. 12, 831–835.
Trincheri, N.F., Follo, C., Nicotra, G., Peracchio, C., Castino, R., Isidoro, C., 2008. Resveratrol-induced apoptosis depends on the lipid kinase activity of Vps34 and on the formation of autophagolysosomes. Carcinogenesis 29, 381–389.
van der Schaar, H.M., Rust, M.J., Waarts, B.L., van der Ende-Metselaar, H., Kuhn, R.J., Wilschut, J., Zhuang, X., Smit, J.M., 2007. Characterization of the early events in dengue virus cell entry by biochemical assays and single-virus tracking. J. Virol. 81, 12019–12028.
Vaughn, D.W., Green, S., Kalayanarooj, S., Innis, B.L., Nimmannitya, S., Suntayakorn, S., Endy, T.P., Raengsakulrach, B., Rothman, A.L., Ennis, F.A., Nisalak, A., 2000. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J. Infect. Dis. 181, 2–9.
Wang, W.K., Chen, H.L., Yang, C.F., Hsieh, S.C., Juan, C.C., Chang, S.M., Yu, C.C., Lin, L.H., Huang, J.H., King, C.C., 2006. Slower rates of clearance of viral load and virus-containing immune complexes in patients with dengue hemorrhagic fever. Clin. Infect. Dis. 43, 1023–1030.
Won, J.C., Jang, P.G., Namkoong, C., Koh, E.H., Kim, S.K., Park, J.Y., Lee, K.U., Kim, M.S., 2009. Central administration of an endoplasmic reticulum stress inducer inhibits the anorexigenic effects of leptin and insulin. Obesity (Silver Spring) 17, 1861–1865.
Yang, Z., Klionsky, D.J., 2010. Mammalian autophagy: core molecular machinery and signaling regulation. Curr. Opin. Cell Biol. 22, 124–131.
Yla-Anttila, P., Vihinen, H., Jokitalo, E., Eskelinen, E.L., 2009. 3D tomography reveals connections between the phagophore and endoplasmic reticulum. Autophagy 5, 1180–1185.
Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., Mori, K., 2001. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 107, 881–891.