INFLUENZA A VIRUS STIMULATES AUTOPHAGY TO UNDERMINE HOST CELL IFN-β PRODUCTION

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ABSTRACT

Autophagy is originally described as the main catabolic pathway responsible for maintaining intracellular nutritional homeostasis that involves the formation of a unique vacuole, the autophagosome, and the interaction with the lysosome. Recent evidence indicated the essential role of the host autophagy, in enhancing influenza A virus (IAV) replication in human lung epithelial cells. Here, findings demonstrate that IAV infection increased levels of the autophagosomal marker "microtubule-associated protein light chain 3-II" (LC3-II), at early stage of infection. Further, knockout of Atg5, the crucial components of autophagosome formation, and siRNA-mediated depletion of autophagy related genes Atg9, Atg12, Atg16, provide further evidence on the essential and supportive role of autophagy in IAV replication. Interestingly, in autophagy-deficient cells (Atg5-/- MEFs), and A549 cells lacking autophagy levels of host protective type I interferons, in particular IFN-β, were increased during infection. Taken together, these data further confirm the beneficial role of autophagy for IAV infection may be via regulation of IFN-β production and highlights autophagosome formation as a potential novel antiviral target.

Keywords: Influenza A virus, Autophagy, IFN-β production.

INTRODUCTION

Influenza A virus (IAV) is a member of the family Orthomyxoviridae (Gatherer, 2009) and like other orthomyxoviruses, has a single-stranded RNA genome with the negative strand type

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(Neumann, Brownlee et al., 2004) and (Gatherer, 2009). IAV is associated with great pandemic infections (Gatherer, 2009; Horimoto & Kawaoka, 2001) and considered one of the significant public health threats, particularly in children and the elderly (Barker & Mullooly, 1980; Singleton, Wortley et al., 2004; Barker & Mullooly, 1982). IAV infection induces a variety of intracellular signaling pathways that are either antiviral or required to ensure efficient replication. A prominent cellular antiviral event is the activation of the cascade that produces type I interferons (IFN): IFN-α and IFN-β (Ludwig & Wolff, 2009a; Garcia-Sastre, 2004). Consequently, an appreciated number of the so-called IFN responsive genes (ISGs) are activated (Haller, Arnheiter et al., 1980; Ehrhardt, Marjuki et al., 2006; Almeida, de Oliveira et al., 2008), such as $Mx1$ gene, which encodes for Mx1 protein, a member of the dynamin-like large guanosine triphosphatases (GTPases). Moreover, virus-supportive signaling processes activated upon infection are the IKK/NF-κB (Pauli, Schmolke et al., 2008; Ludwig & Planz, 2008) and PI3K/Akt (Ehrhardt, Marjuki et al., 2006) and (Ludwig, Wang et al., 2002). Recently, important evidence indicated the essential role of additional cellular machinery, the host autophagy, in enhancing IAV replication, and to facilitate the virus release via the apoptosis process regulated by influenza M2 protein (Mclean, Datan et al., 2009; Zhou, Jiang et al., 2009; Gunnage & Munz, 2009). The autophagy processes is characterized by accumulation of double-membraned cytoplasmic vacuoles regulating degradation events and recycling of cellular contents by delivering cytoplasmic materials, required for degradation, to lysosomes. Moreover, autophagy has been shown to play an important role in cell growth, development and disease pathology (Yorimitsu & Klionsky, 2005). Activation of autophagy machinery is one of the down-stream events of a class III phosphatidylinositol 3-kinase (PI 3-K) complex. The conjugations between more than 20 Atg proteins have been identified in autophagosome formation recruited from either endoplasmic reticulum (ER) or pre-autophagosomal structure (PAS). Atg5 and Atg12 are required to form the autophagy vacuoles by recruiting other proteins to autophagosomal membrane from cytosol. The generation of phosphatidylinositol (3, 4, 5)-triphosphate (PIP-3, 4, 5) which recruits additional Atg proteins such as Atg8 (LC3) in conjugation system is essential for autophagosome elongation and maturation.
Finally, autophagosome fuses with lysosome containing hydrolytic enzymes that degrade the contents which are recycled for use in protein synthesis and energy production (Orvedahl & Levine, 2008; Mijaljica, Prescott et al., 2007; Yen & Klionsky, 2007; Legakis, Yen et al., 2007). The current data reveals the crucial role of autophagic pathway in IAV infection and demonstrates that autophagy plays an essential role in regulating IFN-β corroborated by the strong production of IFN-β in autophagy deficient cells and the possible interaction of viral NS1 and NP proteins with autophagy upon infection.

MATERIALS AND METHODS

Cell lines
Human type II alveolar epithelial A549 cells (ATCC CCL-185), MDCK (CCL-34, ATCC-LGC), 293T, WT MEFs and autophagy-deficient (Atg5-knockout) MEFs, Which are kindly obtained from Thomas Meyer, Max-Planck Institute for Infection Biology, Berlin, Germany, were routinely grown in cell growth medium, which consists of Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen, 10938) supplemented with 200 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin G, 100 µg/ml streptomycin and 10% FBS, at 37 °C and 5% CO₂ in a humidified tissue culture chamber. MEFs were generously provided by Noburo Mizushima (Department of Physiology and Cell Biology, Tokyo Medical and Dental University, Tokyo Japan).

Primary IAV infection and progeny infectivity assays
Host cells were washed with PBS and then infected with the IAV (A/WSN/33) at the indicated MOIs diluted in PBS supplemented with 0.2% BSA, for 45 min at room temperature. Cells were washed again and incubated for the indicated time periods at 37 °C in cell growth medium supplemented with 0.2% BSA, unless otherwise indicated. To quantify infectious viruses released in the supernatant during the primary infection, a luciferase-based reporter system was used to transfect 293T cells (Lutz, Dyall et al., 2005), and then virus-dependent luciferase activity were measured using a firefly luciferase substrate, as previously described (Dyer, Ferrere et al., 2000). For Plaque assay, MDCK cells were seeded in 6well plates at a concentration of 1 million cells per well. Then the cells were infected
for one hour at RT with six different dilutions of the supernatant of virus-infected cells culture. The infectious culture was removed and 2 ml of MEM media contains 500 µl 10% agar solutions, 5 µl dextrane and 5 µl BSA was added to each well. The infected cells were incubated for two days and then were fixed overnight with PBS contains 3.7% formaldehyde. For staining, the agar layer was removed and cells were stained with 0.1% crystal violet dissolved in 20% ethanol. The virus plaques were counted manually and plaque forming units per ml was calculated.

**Quantitative RT-PCR**

Total RNA from ATG9, ATG12, and ATG16 transfected and infected A549 cells was extracted 24 h p.i. and purified using TRIzol (Invitrogen) and the RNeasy Mini Kit (Qiagen). Quantitative RT-PCR (qRT-PCR) was used to detect the relative expression of the previous ATGs and IFN-β in A549 cells upon influenza A virus infection by using the QuantiTect SYBR Green PCR Kit (Qiagen) and the following oligonucleotides specific for ATG9, ATG12, ATG16, IFN-β and GAPDH (Table 1).

**Table 1:** Primers sequences and its targeted genes detected by qRT-PCR.

<table>
<thead>
<tr>
<th>Primers sequences</th>
<th>Gene target</th>
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<tbody>
<tr>
<td>APG9L1_for: 5’- ATGTTCGCCAGCATGGTCA-3’&lt;br&gt;APG9L1_rev:5’- GTTGGGTGATGGCAAAGTGCAT-3’</td>
<td>ATG9</td>
</tr>
<tr>
<td>hAPG12_for:5’-CACGAACCATCAAAGGACTCA-3’&lt;br&gt;hAPG12_rev:5’-TTTGTGGTTCATCCCCACG-3’</td>
<td>ATG12</td>
</tr>
<tr>
<td>ATG16L1_for:5’- TGATGGCACATGGAATGACAA-3’&lt;br&gt;ATG16L1_rev:5’-GAGTCGCTTAGTGGCTGCTC-3’</td>
<td>ATG16</td>
</tr>
<tr>
<td>IFNβ1_for:5’-CAG CTC TTT CCA TGA GCT AC-3’&lt;br&gt;IFNβ1_rev:5’-CAG CCA GTG CTA GAT GAA TC-3’</td>
<td>IFN-β</td>
</tr>
<tr>
<td>GAPDH_for:5’-GGTATCGTGGAAGGACTCATGAC-3’&lt;br&gt;GAPDH_rev:5’-ATGCCAGTGAGCTTCCCGTTCAG-3’</td>
<td>GAPDH</td>
</tr>
</tbody>
</table>

**Fluorescence confocal microscopy**
At indicated time points, host cells plated onto cover slips were fixed with PBS containing 3.7% formaldehyde and permeabilized with PBS containing 0.1% triton X-100 for 10 min at room temperature. Cells were sequentially incubated for 1 h at room temperature with mouse monoclonal antibodies against viral proteins NP and NS1 (AbD Serotech, MCA4688G) or rabbit anti-LC3B (Sigma, L7543) and then incubated for an additional 1 h with the donkey anti-mouse Cy5 (Dianova, 715-175-020), Cy2 (Dianova, 715-225-020) conjugated antibodies and goat anti-rabbit Cy3 (Dianova, 111-165-045), respectively. Cover slips were then mounted onto glass slides using Mowiol and examined by Leica TCS-SP laser scanning confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a krypton/argon laser. Photomicrographs were processed using Adobe Photoshop 6.0 (Adobe Systems) and Microsoft Power-Point.

**ELISA**

IFN-β secretion after IAV infection was assessed using the Mouse IFN-β Elisa Kit (Invitrogen, 42400-1), following the manufacturer’s instructions. Briefly, WT and Atg5 knock-out MEF cells were seeded into 96-well-plates at a concentration of 10 x 10^3 cells/well. Cells were then infected with IAV (MOI 5) for 2, 4 or 8 h. Supernatants were used to measure the concentration of secreted IFN-β using a microplate reader (450 nm). Results were processed using Soft-Max program V5 (Molecular Devices, CA, USA).

**RNA interference**

A549 cells were transfected with siRNA sequences targeting Atg9, Atg12 or Atg16 using the RNAiFect transfection kit (Qiagen, 301605). Two days post-transfection, cells were infected with IAV (MOI equal 1) and incubated for an additional 24 h. The number of infectious viral particles in the culture supernatant was quantified as detailed above. Efficiency of gene silencing was validated using real-time PCR as previously described (Machuy, Thiede et al., 2005). The sequences targeted by siATG5, siATG9, siATG12, siATG16, siRaf-1, siNP, and siLuciferase (human-unrelated siRNA used as a control) and knockdown efficiency, indicated by relative gene expression, are listed in table 2.

**Statistical analysis**

SDS 2.2.2 software was used to analysis the data from the real time PCR which developed to drive delta-delta Ct using the following...
equations; (ΔΔCt) = Ct-sample – Ct-control. (ΔΔCt) =

RESULTS

Autophagy is upregulated by IAV infection
To investigate the kinetics of autophagic activation in response to IAV
infection, the expression of autophagy related genes were monitored at
RNA levels using quantitative real time. Expression levels of Atg9, Atg12, and Atg16 within human lung epithelial A549 cells infected
with IAV (IN) is extremely higher than control noninfected cells (NI)
(Fig. 1). Notably, the highest expression level of the autophagy related
genes was shown in Atg12 expression indicated the crucial role of
Atg12 in the initiation step of autophagosomes formation.

Autophagic LC3 protein associates with IAV proteins
To investigate the possible interaction of autophagy and influenza
virus proteins, NS1, NP proteins and LC3 protein were stained using
immunofluorescence microscopy of A549 cells infected with IAV
(MOI 1) at 2, 4 and 8 h p.i. The result revealed that both viral NS1 and
NP proteins colocalized with autophagosomes at indicated early
infection time points (Fig. 2A and B) suggesting that autophagy
proteins could promote viral protein translation and replication.
Together these data further confirm that IAV induces autophagy
which cooperates with viral proteins NP and NS1 during viral
replication.

Figure (1): Autophagy is upregulated upon IAV infection. Relative
expression of autophagy related genes Atg9, Atg12 and Atg16 in
A549 cells highly infected with IAV (IN) compared with control
noninfected cells (NI). Error bars indicate the stander deviation (SD) of three different replicates.

\[ \text{A.} \quad \text{B.} \]

\[\begin{array}{ccc}
\text{LC3} & \text{NS1} & \text{LC3} \\
\text{NI} & 2\text{hpi} & \text{NI} \\
\text{4hpi} & 2\text{hpi} & \text{4hpi} \\
\text{8hpi} & \text{8hpi} & \text{8hpi}
\end{array}\]

**Figure: (2): Autophagy associates with viral NS1 and NP proteins.** (A and B) Representative confocal images depicting A549 cells infected with influenza A/WSN/33 MOI 1 and revealing the expression of viral NS1 (green), viral NP (blue) and LC3 (red) at the indicated time points. Immunofluorescent images are representative of two independent experiments. Data is representative of two independent experiments.

**Autophagy is required for IAV replication**

Due to the association observed between IAV infection and autophagy, the impact of autophagy related gene Atg5 was determined during viral replication through the infection of Atg5 knockout mouse embryonic fibroblast cells (MEF cells). The number of virus progeny was then quantified by traditional plaque assay using MDCK cells. The result showed more than two folds reduction of virus progeny in Atg5 knockout MEFs compared to wild type cells indicated by plaque forming units per ml (PFU/ml) (Fig. 3).
Figure (3): Autophagy is required for IAV replication. Plaque forming units (per ml) of infectious virus particles produced from Atg5 knock-out MEFs (KO) cells infected with MOI 0.05 compared to wild type MEFs after 24 h p.i using plaque assay. Error bars indicate the standard deviation (SD) of three different replicates.

To further confirm the essential role of autophagy related genes in IAV replication, A549 cells were transfected with siRNAs induced deletion of autophagy genes expression such Atg9, Atg12 and Atg16. The knock-down efficiency of the siRNAs listed in table 2 showed almost 90% inhibition of gene expression indicated by qRT-PCR. Forty eight hours later, the transfected cells were infected with low MOI (0.05) of influenza virus. The number of infectious viral particles was indicated by using an established virus dependent luciferase assay (Lutz, Dyall et al., 2005) and traditional plaque assay of A549 lacking autophagy related genes, Atg9, Atg12 and Atg16 showed strong reduction of viral progeny compared to control infected cells (Fig.4A and B). Taken together, these results indicate that autophagy plays an essential role to facilitate IAV replication in both human and mouse system.

Table 2: Knockdown efficiency and oligonucleotide sequences of siRNAs targeted the indicated autophagy related genes.

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Target sequences</th>
<th>Knockdown efficiency %</th>
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<tbody>
<tr>
<td>ATG9</td>
<td>CCGGCTTATCAAGTTCATCTA</td>
<td>88</td>
</tr>
<tr>
<td>ATG12</td>
<td>CTCAGAACAGTTGTATTTTA</td>
<td>92</td>
</tr>
<tr>
<td>ATG16</td>
<td>CCAACAGAAGTTGATTTGAAA</td>
<td>88</td>
</tr>
<tr>
<td>Luciferase</td>
<td>AACUUACGCUAGUACUUAGA</td>
<td>100</td>
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Figure (4): IAV replication requires autophagosomes formation. (A) Quantitative of infectious virus particles using virus-dependent luciferase assay. Error bars indicate the (SD) of three different replicates (B) Plaque forming units (per ml) of infectious virus particles. Error bars indicate the (SD) of two different experiments. Data is representative of two independent experiments.

Autophagy regulates IFN-β production inspired by IAV

To address the role of autophagosomes formation in regulation of IFN-β production following IAV infection, Atg5 knockout MEFs as well as A549 cells lacking autophagy related genes Atg9, Atg12, and Atg16, were infected with high dose of influenza A virus (MOI =2). Then, levels of IFN-β in wild type and Atg5 -/- MEFs over 8 h were monitored using ELISA assay (Fig. 5A). In response to IAV infection Atg5-deficient cells produced considerably higher amounts of IFN-β compared with the WT (> 25-fold, >15-fold and >10-fold in IFN-β at 2, 4 and 8 h p.i., respectively). Similar result was obtained in A549 cells lacking autophagy related genes Atg9, Atg12, and Atg16 indicated by relative expression of IFN-β at RNA level using qRT-PCR. Interestingly, Cells transfected with mixture of siRNAs against the indicated autophagy related genes showed strong expression of IFN-β upon infection (Fig. 5B).
**Figure (5):** Autophagy regulates IFN-β production stimulated upon infection. (A) Concentration of IFN-β production in MEFs cells infected with MOI 1 of IAV using Mouse IFN-β Elisa Kit. Error bars indicate the SD of two independent experiments (B). Relative expression of IFN-β at RNA level in infected A549 cells lacking autophagy related genes Atg9, Atg12, and Atg16 compared with siLuci transfected and infected cells. Error bars indicate the standard deviation SD of three different replicates. Data is representative of two independent experiments. Collectively, these data suggest that knockdown of autophagy related genes enhance the innate immune response in IAV infected cells and indicate that autophagy is necessary for the completion of the IAV developmental cycle.

**DISCUSSION**

The current data further confirms the essential and supportive role of autophagy on IAV replication through the possible involvement of autophagy in viral NS1-modulated IFN-β transcription. Like other viruses, IAV commandeers host cells and exploits their machineries for successful replication. Recently, Autophagy has been found to play an essential role in enhancing IAV replication (Zhou, Jiang et al., 2009). Autophagy has also been linked to the process of the virus
release by the induction of influenza M2 protein-regulated apoptosis (Zhou, Jiang et al., 2009; Gunnage & Munz, 2009). Autophagy involves maturation of early autophagosome into a degradative vacuole by fusion with lysosomes called the autolysosome. This is essential to degradation of sequestered intracellular molecules and damaged organelles (Mizushima & Klionsky, 2007; Yoshimori & Noda, 2008; Monastyrksa, Rieter et al., 2009). This conserved machinery plays a key role in immuno-protection against different invaders, including pathogenic bacteria, intracellular parasites and some viruses like herpes simplex and tobacco mosaic viruses (Amano, Nakagawa et al., 2006; Gutierrez, Munafò et al., 2004; Huang & Klionsky, 2007; Kirkegaard, Taylor et al., 2004; Levine, 2007; Levine & Deretic, 2007; Nakagawa, Amano et al., 2004; Yap, Ling et al., 2007). In contradiction to these protective roles, various bacteria and viruses, such as poliovirus, mouse hepatitis virus and IAV, ensure efficient replication by hijacking the autophagic machinery (Birmingham, Higgins et al., 2008; Mizushima & Klionsky, 2007; Ogawa, Yoshimori et al., 2005; Taylor & Kirkegaard, 2008; Zhou, Jiang et al., 2009). In the current study, experiments demonstrating the significance of autophagy in the replication of IAV were reproduced. Moreover, additional approaches were used here to corroborate findings related to the role of autophagy and to further analyze IAV-autophagy interaction. These approaches include the use of autophagy-deficient MEFs and autophagy genes knockdown within human A549 cells, in which aberrant reproductive cycle of IAV was observed in.

Notably, some pathogens and viruses that interact with autophagic machinery may disrupt autophagosome maturation, while others not. Autophagosomes can mature into degradative autolysosomes in the case of herpes simplex virus, group A Streptococcus and S. Typhimurium infection. However, the RNA viruses poliovirus and mouse hepatitis virus seem to block maturation process of autophagosomes (Prentice, Jerome et al., 2004; Jackson, 2005). Similarly, a recent study demonstrated the ability of IAV to block autophagosome fusion with lysosomes and also showed that viral M2 alone is sufficient to induce the inhibition of autophagosome maturation into autolysosomes (Gunnage & Munz, 2009). The present study further shows that accumulation of autophagosome structures is a hallmark of IAV infection, shown by autophagy related gene
expression at RNA levels and microscope-aided visualization of LC3 accumulation. These membranes could provide a scaffold for the IAV virus to anchor their protein complexes, as suggested by others for other viruses (Prentice, Jerome et al., 2004; Jackson, 2005). Confocal microscopic analysis revealed a direct association of the IAV proteins (NP and NS1) and autophagosomes. NS1 is encoded by the viral NS gene, which encodes also for the nuclear export protein (NEP), also known as NS2 protein, which mediates the export of viral ribonucleoproteins (vRNPs) (Paragas, Talon et al., 2001). The NS1 protein includes two major domains; the N-terminal RNA-binding domain and the C-terminal effector domain (Krug, Yuan et al., 2003). The RNA-binding domain directly associates with the host factor RIG-I, apparently to induce type-1 IFN during viral infection (Wang, Basler et al., 2002; Pichlmair, Schulz et al., 2006). Based on the observed association of NS1 with autophagosomes, it is reasonable to hypothesize that autophagosomal membrane may serve as a platform to facilitate interaction of NS1 with host cell factors for the benefit of the virus. An attractive host cell candidate that may interact with NS1 at autophagosomes is RIG1. This autophagosome-mediated interaction could be important for the regulation of the cellular immune response. Interestingly, the strong production of IFN-β in autophagy deficient MEFs cells upon IAV infection reveals the essential role of autophagy to regulate the transcription of IFN-β following infection. Therefore, the current result provides a link between the autophagic machinery and innate immune signaling against IAV infection. Additionally, this result provides a framework for the design and development of antiviral therapeutic claims by targeting autophagosomes formation.

The type I interferon, IFN-β, is known to play a pivotal role in host defense against IAV infection (Garcia-Sastre, 2004; Ludwig & Wolff, 2009b). IFN-β-induced genes, such as Mx genes, are known to display a strong antiviral activity against IAV (Haller, Staeheli et al., 2007). Very recent studies achieved that HCV exploits autophagy machinery to escape the innate immune response resulted in supporting viral replication (Ke & Chen, 2011; Estrabaud, De et al., 2011; Shrivastava, Raychoudhuri et al., 2011). Interestingly, disruption of autophagy related genes such as Atg6 and Atg7 in HCV infected hepatocytes activates the interferon signaling pathway, particularly IFN-β production (Shrivastava, Raychoudhuri et al., 2011). In the current
study, similar observations was found via disturbing of Atg9, Atg12 and Atg16 in infected A549 cells indicating that autophagosomes formation plays a major role in regulation of IFN-β production upon influenza virus infection. Additionally, IFN-β was markedly upregulated in autophagy-deficient MEFs cells upon IAV infection. Moreover, knockdown of autophagy related genes in human A549 cells displays an aberrant of developmental cycle of IAV, further reiterating the importance of autophagy for IAV replication.

In summary, the present data indicates that autophagy is upregulated in IAV-infected host cells to ensure a successful replication cycle of the virus may through the regulation of IFN-β production.

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