

## New Phenomenon

# Manipulation of autophagy by HCMV infection is involved in mTOR and influences the replication of virus

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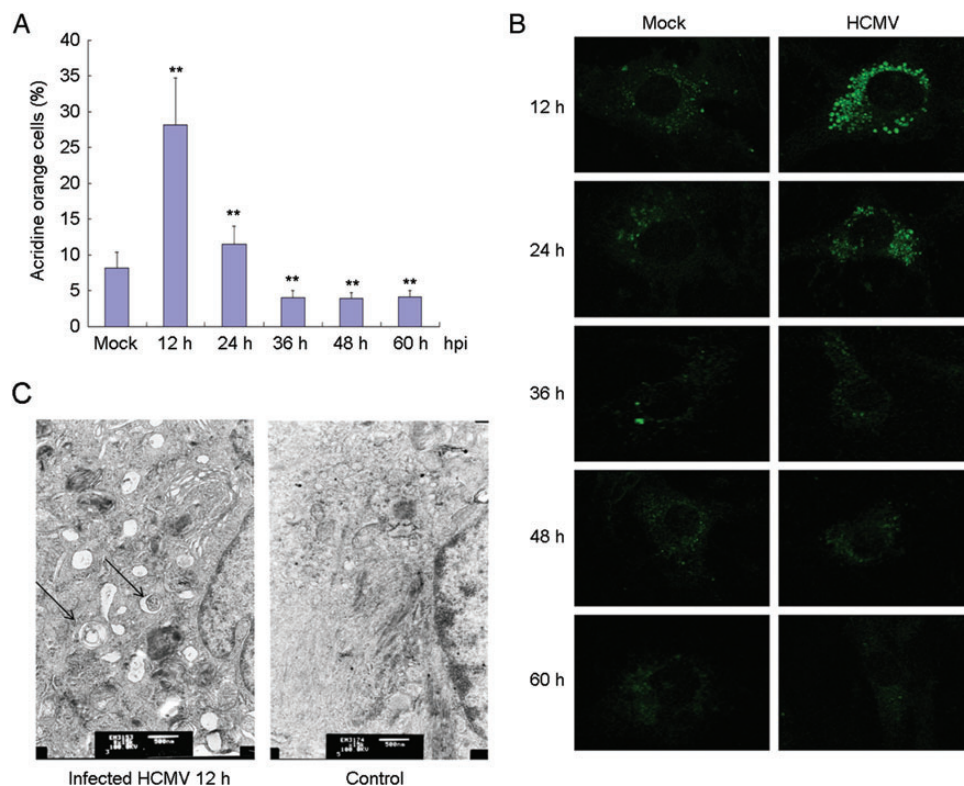
Autophagy is an evolutionarily conserved cellular degradation process, in which intracellular components are digested by the lysosomal pathway. While this process maintains cellular homeostasis by constitutively recycling cytoplasmic organelles and proteins, it can also be stimulated by environmental stress conditions, such as starvation, oxidative stress, and accumulation of misfolded proteins. During viral infection, autophagy can act as a host surveillance mechanism, where viral antigens are delivered to endosomal and lysosomal compartments enriched in immune sensors that can activate autophagy upon stimulation. Viruses, in turn, have adapted several ways in which to evade this antiviral mechanism. While many viruses block various points along the autophagic pathway, some actively subvert autophagy for their own benefit. It has been proposed that viral manipulation of autophagy might directly and/or indirectly facilitate most stages of the viral lifecycle. In some circumstances, autophagy can act as a pro-viral pathway promoting viral replication or exiting from cells [1]. For example, hepatitis C virus (HCV) induces autophagosome accumulation, but then inhibits their ability to fuse with lysosomes [2]. HCV may also require autophagy during early infection [3]. For human cytomegalovirus (HCMV), however, there is controversy surrounding whether it stimulates autophagy during early infection [4] or inhibits autophagy [5,6].

In the present study, we investigated whether and how HCMV modulates autophagy *in vitro* over time. Human erythroleukemia (HEL) cells were infected with HCMV at 1 plaque forming unit (PFU)/cell for 0, 12, 24, 36, 48, and 60 h and stained with acridine orange (Sigma, St Louis, USA) to evaluate autophagosome formation by flow cytometry. HCMV first triggered, then later inhibited, acidic vesicle and autolysosome accumulation, with acridine

orange ratios of  $8.2\% \pm 2.1\%$ ,  $28.2\% \pm 6.5\%$ ,  $11.5\% \pm 2.5\%$ ,  $4.0\% \pm 1.1\%$ ,  $3.9\% \pm 1.2\%$ , and  $4.0\% \pm 0.9\%$  at 0, 12, 24, 36, 48, and 60 h, respectively [Fig. 1(A)].

To further evaluate the effect of HCMV on autophagy, we determined the redistribution pattern of the autophagy marker LC3 in a similar time-course experiment by transfecting a GFP-LC3 plasmid (Origene Technologies, Rockville, USA) into HEL cells. We observed a markedly increased punctate pattern of LC3 at 12 h in HCMV infected cells, and the diffuse and weakly fluorescent pattern at 48 h in control cells [Fig. 1(B)]. Furthermore, HEL cells developed autophagosome-like characteristics at 12 h, including single- and double-membrane vacuoles containing intact and degraded cellular debris [Fig. 1(C)]. Thus, these results suggested that HCMV infection induces autophagy at the early phase of infection, but blocks autophagy at the late phase. LC3-II proteins, which are formed from LC3-I precursor proteins in the cytosol, upon autophagy induction correlate with the autophagosome numbers. We used western blot analysis to evaluate the accumulation of LC3-II proteins, and confirmed these results (antibodies were obtained from Novus Biologicals, Littleton, USA) [Fig. 2(A)].

The regulation of autophagy is complex. Activation of the protein kinase, mammalian target of rapamycin (mTOR), is important not only in the signaling pathway that downregulates autophagy but also for protein synthesis. Since we observed that autophagy decreased at the later phase of HCMV infection, we predicted that mTOR might also be activated at these time points. To monitor mTOR activation, we detected phosphorylation of mTOR substrates involved in protein synthesis, ribosomal S6 protein kinase (p70S6K), and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), at residues Thr389 and Thr37/46, respectively, by



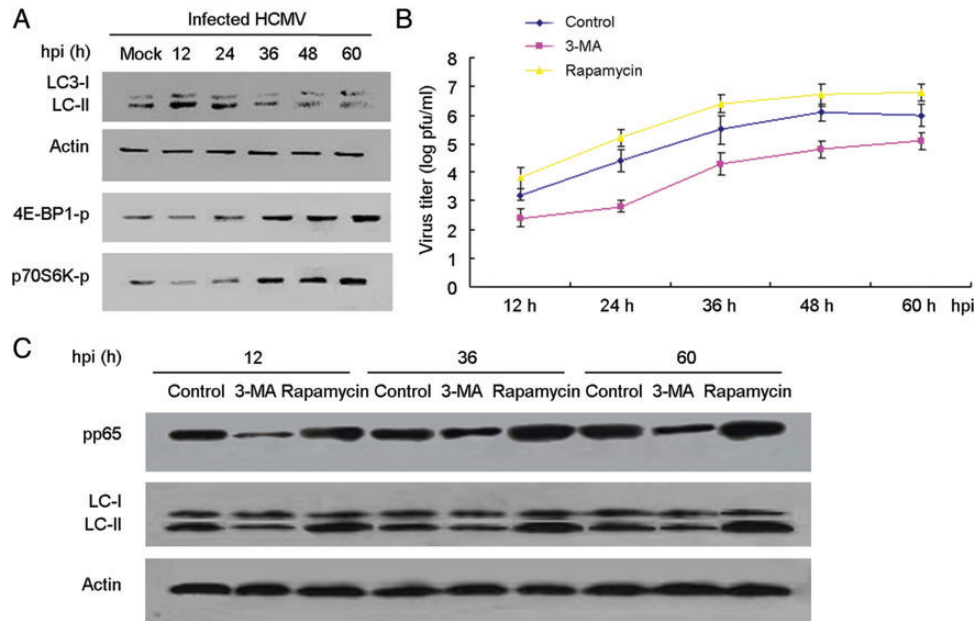
**Figure 1 Modulation of autophagy by HCMV in HEL cells** Cells were routinely infected with 1 PFU/cell of HCMV for 12, 24, 36, 48, and 60 h. Cells were then stained with acridine orange. (A) Autophagic vacuoles were quantified by flow cytometry.  $**P < 0.01$  compared with control. (B) HCMV induced punctate GFP-LC3 distribution in HEL cells. At 24 h after transient transfection of GFP-LC3, HEL cells were routinely infected with 1 PFU/cell of HCMV for 12, 24, 36, 48, and 60 h, and then analyzed for fluorescence. Images were captured using a fluorescence microscope ( $\times 1000$ ). (C) Autophagic vacuoles containing cellular debris appeared at 12 h post-infection. HEL cells were routinely infected with 1 PFU/cell of HCMV for 12 h and examined by transmission electron microscopy. The arrowhead indicates the single- and double-membrane vesicles containing intact and degraded cellular debris ( $\times 15,000$ ).

western blot [Fig. 2(A)]. Indeed, both proteins were more highly phosphorylated at 36 h, suggesting that the mTOR signaling pathway was activated at the later phases of HCMV infection and probably contributed to inhibiting autophagy during this late phase.

We hypothesized that HCMV manipulates the autophagic pathway to benefit its own replication. To address this, we evaluated the influence of autophagy on HCMV replication. We treated the host HEL cells with 10 mM 3-methyladenine (3-MA) (Sigma), an autophagy inhibitor, and found that extracellular viral titers in the culture supernatant decreased at 12, 24, 36, 48, and 60 h after infection compared with untreated infected cells [Fig. 2(B)], suggesting that HCMV-mediated induction of the autophagic pathway is beneficial for HCMV replication in host cells. To further test this, we induced autophagy by inhibiting the mTOR pathway with 100 nM rapamycin (Sigma), and monitored viral titers. Consistent with our previous results, we found that autophagy benefited HCMV replication, as extracellular viral titers markedly increased beginning at 12 h after infection [Fig. 2(B)]. Western blot analysis demonstrated that

3-MA and rapamycin both had similar effects on viral replication (HCMV pp65) (Santa Cruz, Santa Cruz, USA) [Fig. 2(C)]. However, these experiments do not exclude the possibility of other effects of 3-MA or rapamycin, which are independent of regulating autophagy and these will be addressed in the future.

Taken together, these results indicate that HCMV infection of HEL cells induces autophagy in a time-dependent manner. Moreover, they show that HCMV infection induces autophagy as early as 12 h and then inhibits autophagy after 36 h. mTOR signaling is a complex process that affects several crucial cellular functions, such as protein synthesis and autophagy. We observed that HCMV infection induced 4E-BP1 and p70S6K phosphorylation after 36 h, indicating that HCMV stimulated the mTOR signaling pathway. However, there is still controversy as to whether autophagy facilitates viral replication, which requires further investigation, perhaps by manipulating autophagy at specific times during infection. Based on the data shown here, we predict that inducing autophagy by rapamycin will increase viral titers even if administered at the late phase of infection



**Figure 2 Manipulation of autophagy influences the replication of virus** (A) HCMV infection modified LC3 as well as 4E-BP1 (4E-BP1-P) and p70S6K (p70S6K-P) phosphorylation. HEL cells were infected with HCMV, and cell lysates were analyzed for LC3, 4E-BP1-P, and p70S6K-P modification at various times post-infection. (B,C) Inducing or inhibiting autophagy affects extracellular HCMV titers. HEL cells were routinely infected with 1 PFU/cell of HCMV for 12, 24, 36, 48, and 60 h with an autophagy inhibitor, 3-MA (10 mM), or inducer, rapamycin (100 nM). (B) Supernatant was collected, and viral titers were determined by plaque assay. (C) The effect of 3-MA and rapamycin on the expression of HCMV pp65 and autophagy-related LC3 proteins is shown by western blot.

(when mTOR is most active); inhibiting autophagy by 3-MA at this late stage would no longer reduce viral titers. Overall, the data in this study suggest that HCMV infection-induced autophagy does facilitate its own replication in host cells via mTOR, and this conclusion is supported by our results obtained from inhibiting or inducing autophagy by 3-MA or rapamycin treatment, respectively.

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