

# The influence of HCMV infection on autophagy in THP-1 cells

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## Abstract

**Background:** Human cytomegalovirus (HCMV) infection is very common and latency can be reactivated in the future. And it can alter the intracellular environment, similar to other herpesviruses, for viral replication and survival. The aim of this study was to investigate the influence of HCMV infection on autophagy in human acute monocytic leukemia cell line (THP-1 cells).

**Methods:** Reverse transcription polymerase chain reaction (RT-PCR), western blot, and transmission electron microscope (TEM) were used to examine autophagy level. The concentrations of autophagy-related proteins Beclin 1, Atg5, and the light chain three (LC3) were counted when compared with actin level.

**Results:** The expression levels of Beclin1, Atg5, and LC3II mRNAs increased gradually between 1 and 5 days(d) postinfection (p.i.) and subsequently decreased little by little when compared with the control THP-1 cells. However, results of western blot analysis displayed that the level of LC3II increased gradually after 1 day p.i. and decreased at 7 days after infection. But the levels of Atg5 and Beclin1 increased gradually after 2 days p.i. and began to decrease at 5 days after infection, respectively.

**Conclusion:** These results suggested that HCMV infection can facilitate the autophagy and autophagy level may decrease in latent phase. More studies on the relationship between HCMV latency and autophagy are needed to determine the role of autophagy in HCMV latent infection that may help find out a therapeutic approach for clinical treatment.

**Abbreviations:** d = day, HCMV = human cytomegalovirus, IE = immediate-early, LC3 = the light chain three, LC3 = the light chain three, p.i. = postinfection, RT-PCR = reverse transcription polymerase chain reaction, TCID50 = median tissue culture infectious dose, TEM = transmission electron microscope, THP-1 = human acute monocytic leukemia cell line.

**Keywords:** autophagy, HCMV, infection, latency, THP-1

## 1. Introduction

Human cytomegalovirus (HCMV), known as human herpesvirus 5 (HHV-5), belongs to  $\beta$  herpesviruses. HCMV infection is widespread around the world and poses threats to newborns and immunocompromised individuals, such as conductive hearing loss, mental retardation, hepatitis, epilepsy, and even death.<sup>[1-3]</sup> In vivo, HCMV invades many kinds of cells, such as endothelial cells, epithelial cells, and neurons.<sup>[4]</sup> Except that, HCMV exists in

the host in 2 forms: multiplication and latency with no replication of the virus.<sup>[5]</sup> HCMV can enter into the latency after primary infection for lifelong period and can be reactivated under some circumstances such as newborns, HIV, organ transplant patients, or other immunocompromised patients.<sup>[6]</sup> As we all know, immediate-early antigen (IEA), highly expressed in HCMV infection, contains IE1 (encoded by UL123) and IE2 (encoded by UL123) that are produced at 1 hour after infection. And the transcription and expression levels of them are the indications for HCMV lytic and latent infection. Moreover, there are 4 autophagy-related genes: *UL138*, *cmvIL-10*, *US28*, *UL81-82ast*.<sup>[7-10]</sup> *LUNA* encoded by *UL81-82ast* was found to exist in lytic infection and latent infection. *PP71* encoded by *UL82* could transactivate *IE* gene and played an important role in initiating lytic infection. The expression of *UL81-82ast* and *PP71* may contribute to the maintenance of HCMV latency and control the activation of the virus.<sup>[10,11]</sup> More importantly, in vivo, *CD34+* hematopoietic progenitor cells are major reservoir for HCMV latency and HCMV can be reactivated when the cells differentiate into macrophages.<sup>[12]</sup> So, in this study, we used human acute monocytic leukemia cell line (THP-1 cells) to verify the relationship between HCMV latency and autophagy.

Autophagy, an cellular degradation pathway, can digest long-lived proteins and organelles by means of lysosomes to maintain cellular homeostasis.<sup>[13]</sup> It is divided into 3 types: macroautophagy, microautophagy, and chaperone-mediated autophagy.<sup>[14]</sup> Autophagy usually refers to macroautophagy and it can prevent cells from microorganism infection, parasitic infection, and anoxia. Recently, the relationship between HCMV infection and autophagy has been studied in depth and made some important progress. In previous study, in lytic infection, HCMV

Editor: Leyi Wang.

YL and JP contributed equally to this work and should be considered cofirst authors.

Funding: This work was supported by National Natural Science Foundation of China (No: 81370774), Natural Science Foundation of Zhejiang Province (No: LY16H040001), Medical Technology Program of Zhejiang Province of China (grantnumber2014KYA122).

The authors report no conflicts of interest.

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Medicine (2017) 96:44(e8298)

Received: 14 April 2017 / Received in final form: 4 August 2017 / Accepted: 24 August 2017

<http://dx.doi.org/10.1097/MD.00000000000008298>

modified autophagy in 2 opposite directions.<sup>[15]</sup> At the early stage of infection, HCMV could induce autophagy independent of viral proteins. Afterwards, HCMV inhibited autophagy with the aid of viral proteins such as TRS1 and IRS1. And the cells infected with HCMV could resist autophagy induced by rapamycin and lithium chloride<sup>[16]</sup>; these results displayed that HCMV regulated autophagy through several pathways. But currently, all the researches about the relationship between HCMV lytic infection and autophagy have been reported.

So, we conducted this study to investigate the influence of HCMV latency on autophagy. This will help to understand the mechanisms of HCMV latency and provide us a target for a new therapeutic approach of HCMV.

## 2. Materials and methods

### 2.1. Cells and virus

THP-1 cells (ATCC) were grown in RPMI-1640 (Hyclone, Logan, UT), supplemented with 10% fetal calf serum (FBS, Gibco, Gaithersburg, MD), 0.05 mM  $\beta$ -mercaptoethanol (Sigma, St. Louis, MO). HEF cells (ATCC) were grown in Dulbecco modified minimal essential medium (DMEM), supplemented with 10% fetal calf serum (FBS, Gibco). Because the cells used in this study were supplied by ATCC, not directly taken from human beings, so ethical approval was not necessary.

HCMV Towne strain, kindly provided by Prof. Fan (the first affiliated hospital of medical school of Zhejiang university), was used. It was multiplied in HEF cells and obtained by 3 cycles of freezing and thawing. Then, it was collected and conserved in -80°C. The virus infectious titers were expressed as median tissue culture infectious dose (TCID<sub>50</sub>).

### 2.2. Protein extraction and Western blot

THP-1 cells in low attachment 6-well plates (Corning, Corning, NY) and HEF in 6-well plates (Corning) were infected with HCMV at multiplicity of infection (MOI) 5. At 6 hours, 1 day, 2 days, 3 days, 5 days, 7 days, 9 days postinfection (p.i.), HEF cells were scraped after washing twice with PBS and THP-1 cells were collected after centrifugation of 15000 rpm  $\times$  5 min and washing twice with PBS. Cells were dissolved in cell lysis buffer which contained RIPA and 100 mM PMSF (Beyotime Biotechnology, Jiangsu, China) at a ratio of 100:1. Then, the mixture was boiled at 100°C for 10 minutes. Protein concentrations were measured by using BCA protein assay kit (Beyotime Biotechnology). Afterwards, these samples were loaded on bis/acrylamide gels. The PVDF membranes (Millipore, Burlington, MA) were blocked at 5% skimmed milk powder in TBST and incubated with primary antibodies. The primary antibodies used in this study were mouse anti-IE (Cell Signaling Technologies, 1:100 dilution, Danvers, MA), rabbit anti-Beclin-1 (Cell Signaling Technologies, 1:1000 dilution), rabbit anti-LC3II (Cell Signaling Technologies, 1:1000 dilution), rabbit anti-Atg5 (Cell Signaling Technologies, 1:1000 dilution). After washing 3 times, peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (Beyotime Biotechnology) was used for incubation 2 hours at a 1:2000 dilution. Protein concentrations were quantitatively estimated by comparing to the actin level or GAPDH level.

### 2.3. Genome extraction and PCR method

RNA was extracted by Trizol and all procedures were performed according to the instruction. After the extraction, ultraviolet

**Table 1**

**The primers were used in the study.**

GAPDH	Forward: 5'- GAAGGTGAAGGGTCCGGATC-3' Reverse: 5'-GAAGATGGTATGGGATTC-3'
IE1	Forward: 5'- GCCTTCCTAAGACCACCAAT-3' Reverse: 5'- ATTTTCTGGGCATAAGCCATAATC-3'
IE2	Forward: 5'- ATGGTTTTGCAGGCTTTGATG-3' Reverse: 5'- ACCTGCCCTTCACGATTCC-3'
UL81-82ast	Forward: 5'- ATGACCTCTCTCCACACC-3' Reverse: 5'- GGAAAACACGCGGGGA-3'
LC3II	Forward: 5'-AATCCCGGTGATAATAGAAC-3' Reverse: 5'-TTTCATCCCGAAGCTCC -3'
Beclin1	Forward: 5'- CTGGGACCTTTTGACATC-3' Reverse: 5'-TTGCGGTTCTTTCCACGTC -3'
Atg5	Forward: 5'- ATGTCACCCCTTTGCTTCAATC-3' Reverse: 5'- AGCCAGTTGCCCTTATCTGA-3'
Actin	Forward: 5'- CACCCAGCACAAATGAAGTCAAGAT-3' Reverse: 5'- CCAGTTTTTAAATCCTGAGTCAAGC-3'

IE=immediate-early, LC3=the light chain three.

absorption assay was used to analyze the purity of RNA and the ratio of A260/A280 was calculated. All the ratios ranged from 1.7 to 1.9. The primers used in this study were designed and synthesized by Sangon Biotech (Shanghai, China) Co., Ltd (Table 1). HCMV DNA was measured according to the instruction of human cytomegalovirus fluorescence quantitative polymerase chain reaction diagnostic kit (Da'an, Taipei, Taiwan). Moreover, agarose gel electrophoresis was used to verify the concentrations of IE and UL81-82ast.

### 2.4. Transmission electron microscope method

THP-1 cells collected were washed 3 times with PBS and added 2.5% glutaraldehyde for 2 hours incubation at 4°C. Then, the cells were fixed by using 1% osmic acid after washing. And dehydration of gradient ethanol was used at 4°C after washing. Afterwards, the cells added propylene epoxide were embed with epoxy618 at 60°C for 48 hours. The prepared cells were sliced up and stained with lead citrate, and then observed and photographed under H-7650 transmission electron microscope (Hitachi, Tokyo, Japan).

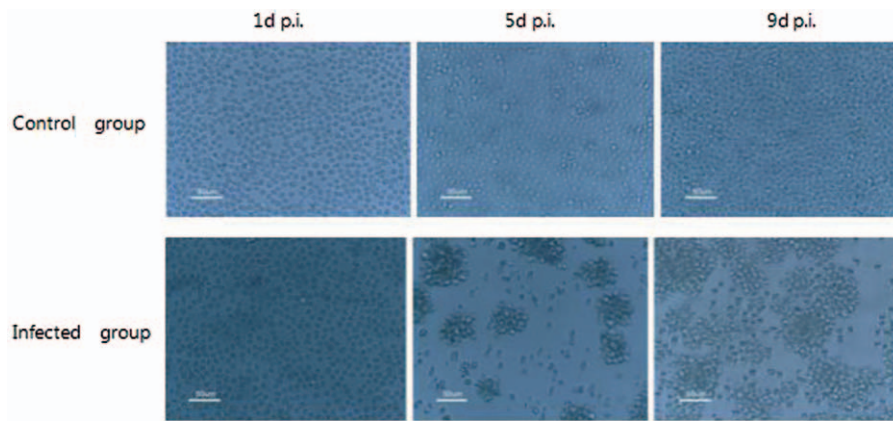
## 3. Results

### 3.1. Establishment of latent infection

In morphology, THP-1 cells infected with HCMV and the control cells were the same in the first few days. Then, the control cells grew normally, but THP-1 cells infected with HCMV began to embrace each other and cytoplasmic vacuoles emerged at 5 days p.i. Furthermore, this phenomenon was particularly evident at 9 days p.i. (Fig. 1).

In THP-1 cells infected with HCMV, about 1.5 HCMV DNA copies were detected in each cell through fluorescent quantitative real time PCR. And HCMV DNA copies increased initially after infection and subsequently began to decrease at 5 days p.i. (Table 2).

In HEF cells infected with HCMV, immediate early gene (IE1 and IE2) and latency-related gene UL81-82ast were expressed at 6 hours, 5 days, 7 days p.i. However, in THP-1 cells infected with HCMV, the mRNA expression level of IE1 was too low to detect by using agarose gel electrophoresis. IE2 mRNA was expressed at 6 hours, 5 days, but not 7 days p.i. Moreover, the mRNA expression level of IE1 was always lower than IE2. In HEF cells,



**Figure 1.** The morphology of THP-1 cells infected with HCMV and the control cells.

the protein expression level of IE was determined after 6 hours p.i. and increased at 5 days, 7 days p.i. However, THP-1 cells infected with HCMV expressed IE at 6 hours, 5 days p.i. and failed to express this protein at 7 days p.i. (Fig. 2).

**3.2. Influence of HCMV infection on autophagy**

Compared with controls, THP-1 cells infected with HCMV emerged double-membraned autophagosomes after 1 day p.i. And the autophagosomes took the predominant at 5 days p.i. At the same time, we could see some cisternal double film structure known as phagophore. At 9 days p.i., autophagosomes or autolysosomes could also be seen. In contrast, autophagy-related vesicles could hardly be seen in the control group. Then, we randomly chose 10 cells in the control group and 10 cells of THP-1 infected with HCMV Towne strain at 1 day, 5 days, 9 days p.i., respectively. Then, we observed and counted autophagy-related vesicles under the transmission electron microscope. About 2 to 3 vesicles defined as autophagosomes or autolysosomes per cell were found in the control group. However, there were approximately 10 to 13, 80, 30 vesicles per cell at 1 day, 5 days, 9 days p.i., respectively (Fig. 3). The mRNA expression levels of autophagy-related genes LC3II, Beclin1, Atg5 obviously increased after 1 day p.i. and achieved the peak at 5 days p.i., then they decreased but were also higher than the controls.

The protein expression levels of LC3II, Beclin1, and Atg5 were different. The level of LC3II increased at 1 day p.i. and reached the top at 3 days p.i. But the concentration of Beclin1 increased at 2 days p.i. and achieved the highest at 7 days p.i., whereas the expression quantity of Atg5 began to increase at 1 day p.i. and reached the highest at 5 day p.i. Moreover, the expression levels

of LC3II, Beclin-1, Atg5 were still higher than the controls (Fig. 4).

So, autophagy level is different after establishing HCMV latency. During latent infection, it is lower than the phase before latency that could be proved by the reduced transcription and the expression levels of LC3, Beclin-1, Atg5 genes. But all the levels were still higher than the control cells. These results suggested that HCMV infection can facilitate autophagy.

**4. Discussion**

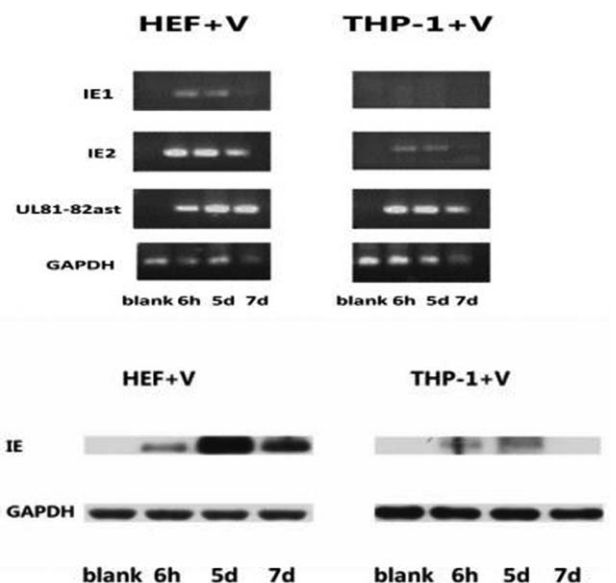
The characteristic of latency is the replication of viral genome and no detection of infectious virus. And the transcription and expression levels of HCMV immediate-early genes (*IE1* and *IE2*) are different in lytic infection and latent infection.<sup>[17]</sup> The expression levels of *IE1* and *IE2* persistently increased in lytic infection. Whereas in latent infection, *IE1* and *IE2* can be expressed at the early stage of latent infection, then decreased obviously and even could not be detected. Recently, *UL81-82ast*

**Table 2**

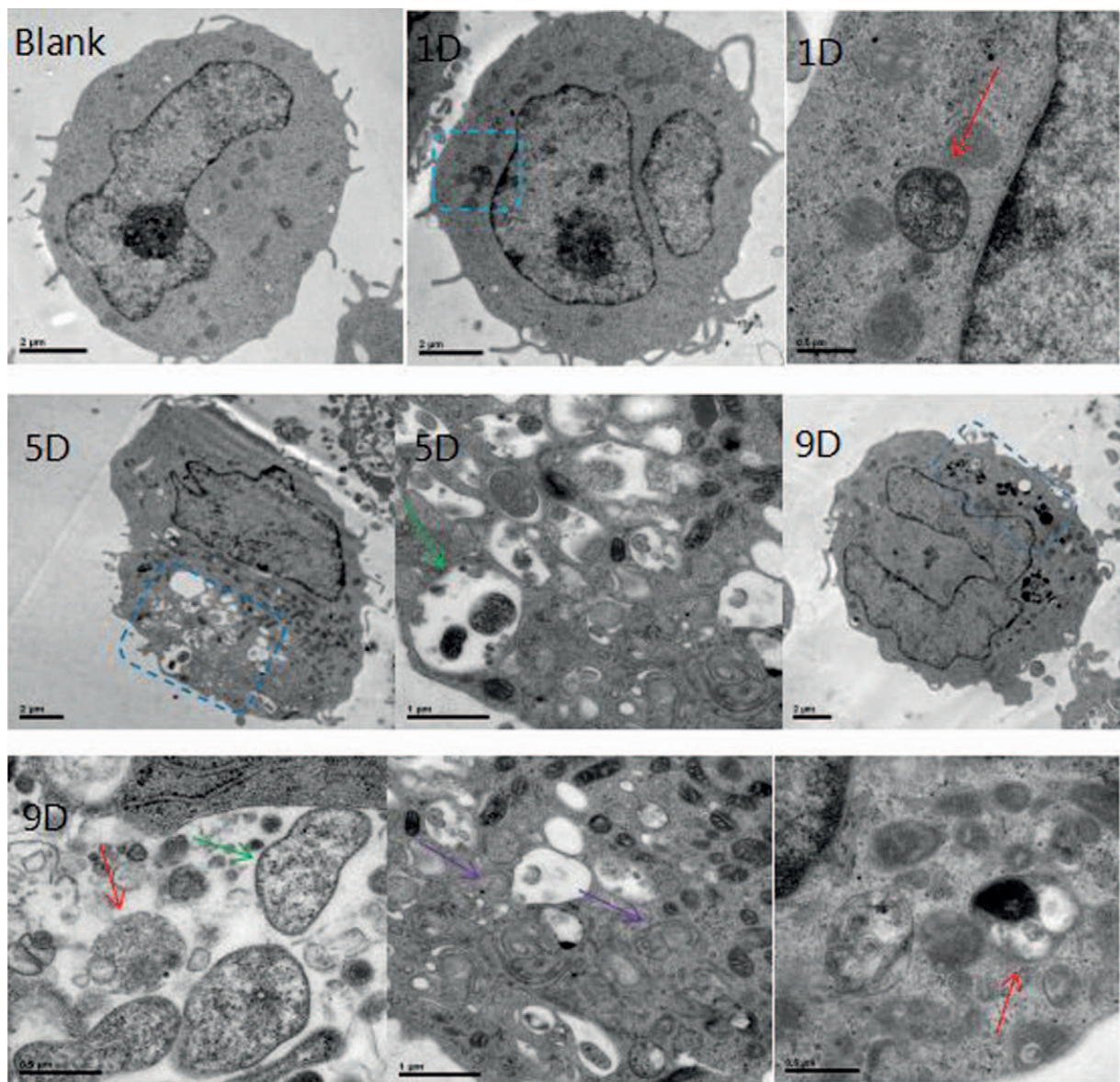
**HCMV DNA copies in THP-1 cells were estimated after infection.**

Postinfection day	Estimated HCMV DNA copies
1 d	1.20
2 d	1.50
3 d	1.68
5 d	2.00
7 d	1.80
9 d	1.55

d=day, HCMV = human cytomegalovirus.



**Figure 2.** The levels of IE and UL81-82ast in lytic infection were different from latent infection.



**Figure 3.** Autophagy-related structures in THP-1 cells infected with HCMV under the electron microscope. Blue box: the area enlarged in next; purple arrow: autophagosome precursor; red arrow: autophagosomes; purple arrow: autophagolysosomes.

was found in lytic and latent infection. Moreover, the expression of *UL81–82ast* gene and no the replication of viral genome were considered as the mark of HCMV latency. In this study, *UL81–82ast* was expressed in HEF cells and THP-1 cells. Moreover, the mRNA expression level of IE2 was very low and IE1 mRNA could not be detected at 7 days p.i. in THP-1 cells. But in HEF cells, IE1 and IE2 were always expressed. So, we concluded that HCMV could establish the latency at 5 days p.i.

Compared with the controls, autophagosomes emerged at 1 day p.i. and significantly increased at 5 days p.i. And the number of autophagosomes and autolysosomes at 9 days p.i. was lower than 5 days p.i., but still more than 1 day p.i. The autophagy-related genes of LC3II, Beclin1, and Atg5 also increased gradually at 1 day p.i. and decreased at 7 days p.i. The protein expression level of LC3II increased at 1 day p.i. and achieved the top at 5 days p.i., and then decreased. However, the protein levels of Beclin1 and Atg5 increased at 2 days p.i., reached the peak at 7

days p.i. and 5 days p.i., respectively, and subsequently decreased. This may be explained as follows: at first, transcription and translation are 2 processes that involve different factors; protein can be regulated not only on transcription and translation level but also post-translationally. In other words, mRNA is not a direct indicator to protein, but a tool for further verifying the protein levels. Moreover, these 2 methods have different sensitivity and they could be affected by the quality of products and the conditions of the study. The results mentioned above displayed that HCMV could induce autophagy and the capacity of promoting autophagy may be weakened when HCMV established the latency. If we draw a similar conclusion in other cell lines, we may use the pharmaceuticals that promote the autophagy level to impede HCMV latency in immune deficiency patients.

In previous studies, HCMV inhibited autophagy for contributing to the replication of the virus in lytic infection,<sup>[18]</sup> but our

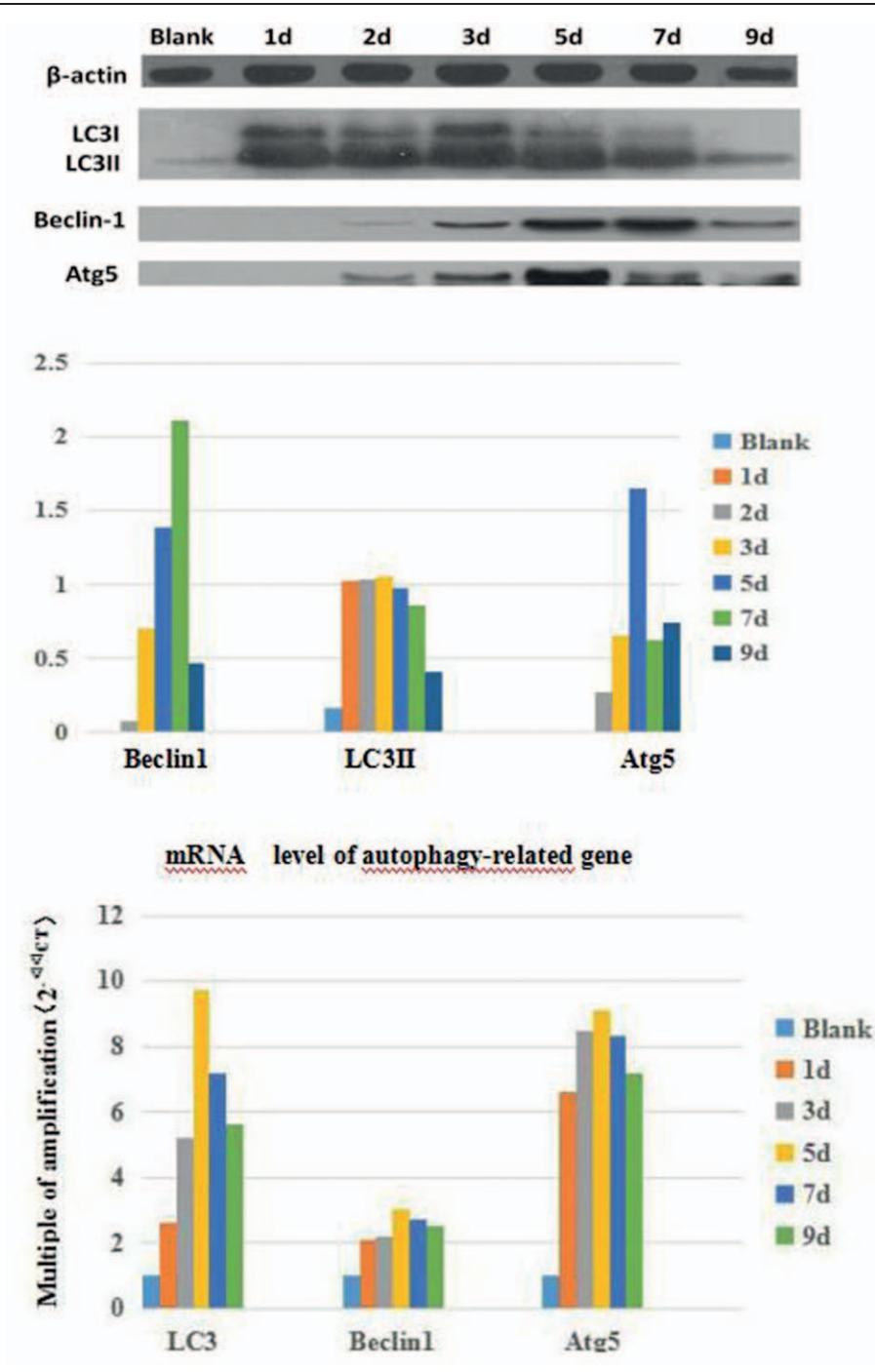


Figure 4. The mRNA and protein levels of LC3, Beclin1, and Atg5 in THP-1 cells.

studies found HCMV could induce autophagy; this may be because autophagy could play a role in the establishment of HCMV latency. The host cells could keep alive by HCMV inducing autophagy and this may result in HCMV latency for a long period. As was reported by Tovilovic et al,<sup>[19]</sup> HSV-1, one kind of herpesvirus, could establish latency in U251 (glioma cells) and keep U251 cells alive by inducing autophagy through AMPK/Akt/mTOR pathway. But in THP-1 cells, what is the mechanism of HCMV inducing autophagy and why the ability of autophagy can be weakened after establishing HCMV latency are needed to be explained by further studies. We speculated that this

phenomenon may be related to the following reasons. First, HCMV DNA could induce autophagy that was confirmed by McFarlane et al.<sup>[20]</sup> Moreover, Rasmussen et al<sup>[21]</sup> reported that HSV-1 (one kind of herpesviruses) DNA was the major factor of inducing autophagy, and HSV-1 could induce the autophagy of bone marrow cells by connecting with STING. In our study, HCMV DNA increased at first and then decreased when HCMV established latency. And previous studies demonstrated that toll-like receptor 9, DNA-dependent activator of interferon (IFN)-regulatory factors, and inflammasome may be involved in the process of HCMV DNA inducing latency.<sup>[22,23]</sup> Second, double-

stranded RNA (dsRNA) and IFN could activate the dsRNA-activated protein kinase (PKR) pathway and induce the autophagy. Recent study reported that dsRNA could induce autophagy through PKR pathway after infecting with HSV-1.<sup>[24,25]</sup> Moreover, PKR could accumulate in the nucleus after HCMV infection that displayed that HCMV could activate the PKR pathway by dsRNA.<sup>[26]</sup> In our study, HCMV actively replicated before the latency and dsRNA would increase correspondingly. The reduction of HCMV DNA led to a decrease in dsRNA. So, the autophagy level could be lowered in latent infection. In previous studies, HCMV could be combined with TLR2 or TLR4 and then induced IFN.<sup>[27,28]</sup> Subsequently, IFN could activate PKR pathway and induce the autophagy.<sup>[29]</sup> Infectious virus was not produced in latent infection and IFN was reduced correspondingly. So, autophagy decreased in HCMV latency. However, the host cells existed in an inflammatory environment, thus the autophagy level was still higher than the normal cells. At last, CD46 could induce autophagy that was confirmed by Joubert et al.<sup>[30]</sup> CD46-Cyt-1 could connect with Golgi-associated PDZ and coiled-coil motif-containing protein and link to autophagosome for the formation of VPS34/Beclin1, and then induced autophagy. No infectious viruses were produced and the simulation of CD46 could be weakened. So, the level of autophagy was lower in latent infection.

In conclusion, HCMV could induce autophagy and the capacity of promoting autophagy may be weakened in the latent infection.

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