Inhibition of Autophagy by 3-Methyladenine restricts Murine Cytomegalovirus Replication

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Abstract

**Background:** Cytomegalovirus (CMV) could induce autophagy early upon infection, which might have an impact on virus replication and the survival of host cells. The purpose of the present study was to determine how autophagy affects virus replication and whether it is associated with caspase-3 dependent apoptosis during murine cytomegalovirus (MCMV) infection.

**Methods:** The eyecup isolated from adult C57BL/6J mice (6-8 weeks old) and mouse embryo fibroblast cells (MEFs) were cultured and infected with MCMV K181 strain, following by treated with 3-methyladenine (3-MA) or rapamycin to block or activate autophagy. Immunofluorescence staining and western blot were used to detect the expression of early antigen (EA) of MCMV, autophagy and cell death related factors. Plaque assay was performed to detect the virus titer in different groups. TUNEL assay was used to measure the percentage of cell death.

**Results:** Results showed that autophagy was induced at 24, 72 and 96 hours post infection (hpi) with MCMV in MEFs. In the eyecup culture, it also showed that autophagy was induced at 4 and 7 days post infection (dpi). In addition, caspase-3 dependent apoptosis and receptor-interacting kinase 1/ receptor-interacting kinase 3/mixed lineage kinase domain-like protein (RIPK1/RIPK3/MLKL) dependent necroptosis were induced by MCMV infection in eyecup. In MEFs, caspase-3 dependent apoptosis was inhibited, while RIPK1/RIPK3/MLKL dependent necroptosis was activated with MCMV infection. Once treatment with 3-MA, there were significantly less active virus particles released in MEFs and eyecup, also EA expression was significantly inhibited in the eyecup. However, treatment with rapamycin have no such significant influence on either virus titer or EA expression in MEFs and eyecup. Furthermore, cleaved caspase-3 was elevated, while RIPK1/RIPK3/MLKL pathway was inhibited with treatment of 3-MA both in MEFs and eyecup.

**Conclusion:** Inhibition of autophagy by 3-MA could both restrict virus replication and promote caspase-3 dependent apoptosis in the eyecup and MEFs with MCMV infection. It can be explained that on the early period of MCMV infection, suppressed autophagy process directly reduced virus release. Thereafter, caspase-3 dependent apoptosis was activated and resulted in decreased virus replication.

**Background**

Autophagy, an evolutionally system, was firstly observed in 1960s. It was described that cells are capable of disrupting its own proteins or organelles by enclosing them in the autophagosomes, a double-membrane vesicles, and then fuse with lysosomes for enzymatic degradation[1]. As a well-studied process, autophagy is characterized by over 35 autophagy-related proteins (ATG) and can be organized into the following main steps. It includes phagophore initiation, membrane elongation, autophagosome formation and autophagosome fusion with lysosomes[2]. Autophagy plays a dual role in virus infection, which not only could modulate the primary antiviral response and prevent prolonged and excessive inflammation, also could control viral infections, and finally result in the clearance of viral antigens and
other pathogens[3]. As to the interaction between autophagy and cytomegalovirus (CMV), a ubiquitous virus that has co-evolved with their mammalian hosts over millennia, it has been demonstrated that one of the important autophagy hallmark, the faster-migrating form of LC3II, increased even at 2 hour post HCMV infection, and it can be detected the vesicles with the characteristic appearance of autophagosomes or autolysosomes at 6 hour post infection of HCMV in human fetal foreskin fibroblasts (HFFF2), which indicated that the autophagy was induced very early after human HCMV infection[4]. In addition, autophagy is able to promote cell survival and limit pathogenesis in human cytomegalovirus (HCMV) infection. However, HCMV might have developed efficient strategies to block the activation of autophagy during infection, as it was also found that HCMV infection could drastically inhibit autophagosome formation in vitro[5] at later time points of infection via viral TRS1 protein interacts with Beclin 1[6]. An mTOR-Independent inducer of autophagy, Trehalose and another autophagy inducing compounds SMER28 could suppress the production of cell-associated virus and viral replication[7, 8], which might provide a novel therapeutic target for the treatment of HCMV related diseases.

Most of the world's population are infected with HCMV, among which the sero-prevalence ranged from 40 to>90%[9], which generally are asymptomatic infection in immunocompetent individuals. However, CMV retinitis occurs predominantly in those patients who are unable to generate a normal primary T-cell response against the virus or in patients who are carriers of CMV but have decreased CMV-specific T-cell response due to disease or immuno-suppressive treatment, such as acquired immunodeficiency syndrome (AIDS) and leukemia[10]. Like Ebola and Zika virus are able to infect the eye and cause severe eye diseases, including optic neuritis and chorioretinal atrophy[11, 12], CMV infection not only resulted in retinitis in immunocompromised individuals, but also systematic CMV infection could transmit to specific eye compartments, including the anterior segment and choroid but not retina in immunocompetent individuals [13]. During this systematic infection involved ocular infection, viral replication was firstly detected in the endothelial cells of the iris followed by the perivascular cells, which indicated that there's need to consider CMV as a pathogen could induce long-lived chronic and low level inflammatory sequelae in the eye, even in the neural retina[13]. Therefore, it interest us that how autophagy regulates virus replication during CMV infection especially in the posterior segment parts of the eye.

It seems that the interaction between autophagy and CMV infection is quite complicated, which still needs more exploration. As CMV is a strictly species specific virus, making it difficult to study HCMV in vivo experiments. Murine CMV (MCMV), which is similar to HCMV in sequence and in vivo pathogenesis, is always utilized to mimic HCMV infection as a widely useful animal model. In our present study, MEFs and the isolated eyecup were cultured with MCMV infection to study the role of autophagy in MCMV replication.

Methods And Materials

Animals and eyecup isolation
C57BL/6J mice used in this study were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed with a 12-hour light cycle alternating with a 12-hour dark cycle and were given standard rodent chow and water and libitum. All eyecups were isolated from adult (6-8 weeks old) mice. The protocol were accorded to that was described previously[14]. Briefly, all mice were sacrificed under anesthesia by CO$_2$ asphyxiation. Then the eyes were removed and immediately placed in phosphate buffered saline (PBS) on ice. Using sterile scissors to remove the iris and lens at the posterior margin of the limbus and the cornea with a dissecting microscope. Lastly, the retina is carefully removed, following by cutting the optic nerve. The remaining choroid/sclera was placed on membrane filter and covered with matrigel, followed by culturing in a 24-well plate with DMEM medium.

**Virus**

MCMV strain K181 used in this study were kindly as a gift from Edward Morcarski, Emory University, Atlanta, Georgia, USA. BALB/C mice (6-8 weeks old) were used to propagate virus. Firstly, all mice were injected intraperitoneally with 2mg steroid, two days later, 1x10$^3$PFU of MCMV were injected intraperitoneally and, meanwhile, steroid was intraperitoneal injected every two days. Two weeks later, all mice were sacrificed and the salivary glands were collected and homogenized within DMEM medium. Virus titer was detected via plaque assay on duplicate cultures of mouse embryo fibroblast cells (MEFs). Virus stocks were stored in liquid nitrogen. Every fresh stock virus could be diluted to the appropriate concentration in serum–free DMEM when used for a single experiment[15].

**Plaque assay**

MEFs were cultured in 24-well plates with Dulbecco's modified Eagle's medium (DMEM; Mediatech, Manassas, VA) containing 5% fetal bovine serum (FBS; Thermoscientific, Waltham, MA) (at 37 °C, 5% CO2). Supernatant of the eyecup culture plate were collected at 4, 7, and 10 days post infection (dpi) and serially diluted. Then add 100 μl of each dilution was to the prepared MEF monolayers, following by which were incubated at 37 °C for 1 hour. At last, 2% agarose solution mixed with 2 ×DMEM (Life Technologies, Grand Island, NY) with ratio 1:1 and added 0.5ml of agarose mixture (0.5% agarose in 1 ×DMEM) to each well, which were incubated for 5 days at 37 °C. Then, 10% formaldehyde was used to fix the cells for 2 hours and stained with 0.13% crystal violet to count plaques via dissecting microscope.

**Antibodies and Reagents**

Anti-MCMV early antigen (EA) was used to identify MCMV-infected cells in the eyecup[16]. Rabbit anti-RPE65 (specific for RPE cells) was from Abcam (Cambridge, MA, USA). Rabbit anti-LC3B, p70 S6 Kinase, anti-cleaved caspase-3, anti-RIPK3, anti-phospho-RIPK3, anti-RIPK1, anti-MLKL, goat anti rabbit IgG-horseradish peroxidase (HRP), and goat anti-mouse IgG-HRP were pursed from Cell Signaling (Cell Signaling Technology, Danvers, MA, USA). Rabbit anti-p62, anti-phospho-MLKL were also from Abcam. Anti-β-actin was from Sigma-Aldrich (St. Louis, MO, USA). Anti-mouse Alexa 488, anti-rabbit Alexa 594 were purchased from Vector Laboratories (Burlingame, CA, USA). BCA assay kit was from Thermo Fisher Scientific.3-MA (5mM) and rapamycin (500nM), which were used to inhibit and induce autophagy, were
purchased from Sigma-Aldrich (St. Louis, MO, USA). TUNEL assay kit was from Roche (Roche Diagnostics, Indianapolis, IN, USA).

**Western Blot Analysis**

Total proteins were extracted from eyecups with MCMV infection or mock infection, 3-MA and Rapamycin treatment group at 4, 7 and 10 dpi with NP40 lysis buffer. The protein concentration was identified using BCA assay kit according to the manufacturer’s instructions. Proteins were separated by SDS-PAGE, following by electro-blotting onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Piscataway, NJ, USA). Then it was blocked with 5% skimmed milk for 1 hour at room temperature and afterwards membranes were incubated overnight with primary antibodies (at the dilution of 1:1000 with 5% BSA) at 4°C. The next day, the membranes were incubated with HRP-conjugated secondary antibody for 1 hour at room temperature. Lastly, the membranes were covered with chemiluminescence completely (ECL; GE Healthcare) and visualized throughChemiDoc Image System (Bio-rad, CA, USA).

**Immunofluorescence Staining**

Cultured eyecups were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 1 hour and then immersed in 25% sucrose overnight at 4°C. The next day, the eyecups were snap frozen, and sectioned using cryostat. For double staining with TUNEL and RPE65, TUNEL assay was initially performed according to the manufacturer’s instructions. Then after washing with PBS and blocking with PBS containing 10% normal goat serum, slides were reincubated with primary antibodies biotinylated anti-EA (1:500) labeled with FITC overnight at 4°C. The next day, Texas redlabeled avidin (1:600) was used to incubate with sections for 1 hour at room temperature. When double staining of RPE65 and viral EA in sections or in whole eyecup mount, firstly, slides were dried for 20 minutes and then washed for 30 minutes in PBS, followed by permeabilization with 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes on ice. Then after blocking in PBS containing 10% normal goat serum, 2% BSA, and 0.5% Triton X-100 for 1 hour at room temperature, sections were incubated with rabbit-derived anti-RPE65 (1:800)and anti-EA (1:500) labeled with FITC antibody overnight at 4°C. The next day, after washing with PBS for three times, sections were incubated with anti-rabbit Alexa 594 (1:1000) for 1 hour at room temperature followed by washing again. Lastly, to visualize nuclei, all slides were mounted with anti-fade medium containing 40,6-diamidino-2-phenylindole (DAPI; Vectashield, Vector Laboratories) and images were captured using a microscope (Zeiss upright 780; Oberkochen, Germany).

**Statistical Analysis**

Data for plaque assay were expressed as means ± SEM (standard error of mean). Statistical significance was determined using either a 2-tailed t-test through the GraphPad Prism 8 analysis (GraphPad Software, Inc., San Diego, CA, USA). $P$ values $<$ 0.05 represents significant difference. *$p$ $<$ 0.05, **$p$ $<$ 0.01, ***$p$ $<$ 0.001.
Results

Time-dependent regulation of autophagy upon MCMV infection

It has been reported that autophagy could be induced by CMV infection at very early period, which is beneficial to virus survival. Here, the influence of MCMV infection on autophagy in cultured eyecup as well as MEFs was investigated. Atg8/LC3 is the most widely used autophagy-related protein, belonging to a ubiquitin-like protein. The two forms of LC3 including nonlipidated and lipidated are usually referred to respectively as LC3-I and LC3-II, which are often an excellent marker for autophagic structures[17]. In our present study, the protein levels of LC3, SQSTM1/p62, an autophagic substrate located within autophagosomes, and phosphor-p70 S6 kinase (T389), a downstream target of mammalian target of rapamycin (mTOR) signaling, were quantified to testify the autophagy levels. As shown in Figure 1A, LC3I/LC3II ratio was decreased (LC3I decreased but LC3II increased relatively) at 24, 72 and 96 hours post infection (hpi) of MCMV in cultured MEFs (Lane 2, 6, 8), meanwhile p62 and phosphor-p70 S6 kinase were also reduced slightly (Lane 2, 6, 8), which indicated that MCMV infection activates autophagy during MCMV infection in MEFs. In the eyecup culture of C57BL/6J mice, MCMV infection also increased LC3II expression at 4 and 7 days post infection (dpi.) (Figure 1B lane 2, 4). However, there’s no significantly difference between MCMV infection and mocks treated eyecups at 10 and 14 dpi (Figure 1B lane 6 and 8) with MCMV infection. Also, the expression of p62 didn’t change a lot with MCMV infection (Figure 1B lane 2, 4, 6 and 8). In addition, phosphor-p70 S6 kinase expression was increasing over time (Figure 1B lane 2, 4, 6, and 8). These results indicated that the autophagy was activated early after MCMV infection both in MEFs and eyecup culture, while without the degradation of substrate p62 completely in the eyecup.

Tissue/cell specific effect of MCMV infection on cell death

In order to determine whether MCMV infection could induce caspase-3 dependent apoptosis or necroptosis, related proteins were detected via western blot. Results show that the cleaved caspase-3 increased over time, but MCMV infection down regulated cleaved caspase-3 protein levels at all time points detected in MEFs (Figure 1A lane 2, 4, 6, and 8). Interestingly, in eyecup culture the cleaved caspase-3 was increased with MCMV infection, especially at 10 dpi (Figure 1C lane 2, 4, 6, and 8). The necroptosis related protein levels of active RIPK1, pRIPK3 (s232), and pMLKL (s345) were slightly increased at 24 and 48 hpi in MEFs (Figure 5B lane 2 and 8). Similarly, in cultured eyecup MCMV infection also increased the protein levels of active RIPK1, RIPK3 and MLKL, to some extent, at 4, 7, 10 and 14 dpi (Figure 1C lane 2, 4, 6, and 8). These results suggested that caspase-3 dependent apoptosis and RIPK1/RIPK3/MLKL dependent necroptosis were induced by MCMV infection in eyecup, while in MEFs infected with MCMV, caspase-3 dependent apoptosis was inhibited and RIPK1/RIPK3/MLKL dependent necroptosis was activated.

Autophagy inhibition by 3-MA restricted viral replication upon MCMV infection
Consistent with the notion that the MCMV infection, replication and even latency are tissue or cell dependent because of the different innate and/or adaptive immune environment (reviewed by [18], our present study show that the tissue-specific effect of MCMV infection on autophagy and cell death pathway. Furthermore, the eye is an immune privileged organ, although there are several studies focused on the relationship between autophagy and CMV infection, it’s still not known how autophagy involved in MCMV virus replication in the eye. In this study, 3-MA, one of the most used autophagy inhibitor, was used to block autophagy induced by MCMV infection in MEFs and eyecup culture. Plaque assay show that 3-MA treatment significantly decreased active virus particle releasing at 48,72 and 96 hours after MCMV infection in MEFs($p<0.05$), but there’s no significant effect of rapamycin treatment on virus replication in MEFs($p>0.05$) (Figure 1D). Meanwhile, in the eyecup culture, the virus releasing was also decreased significantly after treatment with 3-MA($p<0.05$) at4, 7, 10 and 14dpi(Figure 1E). However, treatment with rapamycin just slightly decreased the virus releasing in eyecup cultureat 4 and 7 dpi (Figure 1E).

To confirm the virus titer assay results, MCMV early antigen(EA), indicating the active virus replication within MCMV-infected eyes,was detected in eyecup using immunofluorescence staining.As show in Figure 2A, EA positive stained cells in the retinal pigment epithelium(RPE) or choroid layer treated with 3-MA were significantly reduced, which decreased 78.3% at 4dpi, 88.3% at 7dpi, 89.1% at 10dpi and 52.8% at 14dpi compared with MCMV infection only(Figure 2B).While the EA positive stained cells in rapamycin treated eye cups are comparable with control eyecup at 4 and 10dpi and even increased at 7 and 14dpi(Figure 2B) during MCMV infection. Consistent with the slides staining, EA positively stained cells in the flatmount of eyecup was inhibited significantly by 3-MA treatment but not by rapamycin treatment (Figure 3). These results indicated that autophagy inhibitor, 3-MA could suppress the production of virus particle and EA expression in eyecup with MCMV infection.

**Inhibitory effect of 3-MA suppression on MCMV replication might through caspase-3 dependent apoptosis**

The interrelationship between autophagy and apoptosis is depended on different context [19]. It is well known that the autophagy could mediate cell death, no matter within or beyond the same cell. In most instances, autophagy tends to play an anti-apoptotic but not pro-apoptotic role[20]. As to MCMV infection, although it’s not well investigated, autophagy interacted with apoptosis during MCMV infection of RPE cells[21]. To explore the mechanism of inhibition of MCMV replication by autophagy inhibitor 3-MA, TUNEL assay was used to detect that whether cell death was involved in this process. To our unexpected, TUNEL positive cells were significantly increased with treatment of 3-MA under MCMV infection in eyecup especially at 7 and 14 dpi, while rapamycin treatment did notshow any significantlyeffects (Figure 4A,B). ThenWestern blot was used to determine which mode of cell death plays a leading role, and results showed that when treated with 3-MA, LC3I and p62 expression increased in MEFs (Figure 5A lane 3 and 9), also mTOR and pmTOR, as well as LC3I and p62 increased in eyecup(Figure 6A lane 3 and 6), indicated that autophagy was inhibited. When treated with rapamycin,
LC3I, p62, phosphor-p70 s6 kinase were decreased and LC3II increased in MEFs (Figure 5A lane 5 and 11), also mTOR and pmTOR were reduced in eyecup (Figure 6A lane 4, 8, 12, and 16), indicated that autophagy was induced.

Cleaved caspase-3 significantly increased with 3-MA treatment, while RIPK1/RIPK3/MLKL pathway was inhibited in both MEFs (Figure 5B lane 4 and 10) and eyecup (Figure 6B lane 3, 6, 9 and 12), implied that it is caspase-3 dependent apoptosis but not RIPK1/RIPK3/MLKL dependent necroptosis was involved in 3-MA and autophagy mediated the suppression of MCMV replication in eyecup culture and MEFs.

**Discussion**

It has been demonstrated that CMV infection could induce autophagy shortly after infection. Autophagy, as an important "self-eating" system, could restrict viral infection by direct degradation of viral components, by modulating the intensity of the inflammatory response or by facilitating the processing of viral antigens for presentation by major histocompatibility complex (MHC)[22], whereas multiple viruses utilize components of autophagy and the exosome machinery for the assembly of virions and release of host proteins and RNAs that can affect pathogenesis[23-25].

However, the interplay between MCMV replication and autophagy is poorly understood. Previous studies using chemicals to investigate the impact of stimulation or inhibition of autophagy on HCMV replication have provided controversial results[7, 8, 26]. On the one hand, there's study shown that the induction of autophagy with HCMV infection impaired viral replication, and inhibition of autophagy by a virus expressing ATG4BC74A enhances both viral DNA replication and progeny release[27], demonstrating the antiviral effect of autophagy and supporting the assumption that autophagy serves as a cellular defence against HCMV. For example, Trehalose, one of autophagy inducer, induces changes in the cytoplasmic landscape and in the Rab family of regulatory proteins, limiting virus release from the cell and potentially redirecting virions to acidified, compartments in which they are degraded, while the antiviral activity of SMER28 appears to be independent of cellular trafficking pathways and interferes with the HCMV life cycle at an earlier point, reducing early protein accumulation in both human foreskin fibroblasts (HFFs) and human aortic endothelial cells (HAECs) and delaying viral genome replication in HAECs[8]. On the other hand, activation of autophagy by rapamycin and methyl βcyclodextrin enhanced HCMV infectivity, whereas inhibition autophagy via Spautin 1 and ATG16L1 knockout decreased viral production, in which the pharmacological played similar effects on extra and intracellular viral yields, suggesting that autophagy does not influence release of the virus[26]. In addition, it has been demonstrated that HCMV can directly inhibit the formation and maturation of autophagosomes through the interaction of tegument protein IRS1 and TRS1 with Beclin1, without affecting the synthesis of viral proteins[5, 26], while the host could attenuate autophagy and viral replication in the early stage of HCMV infection through IL-10[28]. These studies suggest that different autophagy inducer might have various roles on viral replication, even they have the same effects on virus growth, it seems like the mechanism may be totally distinct. As to MCMV, it just showed that autophagic vacuole accumulation was detected early during MCMV infection of RPE cells, which could protect retinal cells from MCMV infection induced
apoptosis through mTOR-mediated signaling pathway\cite{21, 29}. In the present study, we find that there's less expression of early viral protein EA and less viral particles released in the eyecup with 3-MA treatment, one of the autophagy inhibitor. However, at the same time, they suffered from increased caspase-3 dependent apoptosis but not RIPK1/RIPK3 mediated necroptosis. These results indicated that caspase-3 dependent apoptosis was involved in 3-MA caused inhibition of MCMV replication.

Apoptosis, necroptosis, and pyroptosis are the three major ways of programmed cell death (PCD) following virus infection\cite{30}. Apoptosis is an evolutionarily conserved process, which is the most extensively investigated PCD during viral infection. However, apoptosis elicited by virus infection has both negative and positive influence on viral replication. On the one hand, host cells could use apoptosis to eliminate virally infected cells, which aborts virus infection. On the other hand, some viruses encode proteins that directly influence the function of core proteins controlling extrinsic or intrinsic apoptotic pathways\cite{31, 32}. As to CMV, caspase-dependent apoptosis has an important role in controlling viruses. It has been demonstrated that CMV infection could induce extrinsic and intrinsic apoptosis, necroptosis, and pyroptosis, and parthanatos during CMV retinitis\cite{33-35}. Inhibition of apoptosis by CMV is mediated by a mitochondria-localized inhibitor of apoptosis, vMIA, a viral inhibitor of caspase activation, vICA, the functional homologs of B-cell lymphoma 2 (Bcl-2) related and c-FLIP proteins, as well as viral proteins including pUL38, IE1 491aa, and IE2 579aa, UL138, US21 can prevent apoptosis induced by various stimuli\cite{36-40}.

The interplay between autophagy and apoptotic proteins is very complicated by the fact that autophagy can act both as a cell survival and cell death process\cite{41}. Although cells can die by autophagic cell death, the main function of autophagy is to promote cell survival during normal tissue homeostasis. In general, cells initiate autophagy under stress as a pro-survival strategy and block apoptosis. However, as stress continues beyond a threshold, cells initiate apoptotic cell death and block autophagy. It seems like that when to switch off from the pro-survival autophagic process to apoptotic state may depend on the level of stress and is regulated by mediator molecules involved in autophagy and apoptosis pathways\cite{41, 42}. While the mechanisms mediating the complex counter-regulation of apoptosis and autophagy are not yet fully understood, important points of crosstalk include the interactions between Beclin-1 and Bcl-2/Bcl-xL and between fas-associated protein with death domain (FADD) and Atg5, caspase- and calpain-mediated cleavage of autophagy-related proteins, and autophagic degradation of caspases\cite{19, 43}. For example, inhibition of or deficiency in caspase 8 results in excessive autophagy in fibroblasts, macrophages, and T cells\cite{44}, while autophagy also could counter-balances the apoptotic response by the continuous sequestration of the large caspase-8 subunit in autophagosomes and its subsequent elimination in lysosomes\cite{45}. The Atg7- caspase-9 complex performs a dual function of linking caspase-9 to the autophagic process while keeping in check its apoptotic activity\cite{46}. Caspase-3 could cleave Beclin-1, which may contribute to inactivate autophagy leading towards augmented apoptosis\cite{47}.

It has been demonstrated that inhibition of autophagy could promote cancer cell caspase-dependent or caspase-independent apoptosis\cite{48-50}, which indicated that autophagy inhibitors as an adjunct to chemotherapy for variety of tumors was worth to be explored. 3-MA, as the most widely used autophagy
inhibitor, is a class III phosphatidylinositol 3-kinase (PtdIns3K) inhibitor[51]. There have been several studies shown that 5-FU-induced apoptosis in colon cancer cells and human skin squamous cell carcinoma can be enhanced by the inhibitor of autophagy, 3-MA[52, 53], also there were studies demonstrated that inhibition of autophagy by 3-MA treatment could increase DDP, IR, 2-DG, purvalanol, IL-24 and hypoxia induced apoptosis in other cancer cells[54-58]. Interestingly, in our present study, we found that autophagy inhibitor, 3-MA, not only restricted viral replication, but also promoted caspase3-dependent apoptosis during MCMV infection in eyecup, which posed us a challenge that whether it’s inhibition of virus replication led by autophagy inhibition that increased apoptosis or enhanced apoptosis caused by 3-MA disrupt normal cell condition in the eyecup, which afterwards restricted virus entry and reduced its replication. Because as it has been described above, autophagy could regulate virus replication directly, and apoptosis could be enhanced by autophagy inhibition. Therefore, we speculate that it depends on the infectious time it has been received. We can see that at 4 and 7 dpi, even when caspase-3 did not be activated, virus replication had been suppressed, implied that during this period of MCMV infection, it is autophagy inhibition itself directly reduced virus growth. However, when caspase-3 was significantly activated at 10 and 14 dpi, it’s more likely that it is increased caspased-3 dependent apoptosis caused by autophagy inhibition that led to interfere virus replication.

Conclusions

This study show that inhibition autophagy by 3-MA could both restrict virus replication and promote caspase-3 dependent apoptosis in the eyecup culture with MCMV infection, and at the early period of MCMV infection suppressed autophagy process directly reduced virus release, while later it was caspase-3 dependent apoptosis that caused decreased virus replication. However, there’re still lots of questions need to be answered by future studies, such as how virus in the eyecup was inhibited by autophagy and how apoptosis was regulated by autophagy during MCMV infection, which might provide useful target for treatment of CMV infectious diseases.

Abbreviations:

CMV, Cytomegalovirus; MCMV, Murine cytomegalovirus; HCMV, Human cytomegalovirus; MEFs, Mouse embryo fibroblast cells; EA, Early antigen; ATG, autophagy-related protein; RIP, Receptor interacting protein; MLKL, Mixed lineage kinase domain-like protein; 3-MA, 3-methyladenine; RPE, Retinal pigment epithelium; mTOR, Mammalian target of rapamycin; PCD, Programmed cell death; FADD, Fas-associated protein with death domain; Bcl-2, B-cell lymphoma 2.

Declarations

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Authors’ contributions

Xinyan Zhang and Feng Fang conceived and designed the experiments. Linlin Zhang, Ting Xi, Yidan Bi, and Zhan Zhang performed the experiments. Yuan Huang, Yuanyuan Lu and Xinglou Liu helped to analyze the data. Xinyan Zhang wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data of this study was analysed and available in this article.

Ethics approval and consent to participate

This study was approved by the Tongji Hospital of Tongji College of Huazhong University of Science and Technology Animal Care and Use Committee.

Consent for publication

Not applicable.

Competing interests

The authors declare that there are no conflicts of interest.

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**Figures**
Figure 1

A

| Lane | 24h m | 48h m | 72h m | 96h m |
|------|-------|-------|-------|-------|
| 70kDa |       |       |       |       |
| 62kDa |       |       |       |       |
| 16kDa |       |       |       |       |
| 14kDa |       |       |       |       |
| 42kDa |       |       |       |       |
| 17kDa |       |       |       |       |
| 42kDa |       |       |       |       |

Phospho-p70 S6 kinase
SQSTM1/p62
LC3 I/II
β-actin
Cleaved caspase 3
β-actin

B

| Lane | d4 m | d7 m | d10 m | d14 m |
|------|------|------|-------|-------|
| 70kDa |     |     |       |       |
| 62kDa |     |     |       |       |
| 16kDa |     |     |       |       |
| 14kDa |     |     |       |       |
| 42kDa |     |     |       |       |

Phospho-p70 S6 kinase
SQSTM1/p62
LC3 I/II
β-actin

C

| Lane | d4 m | d7 m | d10 m | d14 m |
|------|------|------|-------|-------|
| 54kDa |     |     |       |       |
| 78kDa |     |     |       |       |
| 33kDa |     |     |       |       |
| 50kDa |     |     |       |       |
| 19kDa |     |     |       |       |
| 17kDa |     |     |       |       |
| 42kDa |     |     |       |       |

MLKL
RIP1
RIP3
Cleaved caspase 3
β-actin

D

![Graph showing viral titer in MEF cells]

E

![Graph showing viral titer in Eye-cup cells]
MCMV infection induced Autophagy and apoptosis changes in MEFs and eyecup culture MEFs were infected with K181 strain of MCMV (MOI=1). Eyecups were isolated from adult C57BL/6J mice and infected with K181 strain of MCMV (5 X 10^3 PFU). Total protein was extracted at 24, 48, 72 and 96 hours post infection (hpi) in MEFs, followed by autophagy hallmarks LC3I/II and SQSTM1/p62, phosphor-p70 S6kinase (T389), and cleaved caspase-3 in MEFs detection using western blot (A). In eyecup, total protein was extracted at 4, 7, 10, and 14 days post infection (dpi), autophagy hallmarks LC3I/II and SQSTM1/p62, phosphor-p70 S6kinase (T389) (B), and cleaved caspase-3, as well as RIPK1/RIPK3/MLKL pathway was detected via western blot (C). Plaque assay was performed to detect the virus titer in MEFs (D) and eyecup (E) with treatment of autophagy inhibitor 3-MA, or autophagy inducer rapamycin at different time points. Bar graphs are the statistic results presented as mean ± S.E.M of three independently experiments (*p<0.05; **p<0.01; ***p<0.001).
Figure 2

A

B

EA/RPE/DAPI

EA/RPE/DAPI

EA/RPE/DAPI

EA/RPE/DAPI

m

m+V

3-MA+V

Rapamycin+V

D4

D7

D10

D14

m

m+MCMV

3-MA+MCMV

Rapamycin+MCMV

Relative expression of EA

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Figure 2

EA immunofluorescence staining in eyecup with treatment of 3-MA or rapamycin Eyecups were isolated from adult C57BL/6J mice and infected with K181 strain of MCMV (5X10^3PFU). Immunofluorescence staining was used to detect EA positive cells in eyecup with treatment of autophagy inhibitor 3-MA, and autophagy inducer rapamycin at different time points (A). Image J was used to analyze the relative expression of EA in eyecup culture (B). Bar graphs are the statistic results of respective protein levels normalized to controls, results presented as mean ± S.E.M of three independently experiments (*p<0.05; **p<0.01; ***p<0.001).
Figure 3

EA immunofluorescence staining in flat mount of eyecup with treatment of 3-MA or rapamycin. Eyecups were isolated from adult C57BL/6J mice and infected with K181 strain of MCMV (5x10^3 PFU).
Immunofluorescence staining was used to detect EA positive cells in the flat mount of eyecup with treatment of autophagy inhibitor 3-MA, and autophagy inducer rapamycin at 4,7,10, and 14 dpi.
Figure 4

A

M+V

3-MA+V

Rapamycin+V

d4

d7

d14

TUNEL/RPE/DAPI

TUNEL/RPE/DAPI

TUNEL/RPE/DAPI

B

Cell stained with TUNEL (%)

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Figure 4

TUNEL staining in MCMV infected eyecup with treatment of 3-MA or rapamycin Eyecups were isolated from adult C57BL/6J mice and infected with K181 strain of MCMV (5X10^3PFU). TUNEL assay was performed to test the apoptosis positive cells distribution in eyecup with treatment of autophagy inhibitor 3-MA, or autophagy inducer rapamycin at 4, 7, 10, and 14 dpi (A). Image J was used to analyze the percentage of cells stained with TUNEL (B).
Time-dependent effect of 3-MA and rapamycin treatment on cell death pathways in MEFs infected with MCMV. MEFs were infected with K181 strain of MCMV (MOI=1). Total protein was extracted at 24, and 48 hpi in MEFs, followed by autophagy hallmarks LC3I/II and SQSTM1/p62, phosphor-p70 S6 kinase (T389) (A), and cleaved caspase-3, as well as RIPK1/RIPK3/MLKL p(B) detection using western blot.
Figure 6

Time-dependent effect of 3-MA and rapamycin treatment on cell death pathways in eyecup cultures infected with MCMV Eyecups were isolated from adult C57BL/6J mice and infected with K181 strain of MCMV (5 x 10^3 PFU). Total protein was extracted at 4, 7, 10, and 14 dpi, autophagy hallmarks LC3I/II and
SQSTM1/p62, phosphor-p70 S6kinase (T389), mTOR, pmTOR(A), and cleaved caspase-3, as well as RIPK1/RIPK3/MLKL were tested via western blot(B).