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Original article

In vivo induction of autophagy in splenocytes of C57BL/6 and BALB/c mice infected with ectromelia orthopoxvirus

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Abstract

Autophagy is a self-degradation process of cellular components. It plays both antiviral and pro-viral roles in the life cycle of different viruses and the pathogenesis of different viral diseases. In this study, we evaluated autophagy induction in splenocytes of ectromelia virus (ECTV)-resistant C57BL/6 and ECTV-susceptible BALB/c mice during infection with the Moscow strain of the ectromelia virus (ECTV-MOS). Autophagy was analyzed using the Western blot method by assessing type II microtubule-associated protein 1 (MAP1) light chain 3 (LC3) and Beclin 1 expression levels relative to β-actin. Results indicated an increased ratio of LC3-II to β-actin in splenocytes of C57BL/6 mice only at 7 day post infection (d.p.i.) compared to uninfected animals. LC3-II/β-actin and Beclin 1/β-actin ratios in splenocytes of BALB/c mice increased at 5 d.p.i. and remained high until day 14 and 7 p.i., respectively. We confirmed the formation of autophagosome structures in the spleen of BALB/c mice by transmission electron microscopy (TEM). Moreover, autophagy accompanied necrosis in the splenocytes of infected animals. Results suggest that ECTV-MOS induced autophagy, especially in the spleen of the susceptible mouse strain, may support viral replication and promote cell necrosis.

Key words: autophagy, LC3-II, Beclin 1, ectromelia virus, mousepox

Introduction

Autophagy is an essential process responsible for selective degradation of cytoplasmic components. Deregulation of autophagy associates with pathogenesis of neuro- and myodegenerative disorders, systemic lupus erythematosus (SLE) and other autoimmune diseases, cancers, and bacterial and viral infections (Espert et al. 2007, Levine and Kroemer 2008, Yuk et al. 2012). Autophagy participates in involution of the

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bovine mammary gland where it presumably maintains cellular homeostasis in the dry period (Zarzynska et al. 2007).

Three forms of autophagy have been identified: macroautophagy (hereafter referred to as autophagy), microautophagy and chaperone-mediated autophagy. These forms of autophagy are defined by their physiological functions and the mode of cargo delivery to the lysosome (Yang and Klionsky 2010). The Atg1-Atg13 protein kinase complex initiates autophagy, which, in mammals, is activated by the absence of signaling from the nutrient-sensing kinase mammalian target of rapamycin (mTOR). Next, class III phosphoinositide-3-kinases (PI3K) (Vps34 and Vps35)-Beclin 1 (mammalian homolog of Atg6) complexes mediate vesicle nucleation, whereas two ubiquitin-like conjugation pathways [Atg12-Atg5 and Atg8-phosphatidylethanolamine (PE) systems] mediate vesicle expansion. In mammalian cells, a 16 kDa isoform of microtubule-associated protein 1 (MAP1) light chain 3 (LC3) – LC3-II corresponds to Atg8-PE in yeast and stably associates with the autophagosomal membrane. Autophagy culminates in autophagosome-lysosome fusion and the degradation of autophagosomal contents by lysosomal hydrolases (Levine and Kroemer 2008).

Ectromelia virus (ECTV) is an enveloped, double-stranded (ds) DNA virus that belongs to the genus *Orthopoxvirus* within the *Poxviridae* family. The virus causes mousepox in susceptible strains of mice. The mousepox model is used to study the pathogenesis of smallpox in human and other generalized viral infections. Clinical signs and lesions associated with mousepox depend on the strain of mouse infected. Resistant strains such as C57BL/6, C57BL/10 and 129/Sv may show no clinical signs of disease. In contrast, susceptible A, BALB/c, DBA and C3H mice often succumb to disease due to necrosis of the spleen and liver. Resistance to mousepox is associated with *rmp* (resistance to mousepox) genes and recovery from infection requires mobilization of cell-mediated and innate immune responses (Esteban and Buller 2005).

Krzyżowska and colleagues (2002) showed that infection of BALB/c mice with a highly virulent Moscow strain of ECTV (ECTV-MOS) leads to DEVDase-(caspase-3- and caspase-7-) dependent apoptosis in the liver, spleen, conjunctivae and lymph nodes which, presumably, regulates the resolution of viral infection. However, there are no data inducting evidence of autophagy in vital organs of mice during mousepox. Consequently, we studied the induction of autophagy in splenocytes of ECTV-resistant C57BL/6 and ECTV-susceptible BALB/c mice during ECTV-MOS infection.

Materials and Methods

Animals

Eight to twelve-week old male C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were purchased from the Maria Skłodowska-Curie Memorial Cancer Centre and Institute of Oncology in Warsaw, Poland. Experimental procedures on animals were approved by the 3rd Local Ethical Commission for Animal Research at the Warsaw University of Life Sciences-SGGW (permit No. 61/2009).

Virus

Highly infectious ECTV-MOS (ATCC 1374) was grown in permissive Vero cells (African green monkey kidney epithelial adherent cells, ATCC CCL-81) monolayer. The virus was titrated and stored at -80°C until use in experiments. Anesthetized mice were injected with 20 µl of 100 plaque forming units (PFU) of ECTV-MOS into the hind foot pad (f.p.). Uninfected control C57BL/6 and BALB/c mice were injected with sterile PBS (phosphate-buffered saline). The number of animals ranged from 5 to 6 per experimental group.

Reagents

The following antibodies (Abs) were used in the analysis of autophagy: rabbit anti-LC3, rabbit anti-Beclin 1 and goat anti-rabbit secondary Abs conjugated to HRP (horseradish peroxidase). Mouse β-actin was detected with anti-β-actin and rabbit anti-mouse IgG conjugated to HRP. All Abs were purchased from Sigma (USA).

Preparation of single cell suspension

Spleens were aseptically removed at 2, 5, 7, 14 and/or 21 days post infection (d.p.i.) or from uninfected control animals. The isolated organs were mechanically disrupted by passing through stainless steel sieves and erythrocytes were lysed with Tris-NH_4Cl (pH 7.4) buffer. Cells were enumerated in a Neubauer chamber, resuspended in extraction reagent and subjected to total protein extraction.

Electrophoresis and Western blot

Whole cell lysates from splenocytes were prepared using a Mammalian Cell Lysis Kit (MCL1; Sigma,

USA). Protein content in cell lysates was measured using bicinchoninic acid (BCA) assay (QuantiPRO BCA Assay Kit; Sigma, USA) according to the manufacturer's instructions. Subsequently, proteins were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Beclin 1 (60 kDa) and LC3 (16 and 18 kDa) proteins were separated in 12% and 15% resolving gels, respectively. Electrophoresis was carried out at 80V for about 2.5 h. In Western blot analysis β-actin (42 kDa) was used as a loading control.

Protein bands, separated by electrophoresis, were transferred onto polyvinylidene fluoride (PVDF) membrane at 70 V for 60 min in cold transfer buffer. The membranes were then blocked for 1 h in TBST (Tris-buffered saline with Tween 20) containing 5% non-fat dry milk and incubated overnight at 4°C with primary Abs: rabbit anti-LC3 or rabbit anti-Beclin 1 at a final concentration of 1 μg/ml. The membranes were then rinsed in TBST and incubated with anti-rabbit-HRP (1:50000 in TBST/5% not-fat dry milk) for 1 h at room temperature. Chemiluminescent Peroxidase Substrate-1 (Sigma, USA) was applied according to the manufacturer's instructions, and the membranes were exposed to Kodak Retina X-Ray film. Western blot images were recorded on a digital imaging system, KODAK Image Station 4000 MM (USA). Densitometric analysis of the protein bands was performed using ImageJ software (Zanotto et al. 2011).

Histological study

Spleens were aseptically removed from uninfected animals or animals at 7 d.p.i. with ECTV-MOS. Organs were fixed in 10% formalin solution, dehydrated in increasing concentration of alcohol and embedded in paraffin. Samples were cut into 4 μm-thick sections, stained with haematoxylin and eosin (HE) and analyzed in an Olympus BX60 (Japan) microscope equipped with a Color View III cooled CCD camera and Cell^F software (Olympus, Japan).

Transmission electron microscopy

Organ sections were prepared as recommended by the Analytical Center of Warsaw University of Life Sciences-SGGW. Briefly, organs were cut into 1-3 mm-thick pieces and prefixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h at 4°C. The tissue samples were then washed in 0.1 M cacodylate buffer and fixed with 1% osmium tetroxide (OsO_4) for 2 h. Fixed pieces were dehydrated with ethanol/acetone and embedded in Epon 812. Ultrathin sections were cut using a Leica EM UC6 (Germany) ultramicrotome and collected on copper grids with 300 square mesh. Tissue sections were analyzed in a Joel JEM-1220 (Japan) transmission electron microscope equipped with a Morada digital camera (Olympus/SIS, Germany).

Statistical analysis

All quantitative data were expressed as mean ± SD (standard deviation) from at least three independent experiments. Western Blot experiments were analyzed using the Mann-Whitney *U*-test (Statistica 6.0 software; Statsoft Inc., USA). Statistical significance was determined at $P<0.05$.

Results

At 7 d.p.i. with ECTV-MOS, histopathological changes in the spleen of resistant C57BL/6 mice consisted of hyperplastic lymphoid follicles with reactive germinal centers (Fig. 1). Focal necrosis (when present) was observed as small, pale, slightly depressed zones. In susceptible BALB/c mice at 7 d.p.i. with ECTV-MOS, lesions consisted of massive necrosis of lymph follicles, congestion of the red pulp and fibrosis (Fig. 1). Necrosis extended rapidly to efface large segments of parenchyma destroying the normal structure of the spleen. Pyknotic nuclear debris was observed within the necrotic zones (Fig. 1).

Autophagy in splenocytes of C57BL/6 and BALB/c was assessed at different times p.i.: day 2 (primary viraemia), day 5 (secondary viraemia), day 7 (primary lesions), day 14 (developed skin lesions) and/or day 21 (recovery). Analysis of the autophagic process was based on Western blot assessment of LC3-II and Beclin 1 expression levels. LC3-II (16 kDa) and Beclin 1 (60 kDa) levels were measured relative to the corresponding loading control [f-actin (42 kDa)].

Quantitative analysis of LC3-II and Beclin 1 relative to β-actin in splenocytes of C57BL/6 mice during ECTV-MOS infection is shown in Fig. 2. Densitometric analysis revealed a statistically significant ($P<0.05$) increase in the LC3-II/β-actin ratio in splenocytes compared to uninfected control animals (0.70 ± 0.06 and 0.45 ± 0.10, respectively) (Fig. 2B). However, no statistically significant changes in Beclin 1/β-actin ratios (Fig. 2C) were noted during the course of ECTV-MOS infection in splenocytes of C57BL/6 mice.

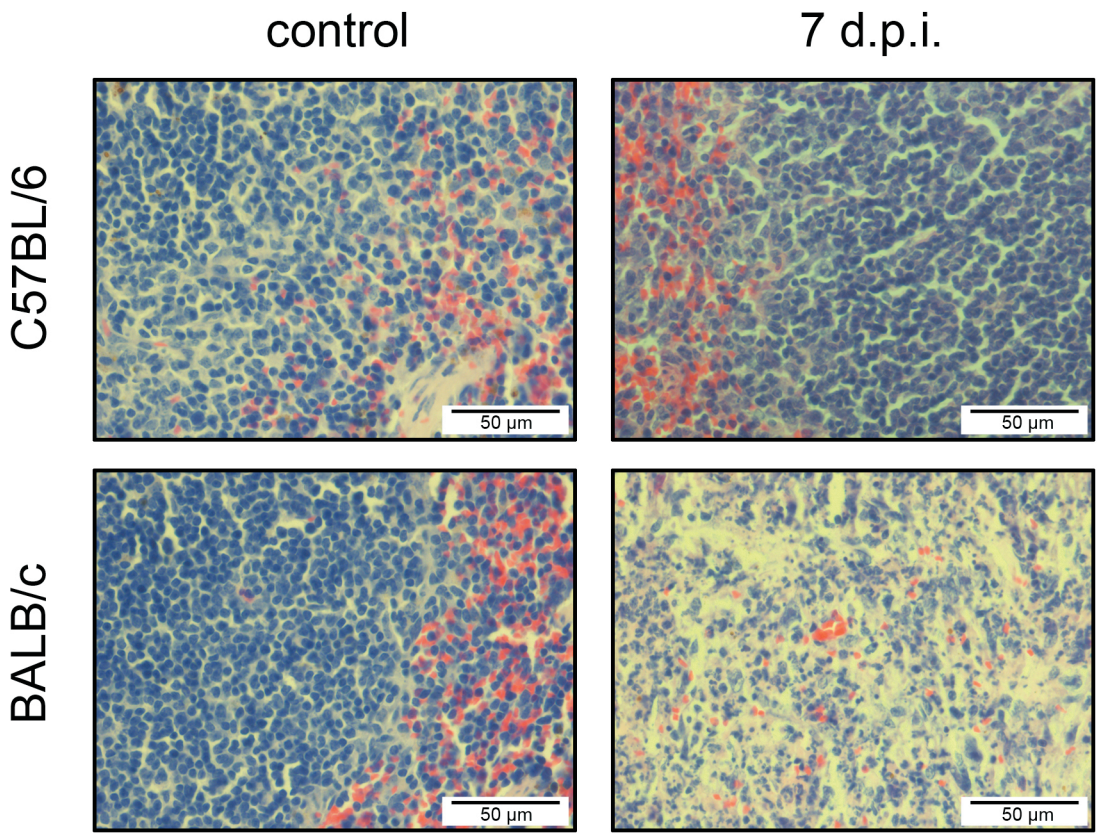

Fig. 1. Histopathology of spleen of resistant C57BL/6 and susceptible BALB/c mouse uninfected and at 7 d.p.i. with ECTV-MOS (HE stain). Splenic white pulp necrosis and fibrosis is observed in BALB/c mouse at 7 d.p.i. with ECTV-MOS.

Analysis of autophagy in splenocytes of BALB/c mice infected with ECTV-MOS was performed on 2, 5, 7 and 14 d.p.i., because susceptible BALB/c mice succumb to disease between 9 and 16 d.p.i. Densitometric analysis of protein bands on Western blot revealed a statistically significant ($P<0.05$) increase in LC3-II/β-actin ratio, in splenocytes of BALB/c mice at 5 d.p.i. compared to uninfected control animals (0.47 ± 0.13 vs 0.22 ± 0.03) (Fig. 3B). The LC3-II/β-actin ratio in splenocytes of BALB/c mice remained high until day 14 p.i. with ECTV-MOS (Fig. 3B). Similarly, at early stages of mousepox (5 and 7 d.p.i.) the Beclin 1/β-actin ratio was statistically significantly ($P<0.05$) higher in splenocytes of infected BALB/c mice than uninfected control animals (Fig. 3C). At 14 d.p.i. the Beclin 1/β-actin ratio started to decrease, reaching values similar to control.

Formation of autophagosomes in splenocytes of BALB/c mice during the later stages of mousepox was confirmed by transmission electron microscopy. Double-membrane autophagosome structures with wrapped organelles were observed in splenocytes of BALB/c mice at 14 d.p.i. (Fig. 4A). At this time point of infection, C-shaped double-membrane structures resembling pre-autophagosomes were also observed in draining lymph nodes (DLNs) of BALB/c mice (Fig. 4B). Moreover, apoptotic cells with chromatin condensation and fragmentation of the nucleus were detected in the spleen of BALB/c mice during the later stages of mousepox (Fig. 4C).

Discussion

Autophagy plays an important role in the host immune response against viral and bacterial infections. During autophagy, viral antigens are sequestrated in autophagosomes and degraded in autolysosomes. Autophagy promotes digestion of endogenously synthesized viral proteins and facilitates their presentation through major histocompatibility complex (MHC) class I or II molecules and thus enhances the adaptive immune response (Dengjel et al. 2005, English et al. 2009). Viruses, however, have evolved different strategies allowing them not only to evade being killed through the autophagy system, but also to divert autophagy to increase their own replication and promote their survival in the autophagic cells (Espert et al. 2007, Orvedahl and Levine 2008).

Here, for the first time, we report that autophagy is induced in splenocytes of C57BL/6 and BALB/c mice during the early stages of ECTV-MOS infection.

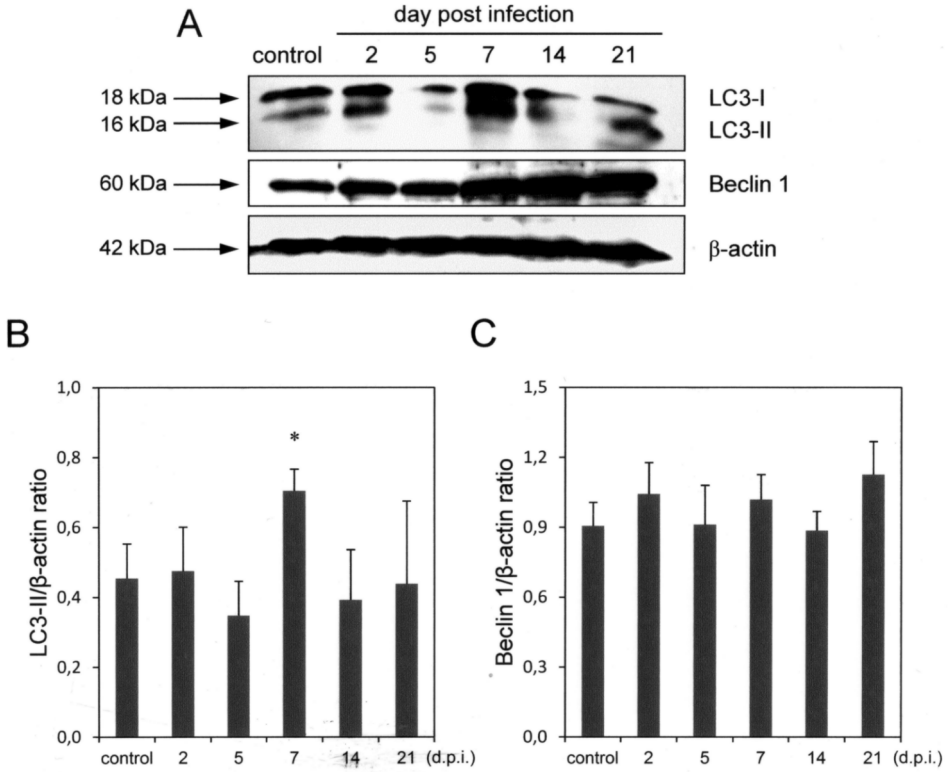

Fig. 2. LC3-II and Beclin 1 expression in splenocytes of C57BL/6 mice during mousepox: representative Western blot analysis of LC3-I (18 kDa), LC3-II (16 kDa), Beclin-1 (60 kDa) and β-actin (42 kDa) expression in uninfected (control) and infected C57BL/6 mice at 2, 5, 7, 14 and 21 d.p.i. with ECTV-MOS (A); quantitative analysis of LC3-II/β-actin (B) and Beclin 1/β-actin (C) ratios in splenocytes of C57BL/6 mice during mousepox. Data were obtained from 3 independent experiments (*P<0.05 vs. control).

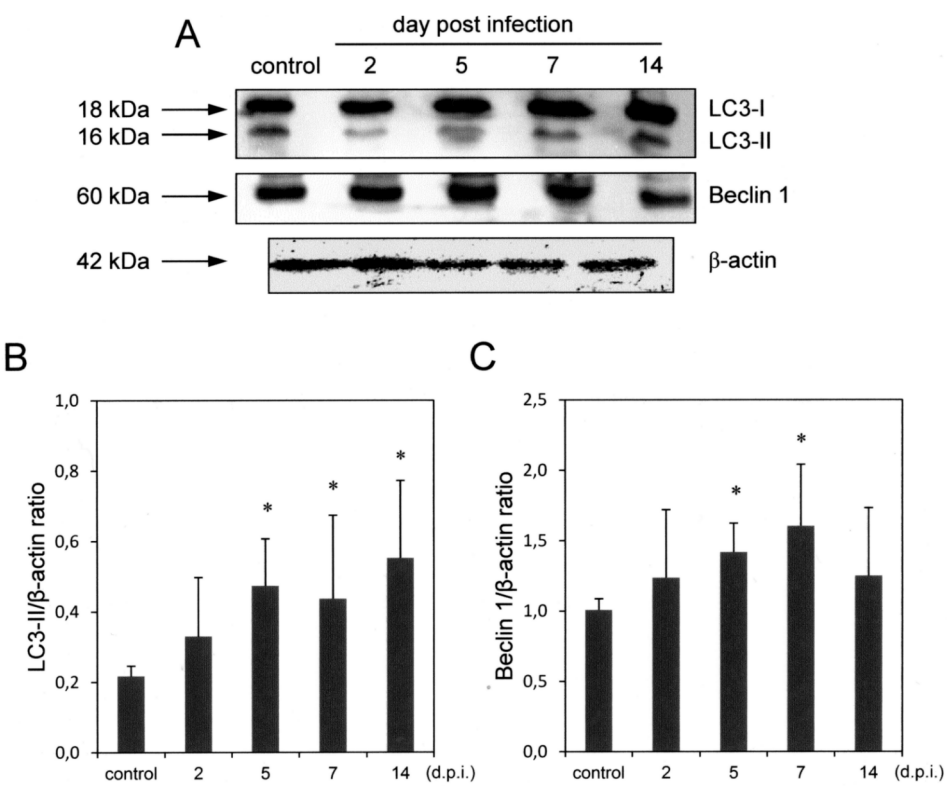

Fig. 3. LC3-II and Beclin 1 expression in splenocytes of BALB/c mice during mousepox: representative Western blot analysis of LC3-I (18 kDa), LC3-II (16 kDa), Beclin-1 (60 kDa) and β-actin (42 kDa) expression in uninfected (control) and infected C57BL/6 mice at 2, 5, 7, and 14 d.p.i. with ECTV-MOS (A); quantitative analysis of LC3-II/β-actin (B) and Beclin 1/β-actin (C) ratios in splenocytes of BALB/c mice during mousepox. Data were obtained from 3 (C) and 4 (B) independent experiments (*P<0.05 vs. control).

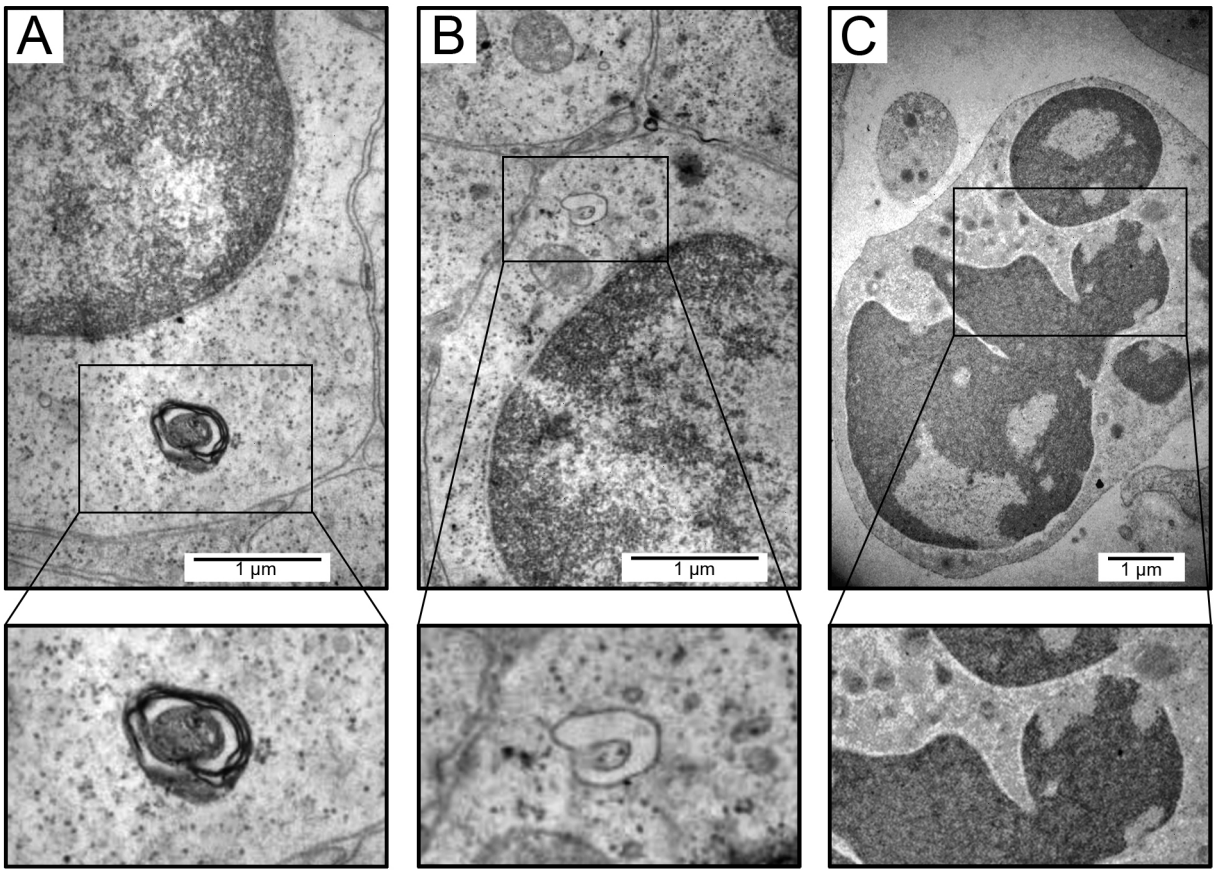

Fig. 4. Transmission electron micrograph of autophagosomal structure in splenocyte (A), pre-autophagosomal structure in cell of DLN(B) and apoptosis in splenocyte (C) of BALB/c mouse at 14 d.p.i. with ECTV-MOS.

However, a higher level of autophagy is observed in splenocytes of susceptible BALB/c mice infected with the virus. In splenocytes of C57BL/6 mice an increased ratio of LC3-II to β-actin was observed only at day 7 p.i., whereas in splenocytes of BALB/c mice increased ratios of LC3-II to β-actin and Beclin 1 to β-actin were detected as early as day 5 p.i. and remained high until day 14 and 7 p.i., respectively (Fig. 2 and 3). Parker et al. (2009) have shown that following f.p. infection of C57BL/6 mice with 100 PFU f.p. of ECTV, very low levels of infectious virus are detected in the liver at day 5 p.i., but none is detected in the spleen or lung. However, in the susceptible A/Ncr strain, infectious virus can be detected in the spleen by day 2 p.i., in the liver by day 5 p.i. and in the lungs by day 6 p.i. This may explain the delayed induction of autophagy in splenocytes of C57BL/6 mice compared to susceptible BALB/c mice. Moreover, splenic necrosis is accompanied by autophagy and correlates with the severity of lesions. It is not excluded that increased levels of autophagy in susceptible inbred strains of mice may have an important role in mousepox pathogenesis.

Autophagy is believed to be a pro-survival mechanism through its capacity to block various forms of necrotic cell death, including necroptosis and poly-(ADP-ribose) polymerase (PARP)-mediated cell death (Shen and Codogno 2012). Moreover, anti-necrosis properties of autophagy have been found in various pathological processes and diseases, such as cancer (Degenhardt et al. 2006), and during ischemic preconditioning of liver grafts, which reduces ischemia/reperfusion injury (Degli Esposti et al. 2011). On the other hand, autophagy has been shown to promote necrosis (Samara et al. 2008, Ullman et al. 2008). Samara et al. (2008) demonstrated that autophagy is required for necrotic cell death and contributes to cellular destruction during necrosis in *Caenorhabditis elegans* neurons. Impairment of autophagosome formation, nucleation, expansion/completion and retrieval by knockdown of the autophagy genes or by pharmacological treatment resulted in suppression of necrosis in *C. elegans* neurons (Samara et al. 2008). Ullman et al. (2008) revealed that autophagy may promote cell death by necrosis in cells with impaired apoptosis. In response to prolonged endoplasmic reticulum (ER)-stress, apoptosis-deficient $bax^{-/-}bak^{-/-}$ mouse embryonic fibroblasts (MEFs) underwent caspase-independent necrotic cell death associated with autophagy (Ullman et al. 2008).

Therefore, the results presented in this study suggest that during the early stages of mousepox in susceptible BALB/c mice, strong induction of autophagy may promote necrosis of splenocytes, which is the leading cause of death in animals infected with ECTV-MOS (Buller and Palumbo 1991).

On the other hand, increased autophagy during the early stages of mousepox, especially in splenocytes of BALB/c mice, may facilitate multiplication and/or survival of ECTV-MOS. Our previous study (Martyniszyn et al. 2011) showed that Beclin 1 strongly accumulates at the sites of intensive ECTV-MOS replication in permissive L929 cells, suggesting a possible role for autophagy in virus replication/survival. It is known that many viruses have evolved mechanisms to divert the autophagic system to their own benefit. For example, human parvovirus B19, a small non-enveloped single-stranded (ss) DNA virus, is able to induce autophagy in cells arrested in the G2 phase and promote survival of infected cells, which presumably allows complete viral replication before destruction of the cell (Nakashima et al. 2006). Dengue virus, an enveloped positive-strand RNA virus, replicates ineffectively in the absence of a functional autophagy pathway (Lee et al. 2008). Enterovirus (EV) 71, a positive ssRNA virus with a non-enveloped capsid, induces formation of autophagosome-like structures both *in vitro* and *in vivo*, allowing viral replication. Inhibition of autophagy by 3-methyloadenine results in decrease of extracellular virus production in human neuroblastoma cells infected with EV71 (Huang et al. 2009).

Recent studies by Ke and Chen (2011) and Shrivastava et al. (2011) have revealed that hepatitis C virus (HCV)-induced autophagic pathway suppresses the innate immune response, leading to HCV replication. HCV, a positive ssRNA enveloped virus, induces complete autophagy through activation of the unfolded protein response (UPR), which positively regulates HCV RNA replication. Inhibition or enhancement of the UPR-autophagic pathway activated or repressed, respectively, production of interferon (IFN)-β mediated by HCV-derived pathogen-associated molecular pattern (PAMP) (Ke and Chen 2011). Moreover, HCV infection in autophagy-knockdown immortalized human hepatocytes (IHHs) enhances the IFN signaling pathway and reduces viral replication. Beclin 1 or Atg7 knock-down IHHs infected with HCV, exhibit increased expression of IFN-regulated genes, including IFN-α/β, 2'5'-oligoadenylate synthetase 1 and IFN-α-inducible protein 27 (IFI27) mRNAs (Shrivastava et al. 2011).

The multiplication of other viruses seems not to be affected by autophagy. A study by Zhang et al. (2006) demonstrated that autophagy is not required for replication and maturation of vaccinia virus (VACV) – another member of the *Poxviridae* family. Morphogenesis of VACV was normal in *atg5*$^{-/-}$ MEFs and *beclin 1*$^{-/-}$ embryonic stem (ES) cells. The kinetics of infectious virion production in autophagy-deficient cell lines was similar to that observed in isogenic wild type counterpart cells (Zhang et al. 2006). Moreover, another study made by Moloughney et al. (2011) revealed that VACV does not exploit autophagy machinery for viral morphogenesis, but instead, disrupts cellular autophagy through a direct Atg12-Atg5 conjugation and inhibition of autophagosome formation. Murine hepatitis virus (MHV), an ssRNA virus of positive polarity, replicates either in primary low passage *atg5*$^{-/-}$ MEFs or bone marrow derived macrophages (BMMø) lacking Atg5 (Zhao et al. 2007).

In conclusion, the results of the present study demonstrate that ECTV-MOS induces autophagy in splenocytes of genetically resistant C57BL/6 and, in particular, susceptible BALB/c mice. However, it still remains undetermined whether the induction of autophagy in splenocytes of infected animals is a process which promotes replication of ECTV-MOS or whether viral replication is not affected positively or negatively by autophagy. The potential role of autophagy and the nature of its function may vary greatly in different types of viral infections. For this reason, the biological significance of ECTV-MOS induced autophagy should be clarified in future studies.

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