Histol Histopathol (2017) 32: 963-975

**http://www.hh.um.es**

**Histology and Histopathology**

*From Cell Biology to Tissue Engineering*

# Ethanol enhances thymocyte apoptosis and autophagy in macrophages of rat thymi

**Tsubasa Betsuyaku[1], Nabil Eid[1], Yuko Ito[1], Yoshihisa Tanaka[1], Yoshinori Otsuki[2] and Yoichi Kondo[1]**

[1]Department of Anatomy and Cell Biology, Division of Life Sciences,
Osaka Medical College, Osaka, Japan and [2]Osaka Medical College, Osaka, Japan

**Summary.** Tingible body macrophages (TBMs) play essential roles in the phagocytosis of apoptotic lymphocytes, specifically under exposure to various stressors. Although excessive ethanol consumption may enhance thymocyte apoptosis, reports investigating the autophagic response of the thymus to ethanol toxicity are still lacking. We investigated apoptosis and autophagy in thymi of an animal model of binge ethanol exposure. Adult male Wistar rats were injected intraperitoneally either with 5 g/kg ethanol or phosphate buffer saline (for the control group) and sacrificed 0, 3, 6 and 24 hours after injection. Light and transmission electron microscopy (TEM) studies revealed enhanced formation of TBMs phagocytosing many apoptotic thymocytes in the thymic cortex of the ethanol-treated rats (ETRs), and this formation was particularly marked at 24 h. The macrophages showed signs of activation under TEM and immunofluorescence double labeling with RM4 (a macrophage marker) and iNOS. Additionally, in comparison to the control group, autophagy was enhanced in ETR thymic TBMs as evidenced ultrastructurally by accumulation of autophagic vacuoles, immunohistochemical increases in LC3 puncta, Western blot analysis of the latter protein, and colocalization of LC3 and RM4 in immunofluorescence double labeling. Immunoelectron microscopy also revealed LC3-labeled autophagic vacuoles and apoptotic cell phagosomes in ETR TBMs, suggesting the possibility of LC3-related phagocytosis. This was confirmed by enhanced colocalization of LC3 with lysosomal cathepsins in double labeling. These results indicate that enhanced autophagy in ETR thymic TBMs is not only a cytoprotective mechanism but could also be involved in the clearance of apoptotic thymocytes, thus preventing autoimmune reactions and suppressing inflammatory response.

**Key words:** Autophagy, LAP, Apoptosis, Ethanol, Thymus, TBMs

*Offprint requests to:* Dr. Nabil Eid, Division of Life Sciences, Department of Anatomy and Cell Biology, Osaka Medical College, 2-7 Daigaku-machi, Takatsuki, Osaka 569-8686, Japan. e-mail: nabil@osaka-med.ac.jp or nabileidm@yahoo.com

DOI: 10.14670/HH-11-861

## Introduction

Autophagy is a cytoprotective mechanism for the removal of damaged cellular components after exposure to various stressors such as oxidative stress and mitochondrial damage (Kroemer et al., 2010; Perrotta et al., 2011; Eid et al., 2013; Shin et al., 2016). Morphologically, autophagy is characterized by the formation of isolation membranes that engulf cellular components producing autophagosomes. The latter then fuse with lysosomes to form autolysosomes for cargo clearance. Autophagosome formation is mediated by microtubule-associated protein 1 light chain 3 (LC3), and is promoted by lipidated LC3-II, which is produced from LC3 (Mizushima et al., 2011; Chen et al., 2012; Eid et al., 2016a). Activation of the autophagy pathway also aids LPS-stimulated macrophages in protection against cell death, presumably by reducing the production of iNOS and suppressing inflammatory response (Han et al.,

2013; Liu et al., 2015; Yang et al., 2015). Based on in vitro studies, LC3 targeting of the phagosomal membrane has also been recently reported to enhance maturation and degradation of phagosomes containing zymosan particles, infectious agents and apoptotic cells via a process called LC3-associated phagocytosis (LAP), which is induced in a different way from autophagy. The membrane of a LAP-engaged phagosome (LAPosome) is single, while autophagosomes have a double membrane. The signal required for LAP is reactive oxygen species (ROS), which can also trigger autophagy (Sanjuan et al., 2007; Shui et al., 2008; Martinez et al., 2011; Sprenkeler et al., 2016). However, some studies have shown that apoptotic cells can be degraded by LC3-mediated autophagosomes in macrophages (Baghdadi et al., 2013) and semiprofessional phagocytes (Brooks et al., 2015), although the authors of these studies insisted that LAP could contribute partially to autophagosome-related degradation of phagocytosed apoptotic cells.

Alcohol (ethanol) consumption is a global health problem, and almost all body organs may be affected by its abuse (Haorah et al., 2008; Liang et al., 2014; Yun et al., 2014; Loftis et al., 2016). Excessive ethanol intake induces lymphocyte depletion in lymphoid organs via mechanisms related to the generation of high levels of corticosteroids, bone marrow depression, lymphocyte redistribution, hepatic dysfunction, and excessive lymphocyte apoptosis (Budec et al., 1992; Han et al., 1993; Collier et al., 1998; Eid et al., 2000; Kapasi et al., 2003). Phagocytosis of apoptotic cells by phagocytes is required for resolution of organ damage and maintenance of immune tolerance (Brooks et al., 2015). The clearance of apoptotic thymocytes after exposure to various stressors such as xenobiotics and viral infection is mediated by a class of macrophages known as tingible body macrophages (TBMs), which exhibit high phagocytic activity, often contain multiple apoptotic nuclei, and can grow relatively large (Eid et al., 2000; Salguero et al., 2004). In a previous study, we found marked accumulation of TBMs laden with apoptotic splenocytes in the splenic white pulp of chronic ethanol-treated rats (ETRs). Transmission electron microscopy (TEM) indicated that these macrophages were resistant to apoptosis and appeared to have protective mechanisms. However, the nature of these mechanisms is not clearly understood (Eid et al., 2000).

A growing body of data has recently indicated that excessive ethanol intake via mechanisms related to oxidative stress, mitochondrial damage and lipid overload may enhance cytoprotective autophagy in hepatocytes of humans and animal models of acute and chronic alcoholism (Dolganiuc et al., 2012; Lin et al., 2013; Eid et al., 2013, 2016a). Acute and chronic ethanol consumption have also been reported to induce iNOS expression in various macrophages and other cells as a result of enhanced endotoxin and cytokine production (Kanuri et al., 2009; Pla et al., 2016). However, the involvement of autophagy in TBM survival and phagocytosis of apoptotic thymocytes in animal models of binge ethanol exposure have not yet been reported. In this study, the authors investigated the involvement of apoptosis, canonical autophagy and LAP in acute ethanol-induced damage to rat thymi using multiple light and electron microscopic techniques in addition to Western blot analysis. The results demonstrated enhanced thymocyte apoptosis in ETRs, which was associated with upregulation of autophagy and induction of LAP of apoptotic thymocytes in ETR thymic TBMs. This could be a mechanism for maintenance of immune homeostasis.

## Materials and methods

### *Rats and experimental design*

Adult male Wistar rats (12 weeks old) were purchased from SLC Japan Co., Shizuoka, Japan. Their body weight was around 250-300 g. They were maintained and treated according to the guidelines set by the Experimental Animal Research Committee of Osaka Medical College. 5 g/kg (40% v/v) ethanol was intraperitoneally injected once to the rats, which is consistent with the animal model of binge ethanol consumption. This dose of ethanol represents binge drinking in humans as recently reported by us and other investigators (Carson et al., 1996; Collier et al., 1998; Zhou et al., 2001; Ding et al., 2010; Eid et al., 2016a). The selection of the intraperitoneal route for ethanol intake is consistent with animal models of acute ethanol exposure. In fact, both the oral and intraperitoneal routes of ethanol administration have been found to have similar outcomes regarding blood concentration and organ damage although the oral route is more physiological (D'Souza El-Guindy et al., 2010; Chen et al., 2013; Eid et al., 2016a). After administration of ethanol, the rats were sacrificed by cervical dislocation at various time points (0, 3, 6 and 24 h). An equal volume of phosphate buffer saline, instead of ethanol, was injected in the control group. Three animals were examined at each time point (Ding et al., 2010; Eid et al., 2016a). The pieces of rat thymi were fixed with either 4% formalin for paraffin embedding, or 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate for epoxy embedding and TEM. The other thymic tissues were frozen at -80°C for protein analysis.

### *Antibodies and kits*

The following antibodies were used: mouse anti-macrophage antibody RM4 (KT014) from Trans Genic Inc, Kobe, Japan; rabbit anti-iNOS antibody (ab15326) from Abcam; rabbit anti-LC3 antibody (PM036) from MBL, Nagoya, Japan; goat anti pan cathepsin antibody (sc-6499) from Santa Cruz; rabbit anti-β actin antibody (#4970) from CST Japan. A Terminal deoxynucleotidyl transferase dUTP-mediated nick-end labelling (TUNEL) FITC-labeled kit, from Roche Diagnostics, Mannheim,

Germany, was used for apoptosis detection.

### Hematoxylin and eosin (H&E) staining

After deparaffinization, 4 $\mu$m-sections were stained with H&E using standard methods for histopathological evaluation of ethanol-induced thymic damage.

### TUNEL for detecting apoptotic cells and analysis

TUNEL was performed according to the manufacturer's protocols. Paraffin sections of thymic tissue were dewaxed, rehydrated in ethanol, then treated with TUNEL reaction mixture (TdT enzyme and fluorescent-labelled nucleotides) which labels the fragmented nuclear DNA for 1 hour at 37°C. The number of TUNEL-positive cortical thymocytes (green labeling) were counted from 10 random high-power fields (HPF) (400x magnification) from each group. The slides were observed under Olympus BX41 fluorescence microscope, Tokyo, Japan (Eid et al., 2016a).

### Immunohistochemical staining of RM4, iNOS and LC3

Paraffin-embedded sections at 4 $\mu$m-thickness were stained with the primary antibodies described above, according to the manufacturer's protocols. In brief, after deparaffinization, antigen retrieval and blocking, antibodies were applied overnight at 4°C. The sections were labeled for 1 h with EnVision+ Single Reagents (K4003) for rabbit antibodies and EnVision+ Dual Link, Single Reagents (K4063) for mouse antibody from Dako. Then, sections were treated with ImmPACT DAB Peroxidase substrate as chromogen (Vector laboratories), and counterstained with hematoxylin. RM4 positive macrophages were counted from 10 random HPF (400x magnification) from each groups. Quantification of LC3 puncta in thymic cortex of control and ETRs were performed with image J (http://imagej.nih.gov/ij/) (Eid et al., 2016a).

### Immunofluorescence double labeling of markers of macrophages and autophagy

In double labeling of RM4 and iNOS, RM4 and LC3, and LC3 and pan cathepsin (lysosomal marker), we used a sequential method for applying the primary antibodies, followed by two secondary antibodies conjugated to different fluorochromes according to standard methods and as previously reported (Eid et al., 2016a,b). The slides were observed under confocal laser scanning microscope (Leica TCS SP8, Manheim, Germany). Quantification of LC3 and pan cathepsin co-localization was performed with image J.

### Western blot analysis

Total protein was extracted from rat thymi, solubilized in SDS lysis buffer, and then centrifuged. Protein samples were loaded on SDS-polyacrylamide gels for electrophoresis and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% BSA, and then incubated with anti-LC3 antibody, followed by peroxidase-labelled goat anti-rabbit IgG antibody as reported (Thomes et al., 2012).

### TEM and quantitative analysis

Ultrathin sections at 70 nm-thickness from blocks embedded in epoxy resin were double stained with uranyl acetate and lead citrate, and observed with H-7650 transmission electron microscope, Hitachi, Japan. For quantification of apoptosis, apoptotic cells were counted from 10 random lower-magnification fields (1,000x). The identification of thymi and localization of apoptotic cortical thymocytes were first identified in semithin sections stained with toluidine blue. Ultrastructurally, Apoptotic cells are characterized by margination of condensed chromatin, nuclear fragmentation, and the formation of apoptotic bodies (Otsuki, 2004; Eid et al., 2016a).

### Immunoelectron microscopy of LC3

Ultrathin sections were etched with saturated sodium metaperiodate and hydrochloric acid, washed with filtered distilled water and incubated in 0.1% bovine serum albumin in Tris buffer for 1 h. After incubation in LC3 antibody solution (1/250 dilution) for 2 h, ultrathin sections were washed with Tris buffer and then incubated in secondary antibody conjugated with 10 nm-gold particles (1/10 dilution). Grids were washed and counterstained with uranyl acetate for a few minutes as reported (Eid et al., 2016a,b).

### Statistical analysis

The Student t test was used for comparison between ethanol-treated and control groups. $P<0.05$ was considered as significant.

## Results

### Ethanol-induced elevation of apoptosis in cortical thymocytes is associated with TBM accumulation

As shown in Fig. 1A, compared to the control group, H&E staining showed TBM accumulation in ETR thymic cortexes, peaking at 24 h after EtOH injection. The TUNEL labeling and TEM shown in Fig. 1B,C demonstrate a significant increase in ETR cortical thymocyte apoptosis compared to the control group. Because accumulations of TBMs and thymocyte apoptosis in ETRs peaked at 24 h compared to other time points (3 h and 6 h) (data not shown), the 24 h time point was chosen for analysis in subsequent experiments. In addition, this time period was characterized by the highest autophagic activity in TBMs as shown below.

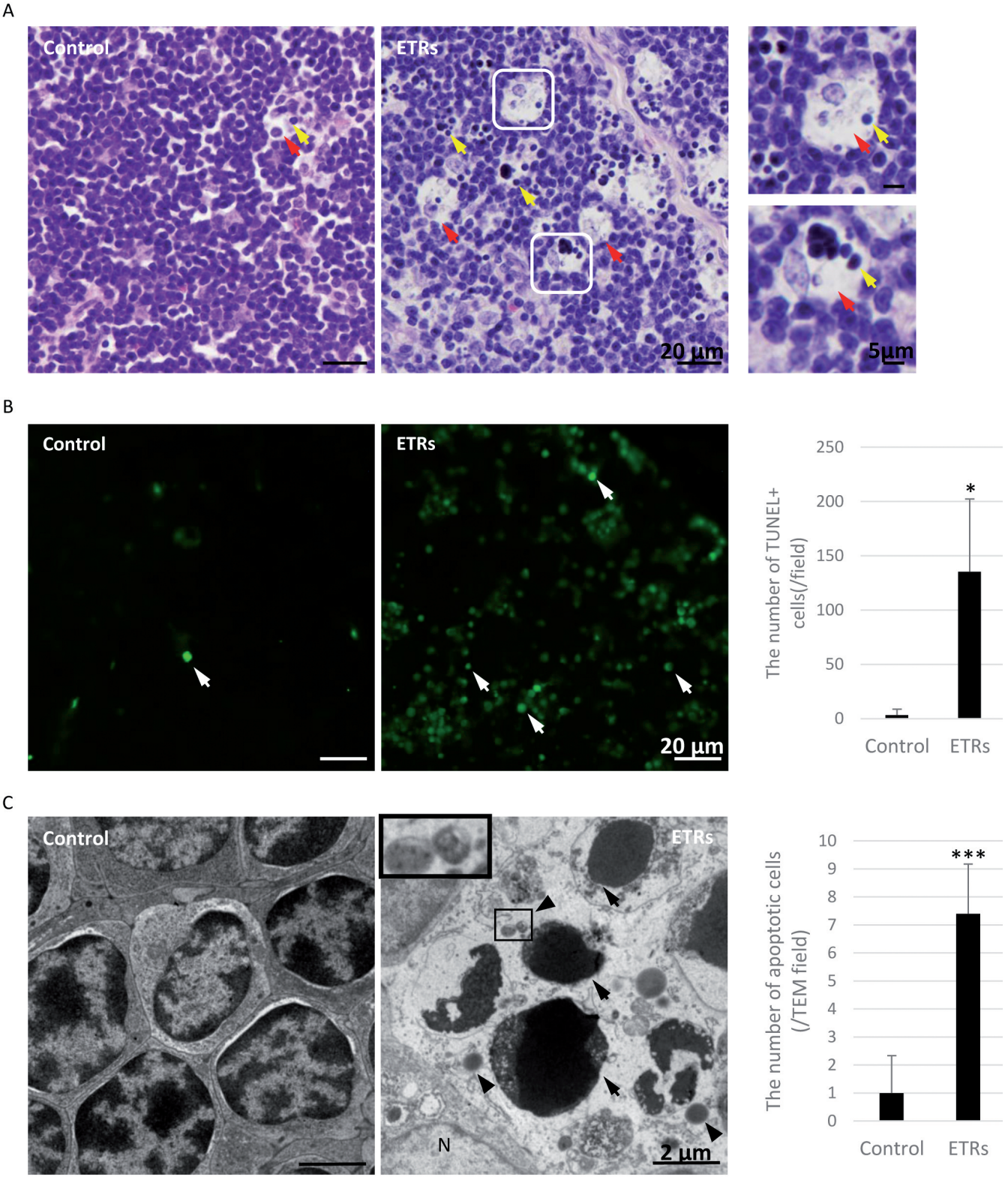


**Fig. 1.** Increased apoptosis of thymocytes associated with accumulation of TBMs in ETR thymic cortexes. **A.** H&E staining showing apoptotic thymocytes (yellow arrows) within TBMs (red arrows) in the thymic cortex. The framed areas are magnified on the right. **B.** TUNEL labeling (white arrows) and quantification of apoptotic cells (the histogram on the right side). **C.** TEM and histogram demonstrating a significant increase of thymocyte apoptosis in ETR thymic cortexes (black arrows). Black arrow heads indicate lysosomes. The inset (a magnification of the framed area) shows two lysosomes with characteristic limiting membranes. N: nucleus of macrophage; *P<0.05; ***P<0.001.

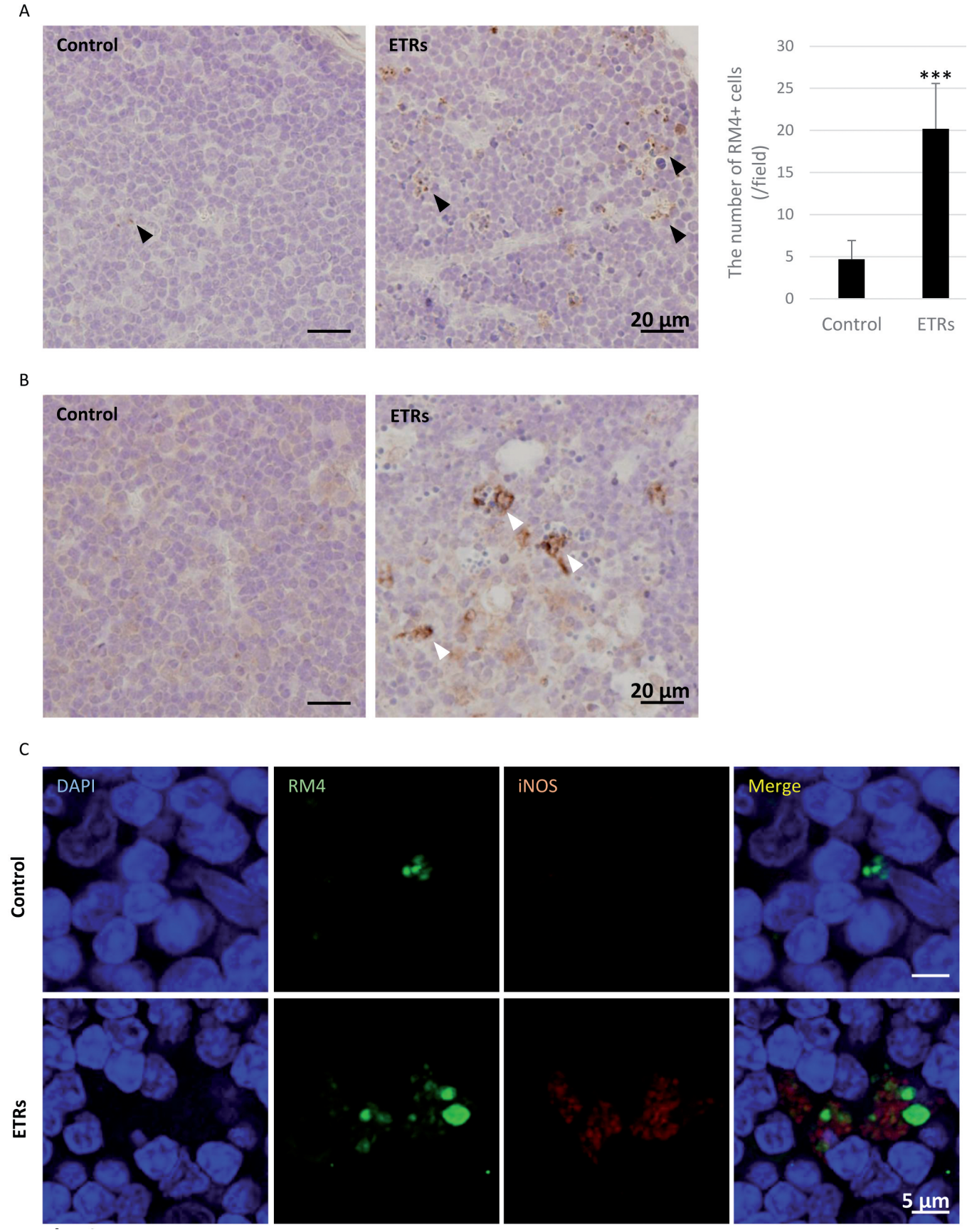


**Fig. 2.** Enhanced expression of RM4 and iNOS in ETR thymic TBMs. **A.** Immunolabeling and quantification of RM4 (a macrophage marker; black arrow heads). **B.** Immunostaining of iNOS (white arrow heads). The brown color (DAB reaction) indicates positive staining. **C.** Immunofluorescence double labeling for RM4 and iNOS ***P<0.001.

*Induction of iNOS in ETR thymic TBMs as revealed by immunohistochemical and immunofluorescence double labeling studies*

Immunohistochemical staining using RM4 (a marker for TBMs and other macrophages) showed enhanced expression and a significant increase of TBM presence in ETR thymic cortexes compared to the control group, with a peak at 24 h in line with H&E staining and TEM findings (Fig. 2A). Immunohistochemical staining also revealed that iNOS was induced and upregulated in ETR cortexes, while the expression of this protein was almost absent in the control group. Cells expressing iNOS have a macrophage-like morphology (Fig. 2B). This was confirmed by iNOS and RM4 immunofluorescence double-labeling (Fig. 2C), where colocalization of these proteins was more frequent in ETR thymi.

*Enhanced autophagic response in ETR thymic TBMs*

As shown in Fig. 3a-f, compared to the control group (a), TEM demonstrated many autophagic vacuoles: autophagosomes (b-d), autolysosomes (e) and multilamellar bodies (f) in ETR TBMs. The autophagosomes engulfed damaged mitochondria, where the remains of cristae are clearly seen. Importantly, the autophagosomes were located close to apoptotic cell phagosomes in TBMs, which may suggest a functional relationship between the autophagic and phagocytic machineries as discussed later (Sanjuan et al., 2007; Shui et al., 2008). In addition, most apoptotic thymocytes in ETRs were in fact observed within TBMs showing signs of phagocytic activity such as smaller nuclear/cytoplasmic ratio (Eid et al., 2000; Jiang et al., 2016). Increased multilamellar body formation was also seen in ETR TBMs, which may indicate repeated sequestration of autophagosome membranes, reflecting enhanced autophagic and phagocytic activity (Brooks et al., 2015). Immunohistochemical staining of LC3 (a specific marker of autophagosomes) showed a significant increase in LC3 puncta formation in the thymic cortex of the ETRs, especially in cells with macrophage-like morphology (Fig. 4A). This indicates the conversion of LC3 to lipidated LC3-II, which is essential for autophagosome formation (Eid et al., 2016a). This was confirmed by Western blot analysis demonstrating the

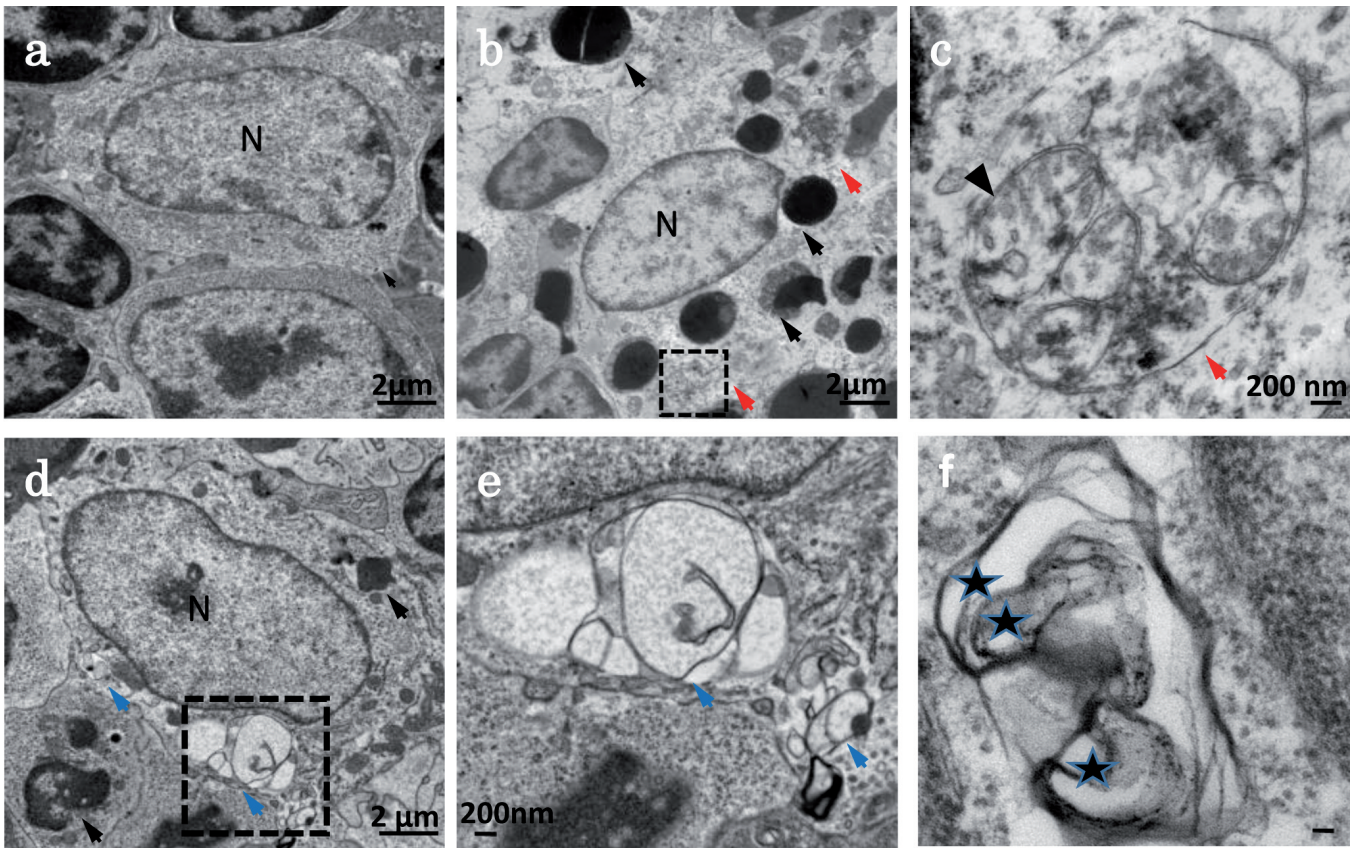


**Fig. 3.** Ultrastructural features of increased autophagy in thymic TBMs of ETRs. Control **(a)**, ETRs **(b-f)**. Black arrows indicate apoptotic cells, red arrows mark autophagosomes and blue arrows show autolysosomes. The framed areas are magnified on the right. N: nucleus of macrophage. Arrow head indicate damaged mitochondria within autophagosome. The stars in f mark multilamellar bodies.

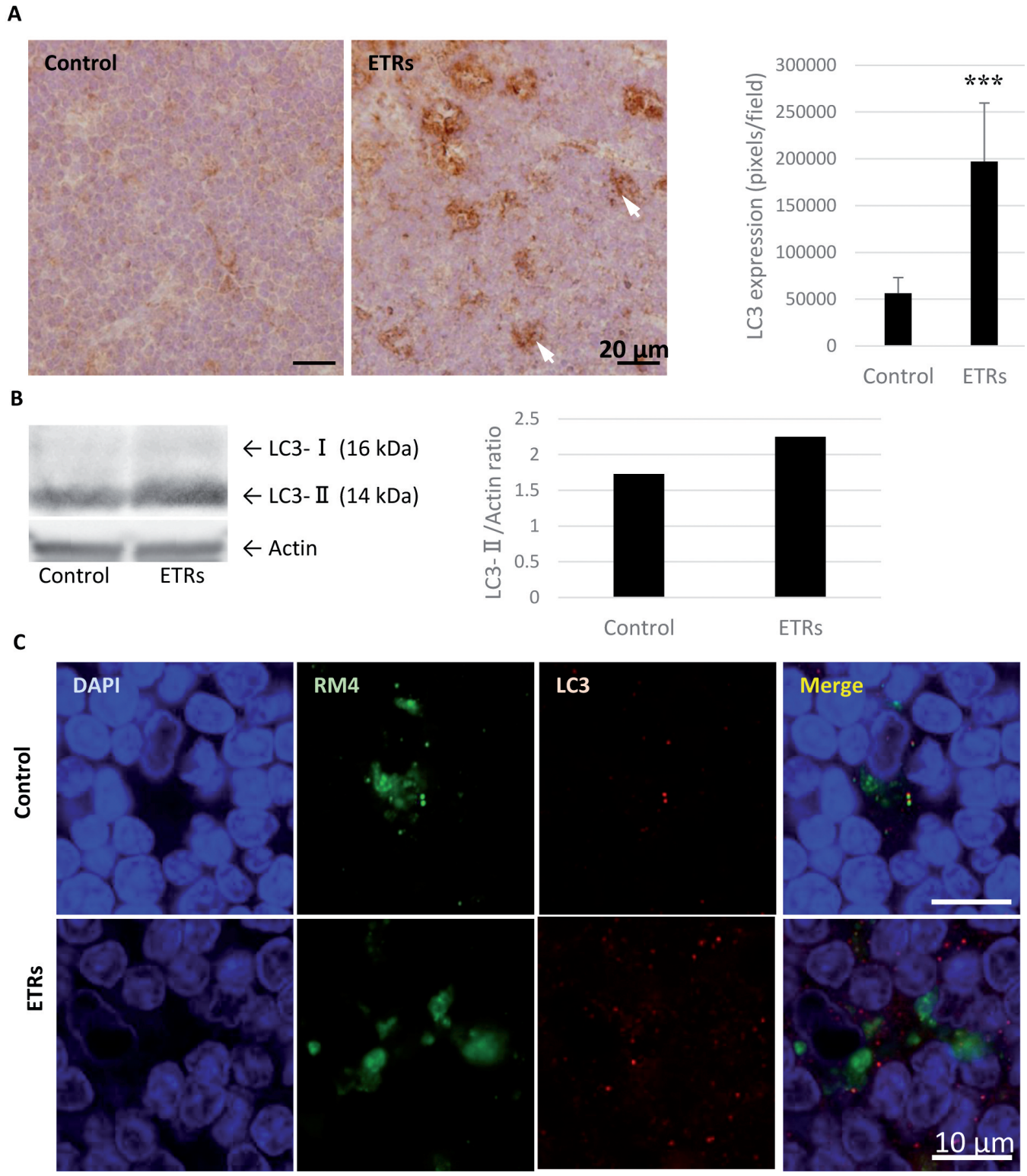


**Fig. 4.** Significant increase of LC3-II in thymic TBMs of ETRs. **A.** Immunohistochemistry of LC3. White arrows mark LC3 puncta in cells with macrophage-like morphology. The histogram on right shows statistical analysis. ***P<0.001. **B.** Western blot analysis showing upregulation of LC3-II in ETR thymi. **C.** Immunofluorescence double labeling for RM4 and LC3 demonstrating enhanced colocalization in ETR thymic TBMs compared to control.

upregulation of LC3-II in the thymi of ETRs compared with the control group (Fig. 3B) (Mizushima et al., 2011; Thomes et al., 2012). LC3 and RM4 immunofluorescence double labeling also showed higher colocalization in the ETR thymi (Fig. 4C), indicating the dominant expression of LC3 by TBMs. Taken together, these data indicate that autophagy was upregulated in ETR thymic TBMs compared to the control group.

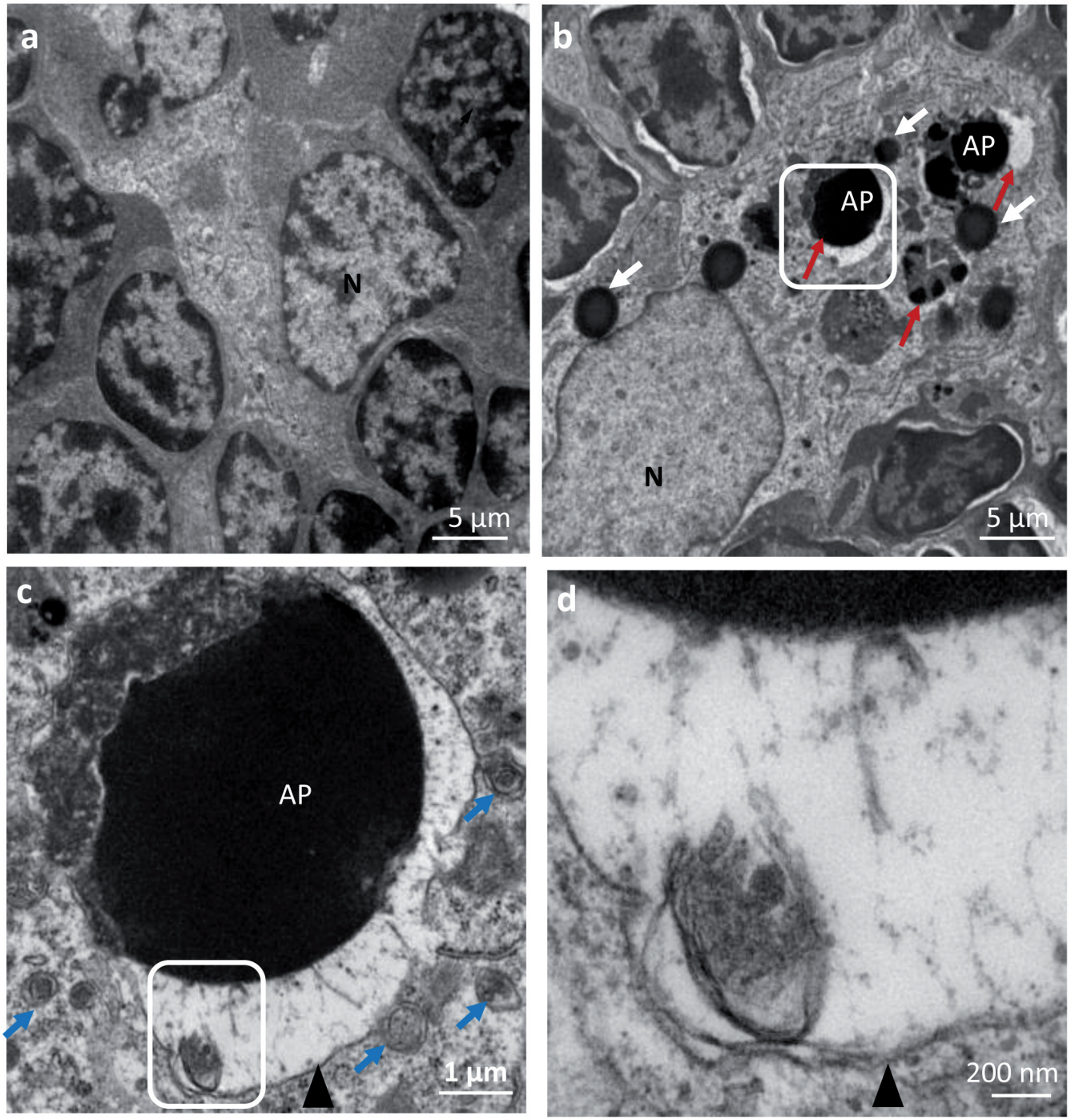


**Fig. 5.** TEM of apoptotic cell phagosomes within TBMs. **a.** Control. **b-d.** ETRs. The framed area in **b** is magnified in **c** and **d**. Red arrows show apoptotic cell phagosomes, white arrows indicate lysosomes, blue arrows mark autophagosomes, and arrow heads mark phagosomal membranes. AP: apoptotic thymocyte. N: macrophage nucleus.

*Involvement of LC3 in phagocytosis of apoptotic thymocytes by TBMs*

As shown in Fig. 5b,c and compared to control (Fig. 5a), there are many apoptotic cell phagosomes in ETR thymic TBMs surrounded by a phagosomal membrane closely related to or almost fused with autophagosomes (also seen in Fig. 3). Moreover, Fig. 5b showed the increase of lysosomal numbers and sizes in ETR thymic TBMs compared to the control group. As LC3 localization to apoptotic cells may stimulate their degradation by phagocytes as reported in LAP (Bandyopadhyay and Overholtzer, 2016), the authors investigated the expression of LC3 using immuno-electron microscopy. As shown in Fig. 6b-d, compared to the control group (a), there was a marked increase of LC3 immunogold labeling in autophagosomes and apoptotic cells within phagosomes of ETR thymic

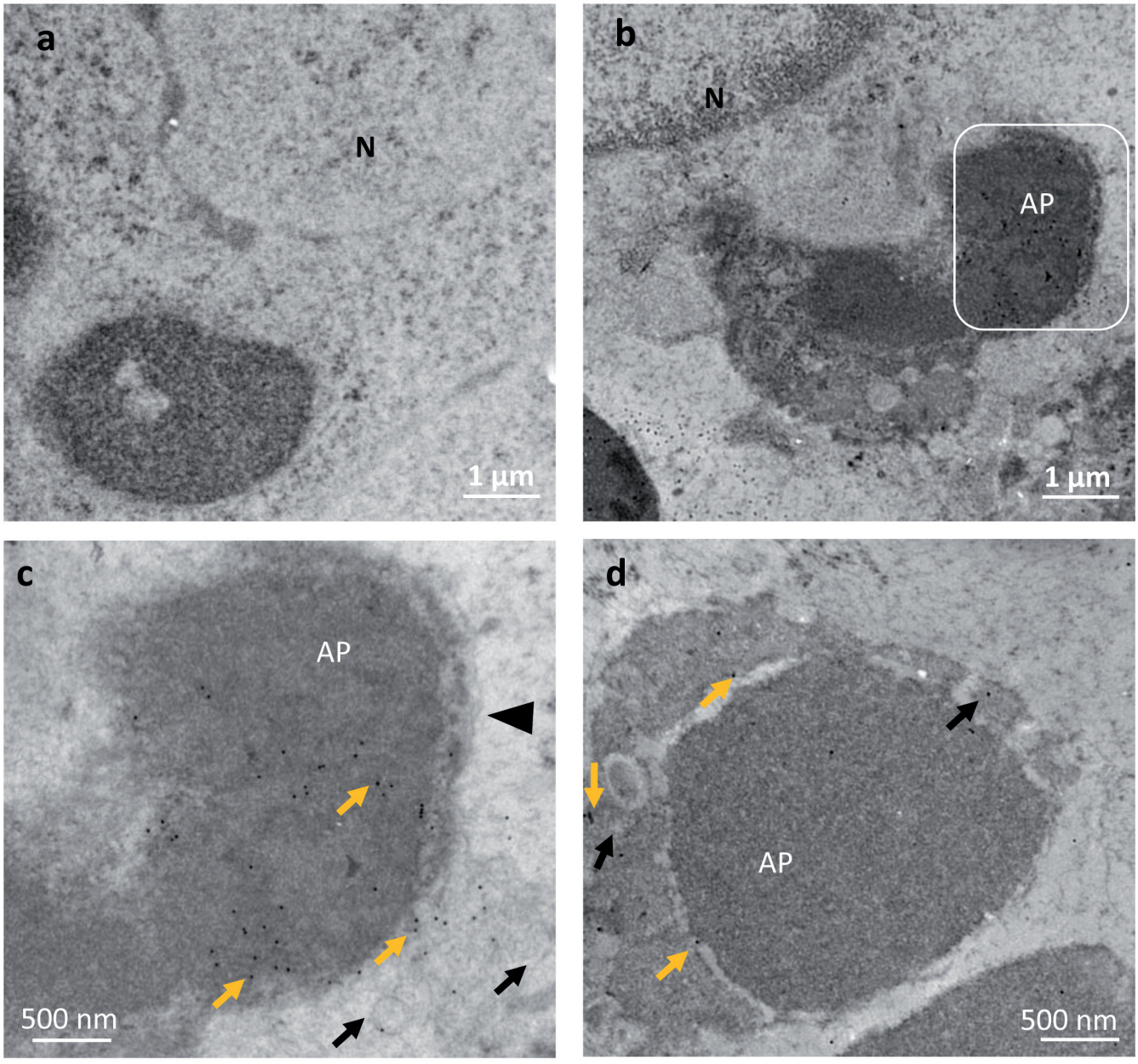


**Fig. 6.** Enhanced LC3 immunogold labeling of autophagosomes and apoptotic cell phagosomes in TBMs of ETRs. The micrographs show control **(a)** and ETRs **(b-d)**. The framed area in **b** is magnified in **c**. Yellow arrows mark LC3 immunogold particles. Black arrows indicate autophagosomes. Arrow head marks phagosomal membranes. AP: apoptotic thymocyte.

TBMs. Collectively, these results indicate that enhanced LC3 expression in ETR thymic TBMs may play important roles in their survival and clearance of apoptotic thymocytes via LC3-related phagocytosis. To confirm the enhanced autophagy and phagocytosis in ETR TBMs, we performed double labeling for LC3 and lysosomal pan cathepsin, with results showing enhanced colocalization of these proteins in ETR TBMs (Fig. 7).

## Discussion

The two major findings of the current study are: firstly, the robust accumulation of TBMs overexpressing RM4 and iNOS proteins associated with phagocytosis of large numbers of apoptotic thymocytes in the thymic cortex of acute ETRs. Secondly, and more importantly, TBMs of ETRs exhibit enhanced autophagic activity

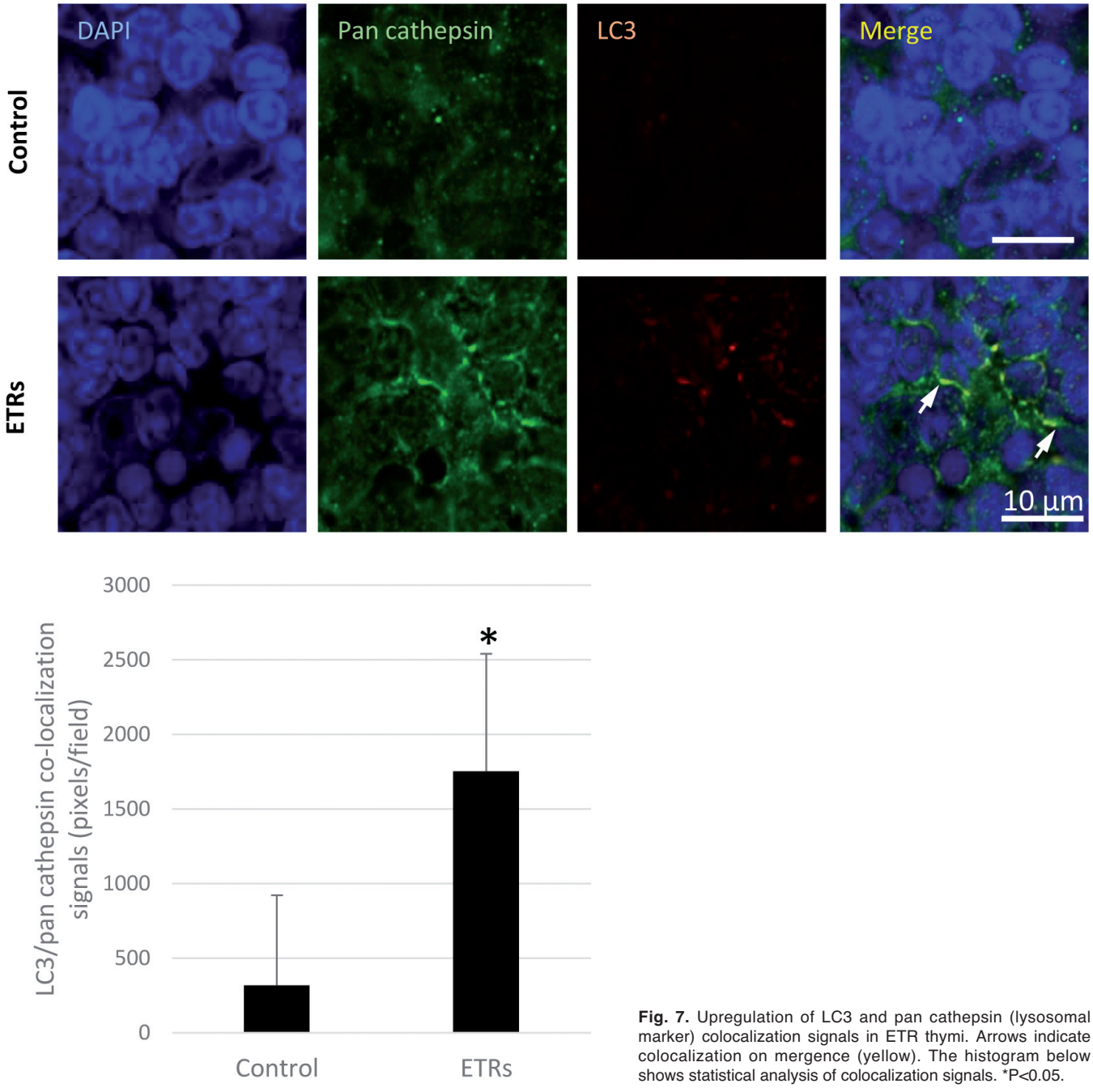


**Fig. 7.** Upregulation of LC3 and pan cathepsin (lysosomal marker) colocalization signals in ETR thymi. Arrows indicate colocalization on mergence (yellow). The histogram below shows statistical analysis of colocalization signals. *P<0.05.

reflecting ethanol toxicity, and show evidence of LAP activation. To the authors' knowledge, this is the first report showing such findings in thymi with an animal model of binge ethanol exposure.

The histopathological findings of the present study demonstrated that the accumulation of thymic TBMs in ETRs give the cortex a "starry sky" appearance, as reported by others (Eid et al., 2000; Salguero et al., 2004). Although some studies have shown evidence of enhanced apoptosis in ETR thymi based on light microscopic techniques (Han et al., 1993; Collier et al., 1998), the ultrastructural evidence of enhanced thymocyte apoptosis and accumulation of TBMs have not been reported in animal models of alcoholism. Therefore, we were keen in the current study to demonstrate the ultrastructural features of enhanced apoptosis of cortical thymocytes and phagocytosis in ETR TBMs (Mcllory et al., 2000; Eid et al., 2000). Enhanced expression of RM4-positive thymic TBMs in ETRs also indicates the acceleration of lysosomal processing of phagocytosed thymocytes, as RM4 antibody is designed for the detection of epitopes present on the membrane of endosomes and lysosomes of macrophages during endolysosomal processing (Iyonaga et al., 1997; Eid et al., 2000).

The induction of iNOS in ETR thymic TBMs may be related to enhanced endotoxin and cytokine production (Kanuri et al., 2009; Pla et al., 2016). Some reports have in fact indicated that limited NO production by iNOS may stimulate the uptake of apoptotic cells by phagocytes (Satake et al., 2000; Fernandez-Boyanapalli et al., 2010). However, as excessive induction of iNOS by ethanol may have detrimental effects on phagocytes due to the production of large amounts of NO, activation of autophagy in ETR thymic TBMs in the current study may be a plausible mechanism to suppress excessive iNOS production as supported by other recent studies (Han et al., 2013; Liu et al., 2015; Yang et al., 2015; Pla et al., 2016; Ilyas et al., 2016).

As the ETR thymic TBMs in the current study appeared to have normal nuclear morphology and intact cell membranes, enhanced autophagic response in these macrophages may be a prosurvival mechanism induced by oxidative and nitrative stress. This enhanced autophagy in ETR thymic TBMs was evidenced by ultrastructural detection of autophagic vacuoles, immunohistochemistry of LC3 puncta, double labeling of LC3 and RM4 and Western blot analysis of LC3-II (Dolganiuc et al., 2012; Han et al., 2013; Lin et al., 2013; Eid et al., 2016a). Importantly, iNOS production may be required for redox-dependent conversion of LC3-I to LC3-II mediated by Atg4 (Gorbunov and Kiang, 2009). However, as mentioned previously, upregulation of autophagy in ETR TBMs may be a mechanism to dampen excessive iNOS and inflammatory response associated with acute ethanol toxicity (Kanuri et al., 2009; Pla et al., 2016). TEM also revealed increased lysosomal numbers and sizes in ETR thymic TBMs compared to the control group (Figs. 1, 5). Ultrastructurally, lysosomes appear as dense bodies with visible content surrounded by a single limiting membrane, and are often observed in a perinuclear pattern (Appelqvist et al., 2013). This supports their differentiation from dark lipid droplets, which are not delimited by conventional unit membranes, are devoid of internal structures and are usually associated with the endoplasmic reticulum (Fujimoto and Parton, 2001). Importantly, TEM identification and accumulation of lysosomes in TBMs of ETRs was confirmed by enhanced expression of pan cathepsin and its colocalization with LC3, indicating the fusion between autophagosomes and lysosomes (Fig. 7) and enhanced autophagic flux (Zughaier et al., 2015; Eid et al., 2016a; Pla et al., 2016). Notably, TEM in the current study revealed damaged mitochondria within autophagosomes in ETR thymic TBMs; this may be an anti-apoptotic mechanism, as these damaged organelles may have a pro-apoptotic effect. The question of whether there is a specific mechanism such as parkin-mediated mitophagy for the clearance of damaged mitochondria in TBMs needs further investigation as we recently reported in hepatocytes of ETRs (Eid et al., 2016a,b).

Immunoelectron microscopy in the current study revealed LC3-labeled phagosomal membranes and apoptotic cells within phagosomes of ETR thymic TBMs in addition to adjacent autophagosomes, indicating LAP activation (Sanjuan et al., 2007; Shui et al., 2008; Martinez et al., 2011; Sprenkeler et al., 2016). In the study that led to the first LAP report, the authors mentioned the possibility of LC3-II translocation from autophagosomes to phagosomes (Sanjuan et al., 2007; Shui et al., 2008). This may explain the observation based on TEM (Figs. 3, 5c) of close association of autophagosomes with apoptotic cell phagosomes. However, the possibility of direct involvement by LC3-mediated autophagic machinery in the degradation of apoptotic cells within phagosomes of ETR TBMs cannot be ruled out, as demonstrated by a number of studies (Baghdadi et al., 2015; Brooks et al., 2015). In fact, the current study's LC3 labeling of apoptotic cells in phagosomes may be essential for lysosomal fusion with phagosomes. This was confirmed by double labeling of LC3 and pan cathepsin, which revealed enhanced colocalization of these proteins in ETR TBMs, indicating elevated formation of phagolysosomes and degradation of cargo as seen in canonical autophagy (Sanjuan et al., 2007; Ferguson and Green 2014; Bandyopadhyay and Overholtzer, 2016). The present study's results may be supported by recent reports indicating that the LGG-1/LC3 autophagy genes may be recruited to internalized apoptotic cells by the phagocyte in *C. elegans*, promoting the formation of autophagosomes and autolysosomes, which may fuse with phagosomes to accelerate corpse degradation (Li et al., 2012; Zou et al., 2012). Collectively, there seems to be a functional convergence of autophagy and phagocytosis, as evidenced by LC3-mediated degradation of phagocytosed cargo based on the stimulation of

lysosome/phagosome fusion (Ferguson and Green, 2014).

It has been reported that TBMs are absent in the germinal centers of lymph nodes in patients with systemic lupus erythematosus (SLE), resulting in a defect in the clearance of apoptotic lymphocytes and progression of the disease (Baumann et al., 2002). Interestingly, a number of recent studies have reported that the defect in LAP for the clearance of apoptotic lymphocytes increases the risk of SLE. (Bandyopadhyay and Overholtzer, 2016; Martinez et al., 2016). Accordingly, it can be concluded that the activation of autophagy and LAP observed in ETR thymic TBMs in the current study may prevent autoimmune diseases such as SLE.

In conclusion, enhanced autophagy in thymic TBMs of acute ETRs may be a prosurvival mechanism and important for the phagocytosis and degradation of apoptotic thymocytes, thus preventing autoimmune reaction and suppressing inflammatory response associated with ethanol toxicity. Further studies are needed to explore the relationship between autophagy and phagocytosis in macrophages for various diseases such as cancer and autoimmune/infectious conditions.

*Acknowledgements.* The authors thank Mr. Tadashi Kanayama for his assistance with the experimental work reported here.
*Conflict of interest.* The authors have nothing to declare.

## References

Appelqvist H., Wäster P., Kågedal K. and Öllinger K. J. (2013). The lysosome: from waste bag to potential therapeutic target. Mol. Cell. Biol. 5, 214-226.

Baghdadi M., Yoneda A., Yamashina T., Nagao H., Komohara Y., Nagai S., Akiba H, Foretz M., Yoshiyama H., Kinoshita I., Dosaka-Akita H., Takeya M., Viollet B., Yagita H. and Jinushi M. (2013). TIM-4 glycoprotein-mediated degradation of dying tumor cells by autophagy leads to reduced antigen presentation and increased immune tolerance. Immunity 39, 1070-1081.

Bandyopadhyay U. and Overholtzer M. (2016). LAP: the protector against autoimmunity. Cell Res. 26, 865-866.

Baumann I., Kolowos W., Voll R.E., Manger B., Gaipl U., Neuhuber W.L., Kirchner T., Kalden J.R. and Herrmann M. (2002). Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus. Arthritis Rheum. 46, 191-201.

Budec M., Cirić O., Koko V. and Ašanin R. (1992). The possible mechanism of action of ethanol on rat thymus. Drug Alcohol Depend. 30, 181-185.

Brooks C.R., Yeung M.Y., Brooks Y.S., Chen H., Ichimura T., Henderson J.M. and Bonventre J.V. (2015). KIM-1-/TIM-1-mediated phagocytosis links ATG5-/ULK1-dependent clearance of apoptotic cells to antigen presentation. EMBO J. 34, 2441-2464.

Carson E.J. and Pruett S.B. (1996). Development and characterization of a binge drinking model in mice for evaluation of the immunological effects of ethanol. Alcohol Clin. Exp. Res. 20, 132-138.

Chen G., Ke Z., Xu M., Liao M., Wang X., Qi Y., Zhang T., Frank J.A., Bower K.A., Shi X. and Luo J. (2012). Autophagy is a protective response to ethanol neurotoxicity. Autophagy 8, 1577-1589.

Chen M.M., Palmer J.L., Ippolito J.A., Curtis B.J., Choudhry M.A. and Kovacs E.J. (2013). Intoxication by intraperitoneal injection or oral gavage equally potentiates postburn organ damage and inflammation. Mediators Inflamm. 2013, 971481.

Collier S.D., Wu W.J. and Pruett S.B. (1998). Endogenous glucocorticoids induced by a chemical stressor (ethanol) cause apoptosis in the spleen in B6C3F1 female mice. Toxicol. Appl. Pharmacol. 148, 176-182.

D'Souza El-Guindy N.B., Kovacs E.J., De Witte P., Spies C., Littleton J.M., de Villiers W.J., Lott A.J., Plackett T.P., Lanzke N. and Meadows G.G. (2010). Laboratory models available to study alcohol-induced organ damage and immune variations: choosing the appropriate model. Alcohol Clin. Exp. Res. 3, 1489-1511.

Ding W.X., Li M., Chen X., Ni H.M., Lin C.W., Gao W., Lu B., Stolz D.B., Clemens D.L. and Yin X.M. (2010). Autophagy reduces acute ethanol-induced hepatotoxicity and steatosis in mice. Gastroenterology 139, 1740-1752.

Dolganiuc A., Thomes P.G., Ding W.X., Lemasters J.J. and Donohue T.M. Jr (2012). Autophagy in alcohol-induced liver diseases. Alcohol Clin. Exp. Res. 36, 1301-1308.

Eid N.A., Ito Y., Li Z., Abe H., Kusakabe K., Shibata M.A. and Otsuki Y. (2000). The relationship between apoptosis and splenocyte depletion in rats following ethanol treatment. Med. Electron Microsc. 33, 89-95.

Eid N., Ito Y. and Otsuki Y. (2013). The autophagic response to alcohol toxicity: the missing layer. J. Hepatol. 59, 398.

Eid N., Ito Y., Horibe A. and Otsuki Y. (2016a). Ethanol-induced mitophagy in liver is associated with activation of the PINK1-Parkin pathway triggered by oxidative DNA damage. Histol. Histopathol. 31, 1143-1159.

Eid N., Ito Y. and Otsuki Y. (2016b). Triggering of Parkin mitochondrial translocation in mitophagy: Implications for liver diseases. Front. Pharmacol. 29, 7:100.

Fernandez-Boyanapalli R., McPhillips K.A., Frasch S.C., Janssen W.J., Dinauer M.C., Riches D.W., Henson P.M., Byrne A and Bratton D.L. (2010). Impaired phagocytosis of apoptotic cells by macrophages in chronic granulomatous disease is reversed by IFN-γ in a nitric oxide-dependent manner. J. Immunol. 185, 4030-4041.

Ferguson T.A. and Green D.R. (2014). Autophagy and phagocytosis converge for better vision. Autophagy 10, 165-157.

Fujimoto T. and Parton R.G. (2011). Not just fat: the structure and function of the lipid droplet. Cold Spring Harb. Perspect. Biol. 3, a004838.

Gorbunov N.V. and Kiang J.G. (2009). Up-regulation of autophagy in small intestine Paneth cells in response to total-body gamma-irradiation. J. Pathol. 219, 242-252.

Han H.E., Kim T.K., Son H.J., Park W.J. and Han P.L. (2013). Activation of autophagy pathway suppresses the expression of iNOS, IL6 and cell death of LPS-stimulated microglia cells. Biomol. Ther. 21, 21-28.

Han Y.C., Lin T.L. and Pruett S.B. (1993). Thymic atrophy caused by ethanol in a mouse model for binge drinking: involvement of endogenous glucocorticoids. Toxicol. Appl. Pharmacol. 123, 16-25.

Haorah J., Ramirez S.H., Floreani N., Gorantla S., Morsey B. and Persidsky Y. (2008). Mechanism of alcohol-induced oxidative stress and neuronal injury. Free Radic. Biol. Med. 45, 1542-1550.

Ilyas G., Zhao E., Liu K., Lin Y, Tesfa L., Tanaka K.E. and Czaja M.J. (2016). Macrophage autophagy limits acute toxic liver injury in mice

through down regulation of interleukin-1β. J. Hepatol. 64, 118-127.

Iyonaga K., Takeya M., Yamamoto T., Ando M. and Takahashi K. (1997). A novel monoclonal antibody, RM-4, specifically recognizes rat macrophages and dendritic cells in formalin-fixed, paraffin-embedded tissues. Histochem. J. 29, 105-116.

Jiang S., Jia Z., Xin L., Sun Y., Zhang R., Wang W., Wang L. and Song L. (2016). The cytochemical and ultrastructural characteristics of phagocytes in the Pacific oyster *Crassostrea gigas*. Fish Shellfish Immunol. 55, 490-498.

Kanuri G., Weber S., Volynets V., Spruss A., Bischoff S.C. and Bergheim I. (2009). Cinnamon extract protects against acute alcohol-induced liver steatosis in mice. J. Nutr. 139, 482-487.

Kapasi A.A., Patel G, Goenka A., Nahar N., Modi N, Bhaskaran M., Reddy K., Franki N., Patel J. and Singhal P.C. (2003). Ethanol promotes T cell apoptosis through the mitochondrial pathway. Immunology 108, 313-320.

Kroemer G., Marino G. and Levine B. (2010). Autophagy and the integrated stress response. Mol. Cell 40, 280-293

Li W., Zou W., Yang Y., Chai Y., Chen B., Cheng S., Tian D., Wang X., Vale R.D. and Ou G. (2012). Autophagy genes function sequentially to promote apoptotic cell corpse degradation in the engulfing cell. J. Cell Biol. 197, 27-35.

Liang Y., Harris F.L. and Brown L.A. (2014). Alcohol induced mitochondrial oxidative stress and alveolar macrophage dysfunction. Biomed. Res. Int. 2014, 371593.

Lin C.W., Zhang H., Li M., Xiong X., Chen X., Chen X., Dong X.C. and Yin X.M. (2013). Pharmacological promotion of autophagy alleviates steatosis and injury in alcoholic and non-alcoholic fatty liver conditions in mice. J. Hepatol. 58, 993-999.

Liu K., Zhao E., Ilyas G., Lalazar G., Lin Y., Haseeb M., Tanaka K.E. and Czaja M.J. (2015). Impaired macrophage autophagy increases the immune response in obese mice by promoting proinflammatory macrophage polarization. Autophagy 11, 271-284.

Loftis J.M., Taylor J, Raué H.P., Slifka M.K. and Huang E. (2016). Alcohol intake alters immune responses and promotes CNS viral persistence in mice. Behav. Brain Res. 4, 1-8.

Martinez J., Almendinger J., Oberst A., Ness R., Dillon C.P., Fitzgerald P., Hengartner M.O. and Green D.R. (2011). Microtubule-associated protein 1 light chain 3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. Proc. Natl. Acad. Sci. 108,17396-17401.

Martinez J., Cunha L.D., Park S., Yang M., Lu Q., Orchard R., Li Q.Z., Yan M, Janke L., Guy C., Linkermann A., Virgin H.W. and Green D.R. (2016). Non canonical autophagy inhibits the autoinflammatory, lupus-like response to dying cells. Nature 533, 115-119.

McIlroy D., Tanaka M., Sakahira H., Fukuyama H., Suzuki M., Yamamura K., Ohsawa Y., Uchiyama Y. and Nagata S. (2000). An auxiliary mode of apoptotic DNA fragmentation provided by phagocytes. Genes Dev. 14, 549-458.

Mizushima N., Yoshimori T. and Ohsumi Y. (2011). The role of Atg proteins in autophagosome formation. Annu. Rev. Cell Dev. Biol. 27, 107-132.

Otsuki Y. (2004). Tissue specificity of apoptotic signal transduction. Med. Electron Microsc. 37, 163-169.

Perrotta I., Carito V., Russo E., Tripepi S., Aquila S. and Donato G. (2011). Macrophage autophagy and oxidative stress: an ultrastructural and immunoelectron microscopical study. Oxid. Med. Cell Longev. 28, 2739.

Pla A., Pascual M. and Guerri C. (2016). Autophagy constitutes a protective mechanism against ethanol toxicity in mouse astrocytes and neurons. PLoS One 11, e0153097.

Salguero F.J., Sánchez-Cordón P.J., Sierra M.A., Jover A., Núñez A. and Gómez-Villamandos J.C. (2004). Apoptosis of thymocytes in experimental African Swine Fever virus infection. Histol. Histopathol. 19, 77-84.

Sanjuan M.A., Dillon C.P., Tait S.W., Moshiach S., Dorsey F., Connell S., Komatsu M., Tanaka K, Cleveland J.L., Withoff S. and Green D.R. (2007). Toll-like receptor signaling in macrophages links the autophagy pathway to phagocytosis. Nature 450, 1253-1257.

Satake K., Matsuyama Y., Kamiya M., Kawakami H., Iwata H., Adachi K. and Kiuchi K. (2000). Nitric oxide via macrophage iNOS induces apoptosis following traumatic spinal cord injury. Brain Res. Mol. Brain Res. 28, 114-122.

Shin J.K., Kang J.W. and Lee S.M. (2016). Enhanced nitric oxide-mediated autophagy contributes to the hepatoprotective effects of ischemic preconditioning during ischemia and reperfusion. Nitric Oxide 31, 10-19.

Shui W., Sheu L., Liu J., Smart B., Petzold C.J., Hsieh T.Y., Pitcher A., Keasling J.D. and Bertozzi C.R. (2008). Membrane proteomics of phagosomes suggests a connection to autophagy. Proc. Natl. Acad. Sci. 105, 16952-16957.

Sprenkeler E.G., Gresnigt M.S. and van de Veerdonk F.L. (2016). LC3-associated phagocytosis: a crucial mechanism for antifungal host defence against Aspergillus fumigatus. Cell Microbiol. 18, 1208-1216.

Thomes P.G., Trambly C.S., Thiele G.M., Duryee M.J., Fox H.S., Haorah J. and Donohue T.M. Jr (2012). Proteasome activity and autophagosome content in liver are reciprocally regulated by ethanol treatment. Biochem. Biophys. Res. Commun. 417, 262-267.

Yang J.Y., Park M.Y., Park S.Y., Yoo H.I., Kim M.S., Kim J.H., Kim W.J. and Jung J.Y. (2015). Nitric oxide-induced autophagy in MC3T3-E1 cells is associated with cytoprotection via AMPK activation. Korean J. Physiol. Pharmacol. 19, 507-514.

Yun J.W., Son M.J., Abdelmegeed M.A., Banerjee A, Morgan T.R., Yoo S.H. and Song B.J. (2014). Binge alcohol promotes hypoxic liver injury through a CYP2E1-HIF-1αdependent apoptosis pathway in mice and humans. Free Radic. Biol. Med. 77, 183-194.

Zou W., Wang X., Vale R.D. and Ou G. (2012). Autophagy genes promote apoptotic cell corpse clearance. Autophagy 8, 1267-1268.

Zhou Z., Sun X. and Kang Y.J. (2001). Ethanol-induced apoptosis in mouse liver: Fas- and cytochrome c-mediated caspase-3 activation pathway. Am. J. Pathol. 159, 329-338.

Zughaier S.M., Kandler J.L., Balthazar J.T., and Shafer W.M. (2015). Phosphoethanolamine modification of *Neisseria gonorrhoeae* lipid A reduces autophagy flux in macrophages. PLoS One 10, e0144347.

Accepted December 27, 2016