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Enhanced autophagy and phagocytosis of apoptotic lymphocytes in splenic macrophages of acute ethanol-treated rats: Light and electron microscopic studies

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Summary. Autophagy is a prosurvival mechanism for the clearance of damaged cellular components, specifically upon exposure to various stressors. In lymphoid organs, excessive ethanol consumption increases lymphocyte apoptosis, resulting in immunosuppression. However, ethanol-induced autophagy and related phagocytosis of apoptotic lymphocytes in the spleen have not been studied yet. Adult male Wistar rats were injected intraperitoneally either with 5 g/kg ethanol or phosphate-buffered saline (as a control group) and then sacrificed 0, 3, 6, and 24 hours after injection. Light and transmission electron microscopy (TEM) findings indicated enhanced T cell apoptosis in the white pulps of ethanol-treated rats (ETRs) compared with the control group, which peaked at 6h and was associated with the accumulation of tingible body macrophages (TBMs). These macrophages exhibited an upregulated autophagic response, as evidenced by enhanced LC3-II (a specific marker of autophagosomes) expression, which peaked at 24h. In addition, double labeling immunofluorescence of LC3-II with lysosomal markers revealed the enhanced formation of autolysosomes in TBMs of ETRs, which was associated with suppression of p62 immunostaining, indicating the enhanced autophagic flux. Interestingly, this elevated autophagic response in ETR TBMs was accompanied by evidence of LC3-associated phagocytosis (LAP) of apoptotic splenocytes. This is based on TUNEL/LC3-II double labeling and TEM observations of phagosomes containing apoptotic bodies, enclosed within phagosomal membranes adjacent to the autophagic vacuoles. It can be concluded that enhanced prosurvival autophagy in splenic TBMs of ETRs and clearing of apoptotic lymphocytes via LAP may contribute to preventing secondary necrosis and autoimmune diseases.

Key words: Autophagy, Apoptosis, Ethanol, LAP, Spleen, TBMs, T cells

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Introduction

The spleen is the largest secondary lymphoid organ, with an extraordinary histological structure consisting of white and red pulp. The white pulp is primarily responsible for initiating immune reactions, lymphocyte recirculation, and B-cell maturation. The white pulp of rat spleen is subdivided into three zones: periarteriolar lymphoid sheath (PALS) (T cell zone), the follicles, and the marginal zone (Cesta, 2006; Steiniger, 2015).

Alcohol (ethanol) consumption is a global health problem, and almost all body organs may be affected by its abuse (Haorah et al., 2008). Excessive ethanol intake induces lymphocyte depletion in lymphoid organs via mechanisms related to corticosteroid overproduction, bone marrow depression, lymphocyte redistribution, hepatic dysfunction, and apoptosis (Eid et al., 2000; Betsuyaku et al., 2017).

Macrophages are critical to the immune homeostatic response and have high phagocytic activity capable of antigen presentation. They can be identified by several macrophage markers, such as the RM4 antibody. (Iyonaga et al., 1997; Eid et al., 2000; Betsuyaku et al.,

2017). The clearance of apoptotic lymphocytes in lymphoid organs after exposure to xenobiotics is induced by a class of macrophages known as tingible body macrophages (TBMs). These macrophages resist apoptosis, exhibit high phagocytic activity, often contain multiple apoptotic nuclei, and can grow relatively large (Eid et al., 2000; Betsuyaku et al., 2017). Particularly in the spleen of malignant lymphoma, the absence of TBMs leads to the survival and hyperproliferation of malignant lymphocytes (Cerroni et al., 2000).

Autophagy is a prosurvival mechanism for the clearance of damaged cellular components upon exposure to various stressors, such as oxidative stress (Kroemer et al., 2010; Chen et al., 2012; Horibe et al., 2017). Morphologically, autophagy is initiated by the formation of isolation membranes, which engulf damaged cellular components, forming double-membrane vesicles called autophagosomes, mediated by microtubule-associated protein 1 light chain 3 (LC3); specifically, its lipidated form, LC3-Ⅱ. The autophagosomes then fuse with lysosomes, forming autolysosomes, which are cleared by lysosomal cathepsins (Perotta et al., 2011; Eid et al., 2013; Klionsky et al., 2021). As an adaptor protein to LC3-II, p62 functions to support autophagosomal maturation and is then digested with internalized organelles after the fusion with lysosomes. Therefore, the decrease in p62 immunostaining is an indication of enhanced autophagic response and flux (Komatsu et al., 2007; Ichimura et al., 2008; Yoshii and Mizushima, 2017).

Based on recent studies, LC3-II can translocate not only to autophagosomes but also to single membrane phagosomes by an alternative mechanism (Sanjuan et al., 2007; Martinez et al., 2011; Brooks et al., 2015; Yefimova et al., 2020; Pena-Martinez et al., 2022). This alternative mechanism is called LC3-associated phagocytosis (LAP), and then the vesicle is named LAP-engaged phagosome (LAPsome). LAP induction requires reactive oxygen species that can also enhance autophagic machinery. In macrophages of the thymic cortex of ETRs, LC3-II was found to target both phagosomes and autophagosomes (Betsuyaku et al., 2017).

However, the involvement of autophagy in the survival of splenic TBMs and phagocytosis of apoptotic splenocytes under ethanol exposure is unreported. Here, we investigated autophagy, apoptosis, and LAP in the spleens of acute ETRs using light and electron microscopic studies. The results indicated that ethanol enhanced the apoptosis of T lymphocytes in the white pulp of ETRs. This was associated with the induction and upregulation of autophagy and LAP in TBMs of ETRs.

Materials and methods

Experiment approval

The animals used in this study were maintained and treated according to the guidelines set by the Experimental Animal Research Committee of Osaka Medical College (this research was approved by the Animal Research Committee of Osaka Medical College under code 25090).

Animal experiments

Twelve-week-old adult male Wistar rats (250-300 g) were purchased from SLC Japan Co. (Shizuoka, Japan). Five g/kg (40% v/v) ethanol was intraperitoneally injected once into the rats, which is consistent with the animal model of binge ethanol consumption (Collier et al., 1998; Eid et al., 2016; Betsuyaku et al., 2017; Horibe et al., 2017). Although the oral route is considered more physiologically relevant, the administration of ethanol through either oral or intraperitoneal routes yields comparable results concerning blood alcohol concentrations and organ damage (D'Souza El-Guindy et al., 2010; Chen et al., 2013). An equal volume of phosphate-buffered saline, instead of ethanol, was injected into the control group. For time-course studies, the animals were sacrificed at 0, 3, 6, and 24 hours after ethanol administration (three animals at each time point). Samples of rat spleen were fixed in either 4% paraformaldehyde for paraffin embedding or in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer for embedding in epoxy resin. The remaining samples were frozen at -80°C for western blot analysis.

Hematoxylin and Eosin (H&E) staining

Following deparaffinization, 4 μm-sections were stained with H&E using standard methods for histopathological analysis of ethanol-induced damage to the rat spleen. The slides were observed using an Olympus BX41 immunofluorescence microscope. The antibodies and kits used in the current research are listed in Table1.

TdT-dUTP Nick End Labeling (TUNEL) assay

TUNEL was performed according to the manufacturer's protocols, as previously reported (Betsuyaku et al., 2017; Horibe et al., 2017). Briefly, paraffin sections were deparaffinized, rehydrated, and then treated with a TUNEL reaction mixture (TdT enzyme and nucleotides conjugated with fluorescent dye) for 1 hour at 37°C. The slides were observed using an Olympus BX41 immunofluorescence microscope. The number of TUNEL-labeled splenocytes was counted from 30 random white pulps from each group.

Immunohistochemical staining of RM4, OX-33, CD3, LC3, FOXO3a, and p62

Deparaffinized 4 μm-sections underwent antigen retrieval, blocking, and incubation with primary antibodies overnight at 4°C. The sections were labeled

for 1h with EnVision+ Dual Link, Single Reagents (K4003) for rabbit antibodies, or Single Reagents (K4063) for mouse antibodies (Dako, Kyoto, Japan). Sections were then stained with ImmPACT DAB Peroxidase substrate as chromogen (Vector Laboratories) and counterstained with hematoxylin. The slides were observed using an Olympus BX41 immunofluorescence microscope. RM4-positive macrophages were counted from 30 random white pulps from each group. Quantification of other molecules in white pulps was performed with Image J (http://imagej.nih.gov/ij/) (Eid et al., 2016; Betsuyaku et al., 2017; Horibe et al., 2017).

Immunofluorescence double staining of apoptotic and autophagy markers

The 4 μm-sections underwent a process of deparaffinization, rehydration, blocking of nonspecific antibody binding, and incubation for 1h at room temperature with the specific primary antibody LC3 (Table 1) in addition to RM4, pan-cathepsin, or TUNEL for double labeling with LC3 (Zhu et al., 2013). Alexa Fluor 594 and VectaFluor™ R.T.U. DyLight® 488 were used for double labeling (sequential method), and DAPI was used for nuclear counterstaining as previously reported (Eid et al., 2016; Betsuyaku et al., 2017; Horibe et al., 2017). The slides were observed on a confocal laser scanning microscope (Leica TCS SP8, Manheim, Germany). Quantification of LC3 and other marker colocalization was performed with Image J.

Western blot analysis

Spleen samples were extracted, solubilized with SDS lysis buffer, and centrifuged. Equal amounts of protein were then subjected to SDS-polyacrylamide electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% BSA and then incubated with an anti-LC3 antibody, followed by peroxidase-labeled goat anti-rabbit IgG antibody as reported in our previous studies (Betsuyaku et al., 2017; Horibe et al., 2017).

Table 1. Primary and secondary antibodies and kits used for the current study.

A. Primary antibodies

Primary Antibody	Company	Catalog Number	Host	Method	Dilution
RM4	Trans Genic inc.	KT014	Mouse	IHC	1:100
				IF	1:100
CD45RA(OX-33)	Bio-Rad	MCA340GA	Mouse	IHC	1:100
CD3(1F4)	Bio-Rad	MCA772GA	Mouse	IHC	1:10
LC3	MBL	PM036	Rabbit	IHC	1:500
				WB	1:1000
				IF	1:250
Actin	Santa Cruz Biotechnology	sc-1616	Goat	WB	1:1000
FOXO3a	Cell Signaling	12829	Rabbit	IHC	1:400
p62/SQSTM1	Novus	H00008878-M01	Mouse	IHC	1:100
pan cathepsin	Santa Cruz Biotechnology	sc-6499	Goat	IF	1:100

B. Secondary antibodies

Secondary Antibody	Company	Catalog Number	Host	Method	Dilution
AlexaFluor 488	Thermofisher	A11001(anti-Mouse)	Goat	IF	1:250
AlexaFluor 594	Thermofisher	A11012(anti-Rabbit)	Goat	IF	1:250
Envision + Dual link	DAKO	K4063		IHC	1:1
Envision + Single	DAKO Aurion	K4002		IHC	1:250
Rabbit immunoglobulins	DAKO	E0432	Rabit	IHC	1:250
donkey ant-goat IgG-HRP	Santa Cruz Biotechnology	SC-2304	Donkey	WB	1:10,000
donkey anti-mouse IgG-HRP	Santa Cruz Biotechnology	SC-2096	Donkey	WB	1:10,000
donkey anti-rabbit IgG-HRP	Santa Cruz Biotechnology	SC-2077	Donkey	WB	1:10,000

C. Kits

Kit	Company	Catalog Number
Vectastain ABC standard Kit	Vector	PK-4000
ImmPACT DAB	Vector	SK-4105
In Situ Cell Death (TUNEL) Detection Kit	Roche Diagnotics	11684817910
Western Lightning ECL Pro	PerkinElmer	NEL120001EA
VectaFluor™ R.T.U. DyLight® 488	Vector	DI-3788

IHC: immunohistochemistry; IF: Immunofluorescence; WB: western blot.

Ultrastructural studies with transmission electron microscope (TEM)

For histopathological studies, 1-micron semithin sections from blocks embedded in epoxy resin were cut and stained with toluidine blue. For TEM, 70-nm-thick ultrathin sections were double stained with uranyl acetate and lead citrate and observed with an H-7100 transmission electron microscope (Hitachi, Japan). The identification of white pulps and the localization of apoptotic cortical splenocytes were first identified in the semithin sections stained with toluidine blue. Ultrastructurally, apoptotic cells are characterized by the margination of condensed chromatin, nuclear fragmentation, and the formation of apoptotic bodies (Eid et al., 2016; Betsuyaku et al., 2017; Horibe et al., 2017).

Statistical analysis

The student's t-test was used for comparison between the ETR and control groups. $P<0.05$ was considered significant.

Results

The accumulation of apoptotic splenocytes and TBMs in white pulps of ETRs

As shown in Fig. 1A,B, compared with the control group, toluidine staining of 1-micron semithin sections showed greater splenocyte apoptosis and TBM accumulation in ETR white pulps, specifically in the T cell-dependent zone around the central artery (PALS), peaking 6 h after ethanol injection. These findings were also observed in H&E-stained sections (data not shown). TEM imaging (Fig. 1C) demonstrated the ultrastructural features of TBMs and apoptotic splenocytes in ETR white pulps. Compared with the control (Fig. 1C), many apoptotic splenocytes were observed within the TBMs; these macrophages appeared to be normal. TUNEL labeling confirmed the enhanced splenocyte apoptosis in ETR white pulp, which was statistically significant (Fig. 2A). Otherwise, immunohistochemical staining of RM4 (a marker of TBMs and other macrophages) showed that ethanol increased TBM accumulation following splenocyte apoptosis, and the peak was at 24h (Fig. 2B).

Marked depletion of T cells in ETR white pulps

As shown by immunohistochemical staining of B-cell marker CD-45RA (Fig. 3A), the expression of CD45RA in ETR white pulps was slightly reduced. On the other hand, the expression of CD3, a specific marker of T cells (Fig. 3B), was markedly reduced in ETR white pulps. Quantitative analysis of immunostaining confirmed the depletion of T cells. The downregulation in CD3 immunostaining can be attributed to the enhanced apoptosis and phagocytosis of T cells by splenic TBMs. Conversely, CD45RA(OX-33) immunostaining, a B-cell marker, exhibited a slight reduction. This suggests that T cells are more susceptible to the apoptotic effects of ethanol. This specific ethanol-induced reduction in T cells compared with B cells has been documented in earlier studies using various methods, including flow cytometry (Shellito and Olariu, 1998). Furthermore, these results align with our previous investigation in the thymus of ETRs, demonstrating increased apoptosis in cortical thymocytes (Betsuyaku et al., 2017).

Enhanced autophagy in TBMs of ETRs

Immunohistochemical staining of LC-II (a marker of autophagic vacuoles or autophagosomes) demonstrated a significant increase in LC3 puncta formation (indicating the formation of LC3-II-mediated autophagosomes) in ETR white pulps (Fig. 4A,B). Western blot analysis also supported this result (Fig. 4C), as evidenced by the upregulation of LC3-II. Double-labeling immuno-fluorescence revealed elevated LC3-II expression and puncta formation, which co-localized with RM4 (a marker of macrophages recognizing lysosomes) in ETR white pulp TBMs (Fig. 4D), indicating that autophagy is specifically marked in ETR TBMs. TEM is the gold standard for the detection of autophagic vacuoles, including autophagosomes and autolysosomes. As shown in Figure 5 and compared with control white pulp macrophages (Fig. 5a), multiple autophagic vacuoles, including autophagosomes (marked by yellow arrows), autolysosomes (marked by black arrowheads), and multilamellar bodies (indicated by red arrows), were observed in ETR TBMs (Fig. 5b,c). Autophagosomes in these macrophages were found to contain damaged mitochondria and many lysosomes located close to the autophagosomes. Enhanced autophagy in TBMs of ETRs is not related to the transcription factor FOXO3a based on immunohistochemical analysis (data not shown). FOXO3a is a transcription factor for autophagy genes such as *LC3* (Horibe et al., 2017), However, immunostaining showed no differences between control and ETR TBMs. To confirm the formation of autolysosomes in ETR TBMs, double-labeling immunofluorescence of LC3-II with pan cathepsin (a lysosomal marker) (Fig. 6A,B) showed the enhanced expression of these proteins and their co-localization, which was statistically significant, indicating increased autophagic activity compared with the control group. Likewise, immunohistochemical staining of p62 (an adaptor protein of autophagy and substrate of LC3) demonstrated the downregulation of p62 expression in ETR TBMs (Fig. 6C), indicating the enhanced autophagic flux.

Activation of LC3-associated phagocytosis (LAP) in ETR TBMs

To test LAP activation in ETR TBMs, we performed

TUNEL/LC3 double labeling, as shown in Figure 7A. LC3 puncta were found to decorate TUNEL-positive apoptotic splenocytes. Thus, LC3-II could be involved not only in canonical autophagy (for maturation of autophagosomes and fusion with lysosomes, forming autolysosomes) but also in LAP via LC3-mediated fusion between phagosomes and lysosomes (Fazeli and Wehman., 2017). The enhanced formation of

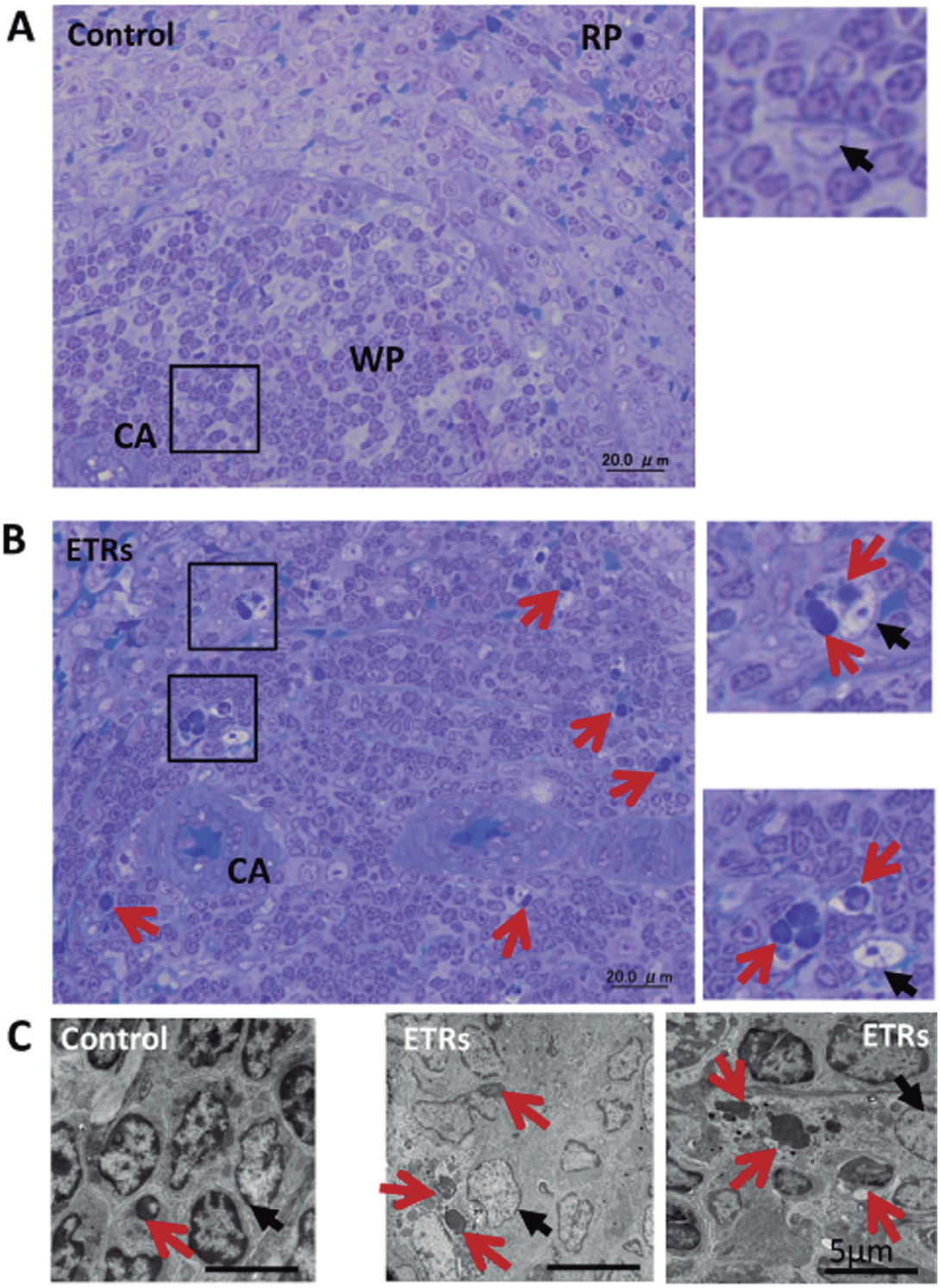

Fig. 1. Enhanced splenocyte apoptosis and phagocytosis by tingible body macrophages (TBMs) in the white pulp of ethanol-treated rats (ETRs). **A, B.** Toluidine-stained semithin sections reveal apoptotic splenocytes (red arrows) within TBMs (black arrows) in the white pulp. The framed areas are magnified on the right. **C.** TEM images show a marked increase of apoptotic splenocytes and TBMs in ETRs, confirming A&B. CA, central artery; WP, white pulp; RP, red pulp.

autolysosomes and phagolysosomes in ETR TBMs is evidenced by double-labeling immunofluorescence of LC3-II with pan cathepsin (a lysosomal marker) (Fig. 6A,B). According to TEM (Fig. 7B), phagocytosed apoptotic splenocytes in ETRs were encapsulated by phagosomal membranes adjacent to autophagosomes, confirming the induction of LAP.

Discussion

The results of this study include three major findings: enhanced T-cell apoptosis by ethanol exposure and concomitant accumulation of TBMs; an increased autophagic response in TBMs; and phagocytosis of apoptotic lymphocytes by these macrophages via LAP.

Several studies have shown that excessive ethanol intake induces T-cell apoptosis in various tissues, including the spleen, resulting in immunosuppression (Shellito and Olariu, 1998; Kapasi et al., 2003; Hote et al., 2008). The results of our study are consistent with these findings; in addition, we provided light microscopic and ultrastructural evidence of TBM-mediated phagocytosis of apoptotic T cells. A previous study by Betsuyaku et al. (2017) demonstrated ethanol-induced thymocyte apoptosis, and here we report ethanol-induced splenic T-cell apoptosis based on the immunohistochemistry of T cells and the localization of apoptotic cells in the PALS. However, the reduction of splenic B-cell expression was not as drastic as that of T cells. The difference in apoptotic response may be dependent on the sensitivity to some toxins and regulators, such as lymphocyte homing, ethanol metabolites, and corticosterone, in addition to other mechanisms (Eid et al., 2000; Pasala et al., 2015).

The observed enhanced apoptosis in spleen T cells of ETRs in the current study is consistent with the apoptotic impact of ethanol documented in various organs using the same animal model, such as thymocytes

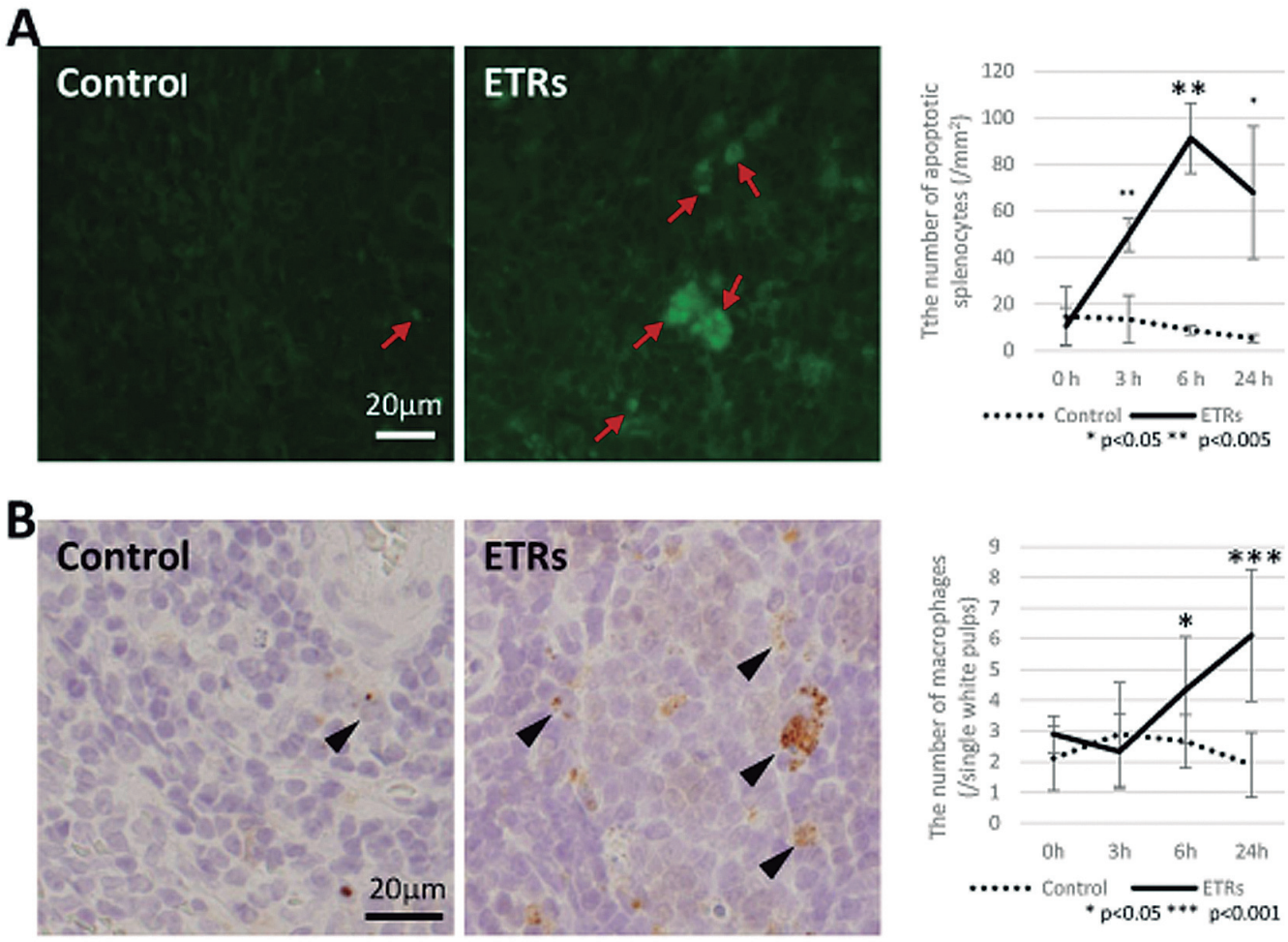

Fig. 2. Quantitative analysis of apoptotic splenocytes and TBMs using the TUNEL method and immunohistochemistry. **A.** TUNEL-positive splenocytes (maximum at 6h in ETRs) (red arrows) and quantification (the histogram on the right). **B.** Immunohistochemical staining and quantification of RM4-positive macrophages (maximum at 24h in ETRs) (black arrowheads) *p<0.05, **p<0.005, ***p<0.001.

(Betsuyaku et al., 2017). This apoptotic effect of ethanol has been reported to be mediated by the mitochondrial pathway, involving mechanisms linked to oxidative stress, induction of inducible nitric oxide synthase, and other factors (Eid et al., 2000; Kapasi et al., 2003; Hote et al., 2008; Horibe et al., 2017).

In the current study, immunofluorescence labeling of pan cathepsin, and electron microscopy revealed an increase in the number of lysosomes in TBMs of ETRs. Enhanced lysosomal formation has also been reported in thymic TBMs (engulfing apoptotic thymocytes) (Betsuyaku et al., 2017) and Sertoli cells (phagocytosing apoptotic germ cells) of ETRs (Horibe et al., 2017). Therefore, splenic TBMs may be activated by ethanol exposure, producing many lysosomes required for autolysosome and phagolysosome formation.

The enhanced prosurvival autophagic response to acute ethanol exposure in various cells, including macrophages, has already been demonstrated in other organs such as the liver, testis, and thymus. (Eid et al., 2016; Betsuyaku et al., 2017; Horibe et al., 2017). Electron microscopy in the current study revealed ultrastructural features of enhanced autophagosome and autolysosome formation in TBMs of ETRs. This is confirmed by enhanced expression of LC3-II using immunohistochemistry and LC3-II/RM4 double-labeling immunofluorescence. This is consistent with a recent study in castrated rats showing macrophages phagocytosing apoptotic cells expressed LC3-II/CD68 (a macrophage marker), suggesting autophagy's prosurvival role as well as the induction of LAP (Silva et al., 2018). In the thymi of ETRs, both the direct effect of ethanol and the phagocytosis of many apoptotic T cells can induce damage to TBMs through oxidative or nitrative stress, which may require autophagy induction as a survival mechanism (Betsuyaku et al., 2017). In the current study, such a cytoprotective cascade can also be induced in splenic ETR TBMs, where LC3-II

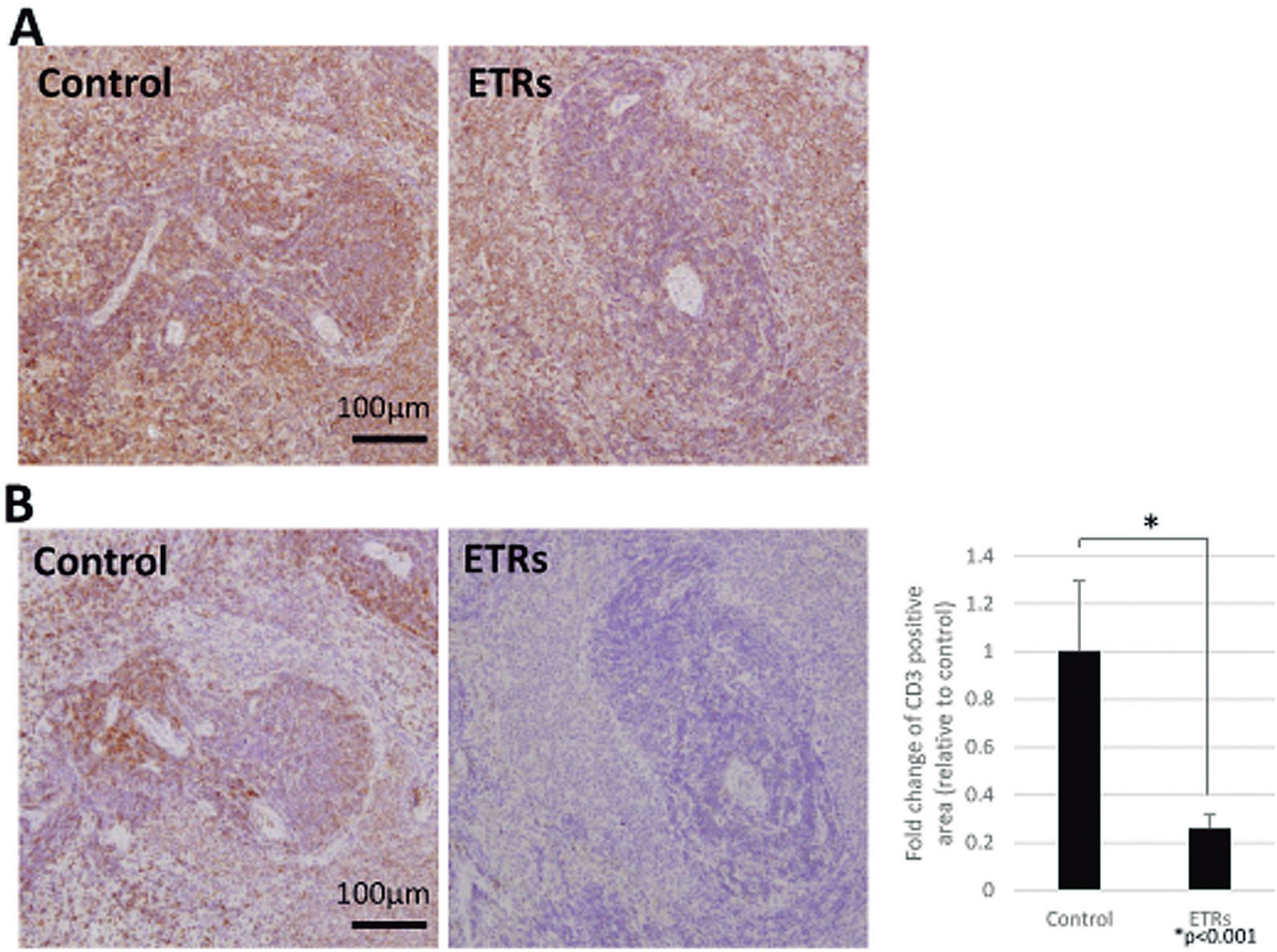

Fig. 3. Ethanol enhances splenic T-cell apoptosis. **A.** Immunohistochemical labeling of OX-33 (CD45RA) (B cell marker) **B.** immunostaining of CD3 (T-cell marker) and quantification on the right side. The brown color (DAB reaction) indicates positive staining. *p<0.001

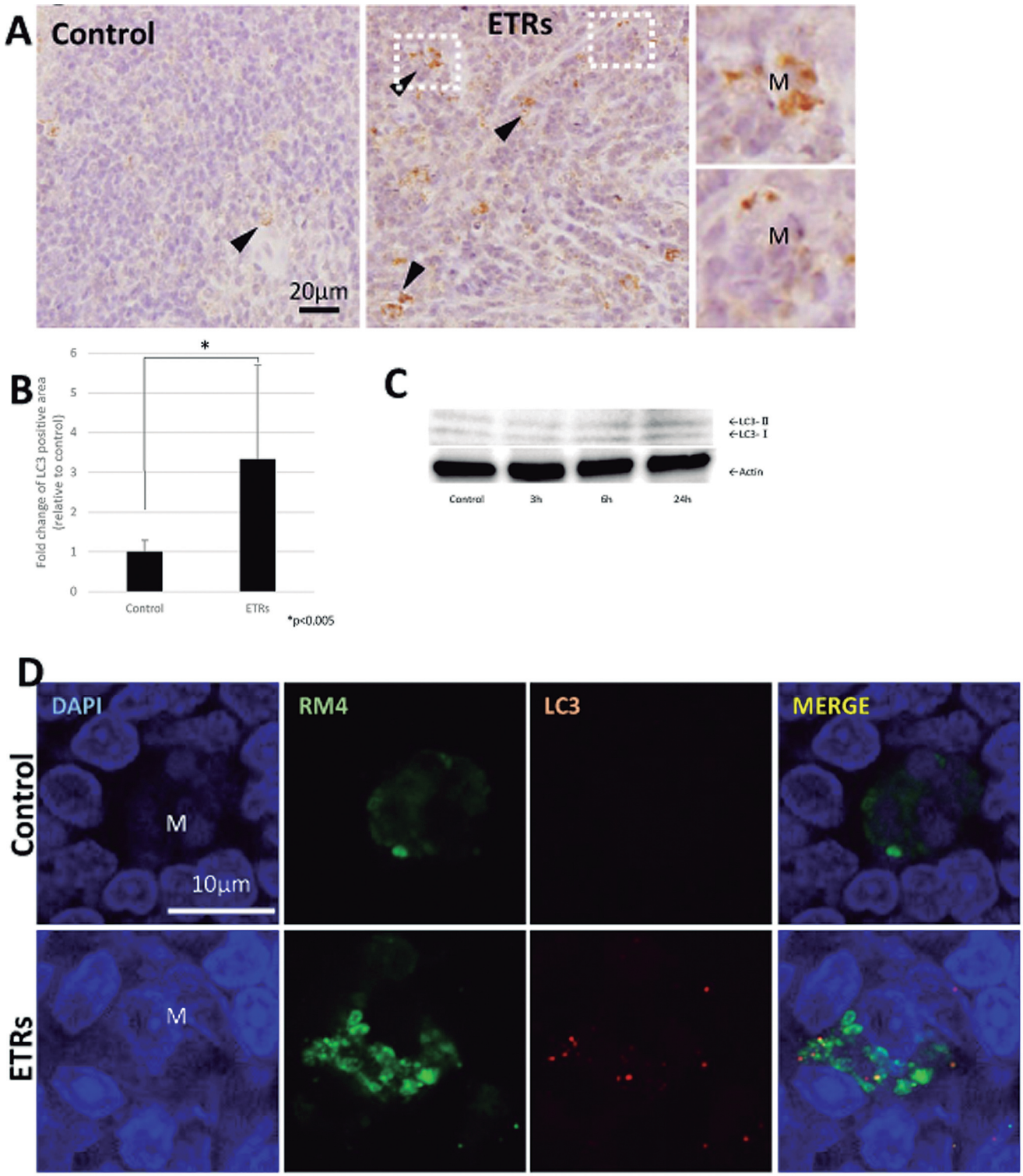

Fig. 4. Significant upregulation of the autophagosome marker LC3-II in ETR TBMs **A, B.** Immunohistochemical staining and quantification of LC3 dots (black arrowheads). **C.** Quantification of LC3-II in rat spleen using western blot. **D.** Immunofluorescence double labeling of RM4 (a macrophage marker) and LC3 indicates enhanced co-localization in ETR white pulp TBMs. *p<0.005. M: macrophage nucleus.

upregulation and p62 downregulation support this possibility. Furthermore, autophagy has been demonstrated to play a prosurvival role in peritoneal macrophages engulfing apoptotic lymphocytes *in vitro*, as demonstrated by the reduced viability of these macrophages when autophagy is blocked (Zhou et al., 2016).

The results of the current study revealed the excessive formation of multilamellar bodies in ETR TBMs, which are thought to be multi-membrane autophagosomes undergoing repeated sequestration (Brooks et al., 2015). In addition, autophagosomes were

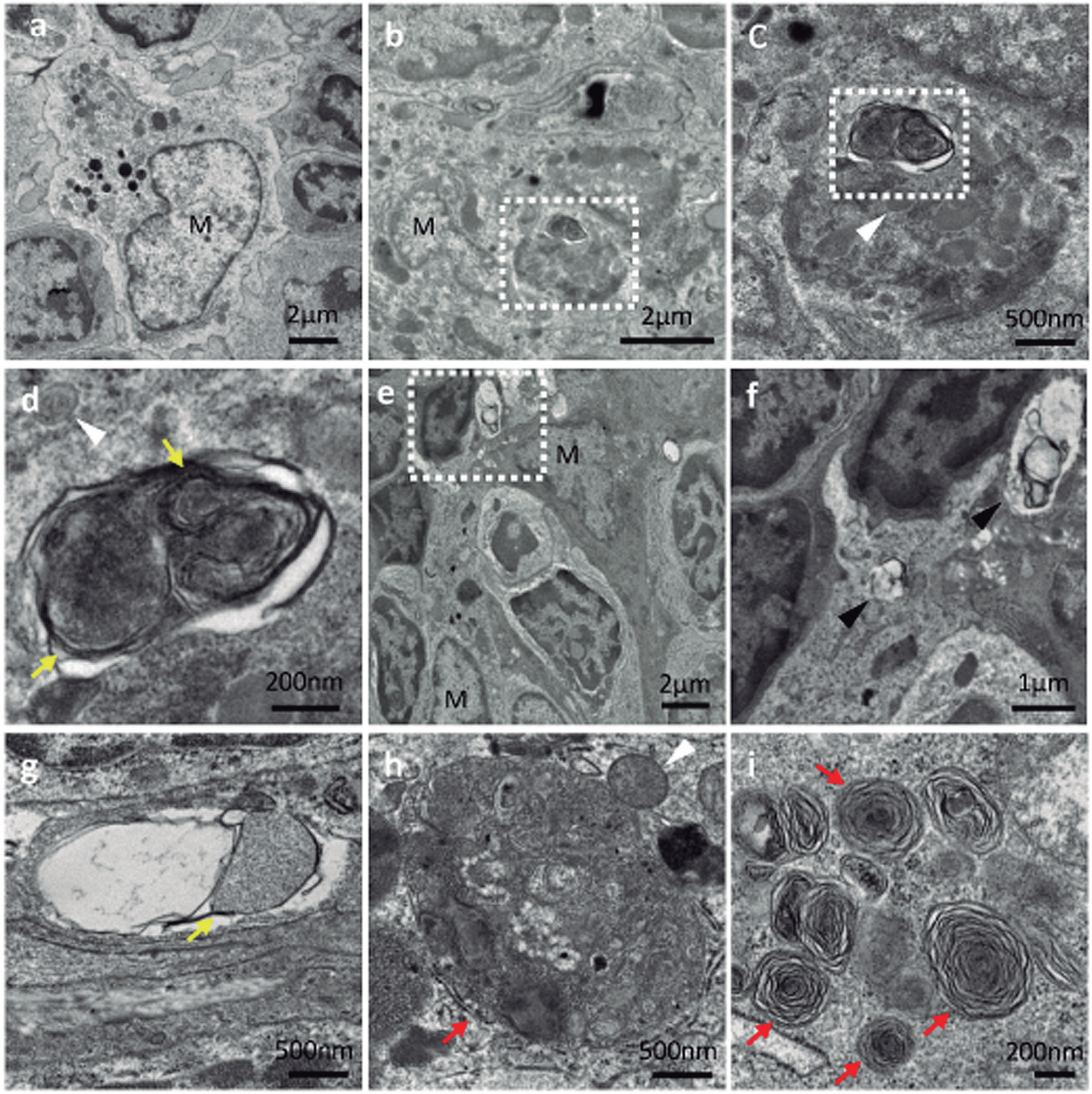

Fig. 5. Ultrastructural characteristics of enhanced autophagy in TBMs in Control **(a)** and ETRs **(b-i)** The framed area in **b** is magnified in **c**, that in **c** is still magnified in **d**, and that in **e** is magnified in **f**. Yellow arrows (autophagosomes); black arrowheads (autolysosomes); white arrowheads (lysosomes); and red arrows (multilamellar bodies). M: macrophage nucleus.

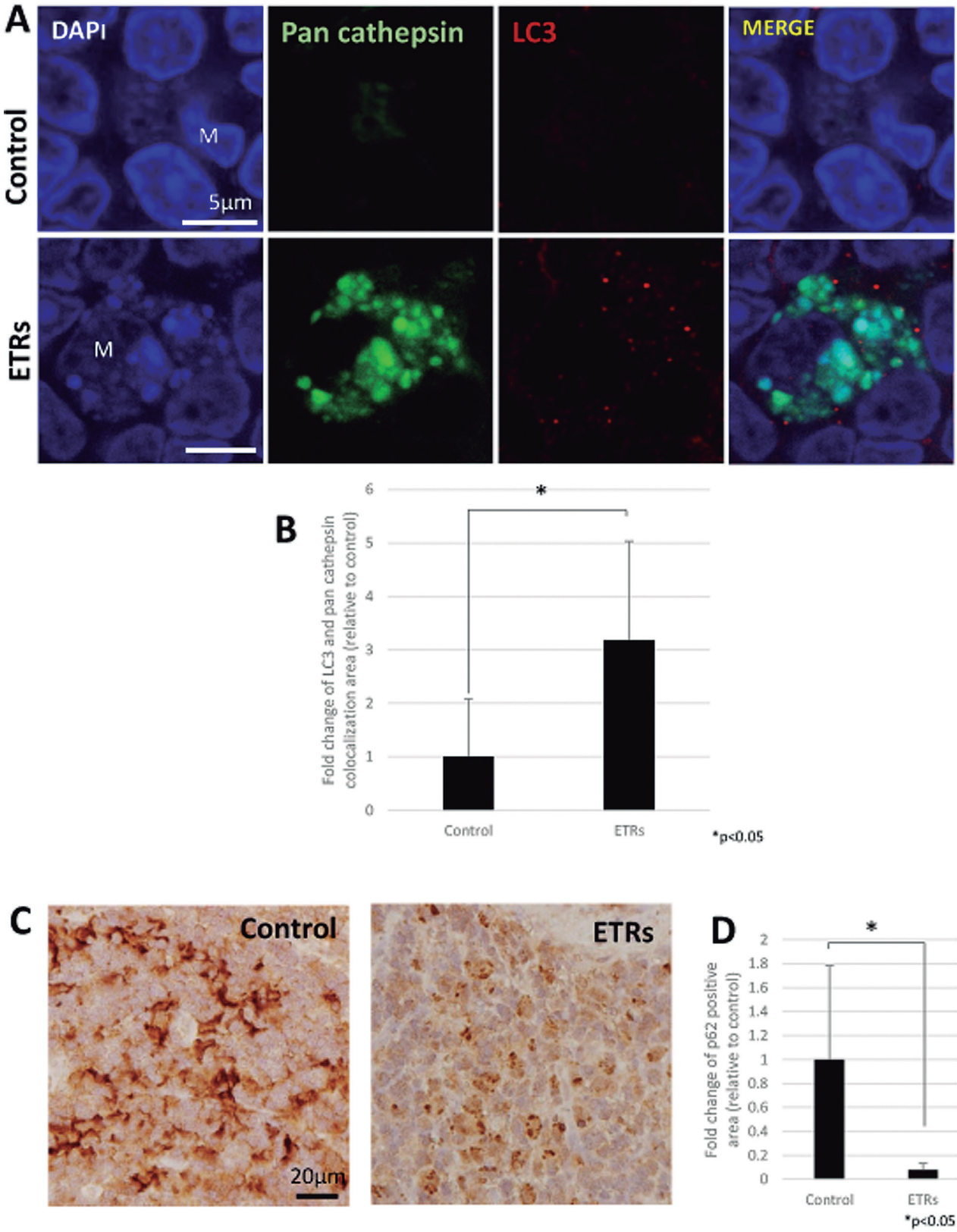

Fig. 6. Increased autolysosome formation and enhanced autophagic flux in ETR TBMs. **A, B.** Immunofluorescence double labeling of LC3 with pan-cathepsin (a lysosomal marker) and quantitative analysis. **C, D.** Immunohistochemistry of p62 and quantification. M: macrophage nucleus.

found to contain damaged mitochondria, suggesting the induction of selective autophagy of mitochondria (mitophagy), an important anti-apoptotic mechanism. Further studies are required to investigate the specific mechanisms of mitophagy in TBMs, such as the PINK1-Parkin pathway (Eid et al., 2016).

One of the key molecules in autophagy induction is LC3, and its upregulation was identified in the current study. Transcription factor FOXO3a was reported to enhance the expression of autophagy genes, including *LC3* (Tran et al., 2002; Zhou et al., 2012; Czarny et al., 2015; Li et al., 2017). We investigated the expression of

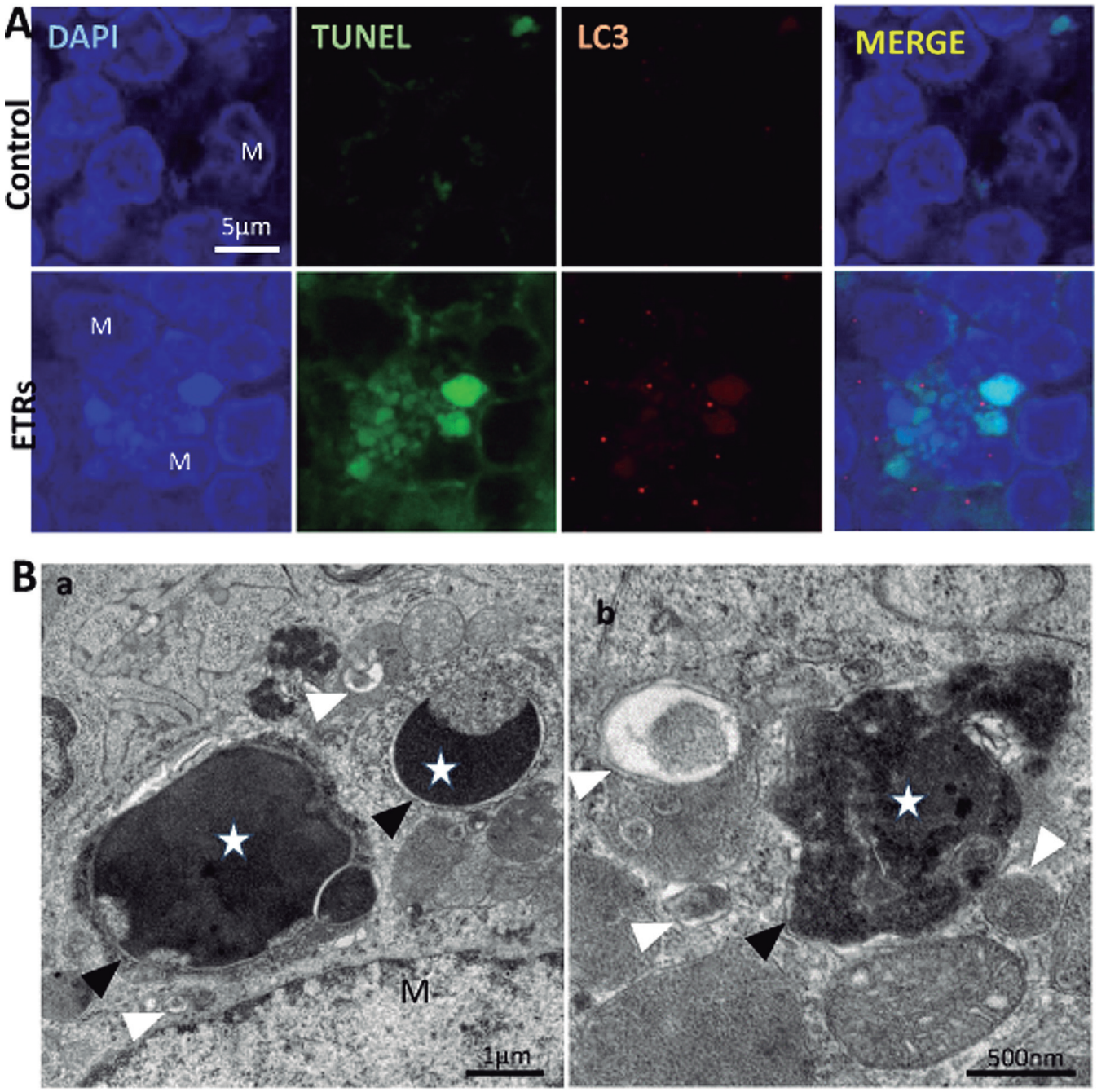

Fig. 7. Light and ultrastructural features of the activation of LC3-associated phagocytosis (LAP) in ETR TMBs. **A.** TUNEL/LC3 double labeling showing decoration of apoptotic cells with LC3. **B. (a, b)** TEM showing phagosomal membranes (black arrowheads) surrounding phagocytosed apoptotic solenocytes (white stars) by TBMs in ETRs. The white arrowheads indicate autophagosomes. M, macrophage nucleus.

this transcription factor by immunohistochemistry, however, its expression was not significant (data not shown). Thus, LC3 expression may be regulated by other transcription factors, such as TFEB, a master regulator of autophagy and lysosomal proteins (Horibe et al., 2017).

Recent studies show that LAP clears apoptotic cells more rapidly than classical phagocytosis, thus preventing secondary necrosis and inflammation (Li et al., 2012; Kim et al., 2013; Lam et al., 2013; Ferguson and Green, 2014; Matte et al., 2016). In the current research, LC3 puncta were found to be closely related to apoptotic TUNEL-positive splenocytes engulfed by ETR TBMs. This indicated the possibility of the induction of LAP. This possibility was confirmed by TEM studies, which revealed the presence of LAPsomes enclosed by single membranes adjacent to autophagosomal vesicles in these macrophages, indicating the activation of LAP. A recent study found that LC3-II-positive cells were simultaneously TUNEL-positive T cells in the spleens of infected pigs, suggesting the involvement of apoptosis and autophagy in the clearance of apoptotic lymphocytes. (Gou et al., 2017). Further studies are needed to confirm the induction of LAP in splenic TBMs by studying specific proteins such as Rubicon and Beclin-1 (Yefimova et al., 2020; Pena-Martinez et al., 2022).

The current study revealed enhanced autophagy in splenic TBMs of ETRs. This autophagic activity seems to function as an anti-apoptotic mechanism, countering the detrimental effects of ethanol, including oxidative stress. Notably, no apoptotic TBMs were detected using the TUNEL method, TUNEL/LC3 double labeling, and TEM. Furthermore, this phenomenon may be associated with an increased occurrence of T-cell apoptosis, thereby accelerating LAP. The observed enhancement of autophagy and LAP in splenic TBMs has also been reported in thymic macrophages (Betsuyaku et al., 2017) and Sertoli cells (Horibe et al., 2017) using the same animal model of acute ethanol toxicity.

The contribution of the defect in LAP to the clearance of apoptotic lymphocytes and the increased risk of systemic lupus erythematosus (SLE) was highlighted recently (Bandyopadhyay and Overholtzer, 2016; Pena-Martinez et al., 2022). SLE is commonly marked by anti-double-stranded DNA antibodies from B cells, however, pathologically, autoreactive T cells are considered as important to its pathogenicity as B cells. Moreover, the importance of the clearance of apoptotic cells by TBMs in SLE is also reported (Baumann et al., 2002). Our two studies collectively demonstrate the role of the autophagic machinery in clearing apoptotic T cells, both in primary lymphoid organs such as the thymus (Betsuyaku et al., 2017) and secondary lymphoid organs, as observed in the current study focusing on the spleen. Consequently, specific macrophages engaging in phagocytosis, canonical autophagy, and LAP play roles in eliminating damaged T lymphocytes induced by ethanol and other xenobiotics within central and peripheral lymphoid organs. This process holds potential to prevent diseases such as SLE. Additionally, the activation of autophagy and LAP in the spleen may play a crucial role in the phagocytosis of various microbes, including viruses, bacteria, fungi, and parasites (Upadhyay and Philips, 2019).

In conclusion, we provided for the first time light and electron microscopic evidence indicating enhanced autophagy in splenic TBMs of ETRs. This appears to be a cytoprotective mechanism, required for phagocytosis, clearance of many apoptotic T cells, and inhibition of autoimmunity and the inflammatory response.

Acknowledgements and conflict of interest. The authors have nothing to declare.

References

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.

Betsuyaku T., Eid N., Ito Y., Tanaka Y., Otsuki Y. and Kondo Y. (2017). Ethanol enhances thymocyte apoptosis and autophagy in macrophages of rat thymi. Histol. Histopathol. 32, 963-975.

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.

Cerroni L., Arzberger E., Pütz B., Höfler G., Metze D., Sander C.A., Rose C., Wolf P., Rütten A., McNiff J.M. and Kerl H. (2000). Primary cutaneous follicle center cell lymphoma with follicular growth pattern. Blood 95, 3922-3928.

Cesta M.F. (2006). Normal structure, function, and histology of the spleen. Toxicol. Pathol. 34, 455-465.

Chen G., Ke Z., Xu M., Wang X., Zhang T., Frank JA., Bower KA., Shi X. and Luo J. (2012). Autophagy is a protective response to ethanol toxicity. 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. Toxicl. Appl. Pharmacol. 148,176-182.

Czarny P., Pawlowska E., Bialkowska-Warzecha J., Kaarniranta K. and Blasiak J. (2015). Autophagy in DNA damage response. Int. J. Mol. Sci. 16, 2641-2662.

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.

Eid NA., 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. (2016). Ethanol-induced mitophagy in liver is associated with activation of the PINK1-Parkin pathway triggered by oxidative DNA damage. Histol. Histophathol. 31, 1143-1159.

Fazeli G. and Wehman AM. (2017). Safely removing cell debris with LC3-associated phagocytosis. Biol. Cell 109, 355-363.

Ferguson T.A. and Green D.R. (2014). Autophagy and phagocytosis converge for better vision. Autophagy 10, 165-167.

Gou H., Zhao M., Fan S., Yuan J., Liao J., He W., Xu H. and Chen J. (2017). Autophagy induces apoptosis and death of T lymphocytes in the spleen of pigs infected with CSFV. Sci. Rep. 19, 13577.

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.

Horibe A., Eid N., Ito Y., Tanaka Y. and Kondo Y. (2017). Upregulated autophagy in Sertoli cells of ethanol-treated rats is associated with induction of inducible nitric oxide synthase (iNOS), androgen receptor suppression and germ cell apoptosis. Int. J. Mol. Sci. 18, 1061.

Hote P.T., Sahoo R., Jani T.S., Ghare S.S., Chen T., Joshi-Barve S., McClain C.J. and Barve S.S. (2008). Ethanol inhibits methionine adenosyltransferase II activity and S-adenosylmethionine biosynthesis and enhances caspase-3-dependent cell death in T lymphocytes: relevance to alcohol-induced immunosuppression. J. Nutr. Biochem. 19, 384-391.

Ichimura Y., Kumanomidou T., Sou YS., Mizushima T., Ezaki J., Ueno T., Kominami E., Yamane T., Tanaka K. and Komatsu M. (2008). Structural basis for sorting mechanism of p62 in selective autophagy. J. Biol. Chem. 283, 22847-22857.

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.

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.

Kim J.Y., Zhao H., Martinez J., Doggett T.A., Kolesnikov A.V., Tang P.H., Ablonczy Z., Chan C.C., Zhou Z., Green DR. and Ferguson T.A. (2013). Noncanonical autophagy promotes the visual cycle. Cell 154, 365-376.

Klionsky D.J., Abdel-Aziz A.K., Abdelfatah S., Abdellatif M., Abdoli A., Abel S., Abeliovich H., Abildgaard M.H. and Tong C.K. (2021). Guidelines for the use and interpretation of assays for monitoring autophagy (4th edition). Autophagy 17, 1-382.

Komatsu M., Waguri S., Koike M., Sou Y.S., Ueno T., Mizushima N., Iwata J., Ezaki J. and Tanaka K. (2007). Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell 131, 1149-1163.

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

Lam G.Y., Cemma M., Muise A.M., Higgins D.E. and Brumell J.H. (2013). Host and bacterial factors that regulate LC3 recruitment to Listeria monocytegenes during the early stages of macrophage infection. Autophagy 9, 985-995.

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

Li L., Zviti R., Ha C., Wang Z.V., Hill J.A. and Lin F. (2017). Forkhead box O3 (FoxO3) regulates kidney tubular autophagy following urinary tract obstruction. J. Biol. Chem. 292, 13774-13783.

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 alpha (LC3)- associated phagocytosis is required for the efficient clearance of dead cells. Proc. Natl. Acad. Sci. USA 108, 17396-17401.

Matte C., Casgrain P.A., Séguin N., Hong W.J. and Descoteaux A. (2016). *Leishmania major* promastigotes evade LC3-associated phagocytosis through the action of GP63. PLoS Pathog. 12, e1005690.

Pasala S., Barr T. and Messaoudi I. (2015). Impact of alcohol abuse on the adaptive immune system. Alcohol Res. 37, 185-197.

Pena-Martinez C., Rickman A.D. and Heckmann B.L. (2022). Beyond autophagy: LC3-associated phagocytosis and endocytosis. Sci. Adv. 8, eabn1702.

Perotta 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.

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.

Shellito J.E. and Olariu R. (1998). Alcohol decreases T-lymphocyte migration into lung tissue in response to Pneumocystis carinii and depletes T-lymphocyte numbers in the spleens of mice. Alcohol Clin. Exp. Res. 22, 658-663.

Silva J.A.F., Bruni-Cardoso A., Augusto T.M., Damas-Souza D.M., Barbosa G.O., Felisbino S.L., Stach-Machado D.R. and Carvalho H.F. (2018). Macrophage roles in the clearance of apoptotic cells and control of inflammation in the prostate gland after castration. Prostate 78, 95-103.

Souza H.S., Elia C.C., Braulio V.B., Côrtes M.Q., Furtado V.C., Garrofé H.C. and Martinusso C.A. (2003). Effects of ethanol on gut-associated lymphoid tissues in a model of bacterial translocation: a possible role of apoptosis. Alcohol 30, 183-191.

Steiniger B.S. (2015). Human spleen microanatomy: why mice do not suffice. Immunology 145, 334-346.

Tran H., Brunet A., Grenier J.M., Datta S.R., Fornace A.J. Jr., DiStefano P.S., Chiang L.W. and Greenberg M.E. (2002). DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. Science 296, 530-534.

Upadhyay S. and Philips J.A. (2019). LC3-associated phagocytosis: host defense and microbial response. Curr. Opin. Immunol. 60, 81-90.

Yefimova M.G., Lefevre C., Bashamboo A., Eozenou C., Burel A., Lavault M.T., Meunier A.C., Pimentel C., Veau S., Neyroud A.S., Jaillard S., Jégou B., Bourmeyster N. and Ravel C. (2020). Granulosa cells provide elimination of apoptotic oocytes through unconventional autophagy-assisted phagocytosis. Hum. Reprod. 35, 1346-1362.

Yoshii S.R. and Mizushima N. (2017). Monitoring and measuring

autophagy. Int. J. Mol. Sci. 18, 1865.
Zhou J., Liao W., Yang J., Ma K., Li X., Wang Y., Wang D., Wang L., Zhang Y., Yin Y., Zhao Y. and Zhu W.G. (2012). FOXO3 induces FOXO1-dependent autophagy by activating the AKT1 signaling pathway. Autophagy 8, 1712-1723.
Zhou P., Tan Y.Z., Wang H.J., Li T., He T., Yu Y., Zhang J. and Zhang D. (2016). Cytoprotective effect of autophagy on phagocytosis of apoptotic cells by macrophages. Exp. Cell Res. 348, 165-176.
Zhu L., Yang J., Zhang J. and Peng B. (2013). The presence of autophagy in human periapical lesions. J. Endod. 39, 1379-1384.

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