Cytokine 123 (2019) 154726

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Ursolic acid exhibits anti-inflammatory effects through blocking TLR4-MyD88 pathway mediated by autophagy

Jun Zhao[a,1], Haoyi Zheng[c,1], Zhongguo Sui[a], Fanbo Jing[a], Xianghua Quan[a], Wenwen Zhao[c,*], Guangwei Liu[b,*]

[a] Department of Pharmacy, Affiliated Hospital of Qingdao University, Qingdao, Shandong, China
[b] Outpatient Department, Affiliated Hospital of Qingdao University, Qingdao, Shandong, China
[c] Qingdao University Medical College, 308 Ningxia Road, Qingdao, Shandong 266021, China

ARTICLE INFO

Keywords:
Inflammation
Autophagy
Macrophagy
Acute kidney injury

ABSTRACT

There is an urgent need for effective treatments to reduce the large and growing burden of acute kidney injury (AKI) and its consequences. Inflammation is believed to play a vital role in the pathophysiology of AKI. Macrophage autophagy is considered protective against inflammation. Previous study discovered that ursolic acid (UA), a natural pentacyclic triterpene carboxylic acid found in many plants as apples, bilberries, cranberries and so on, promoted cancer cell autophagy. In the present study, we aimed to explore the effect of UA on ameliorating AKI and the role of macrophage autophagy in the context of inflammation. The data from in vivo experiments showed that pretreatment of mice with UA significantly suppressed xylene-induced ear oedema as well as protected against LPS-induced AKI. Related mechanisms were further studied through *in vitro* experiment. As expected, UA decreased inflammatory factors TNF-α, IL-6 and IL-1β secretion in macrophages in response to lipopolysaccharide (LPS) stimulation. Furthermore, UA blocked LPS-induced TLR4/MyD88 pathway. More importantly, enhanced autophagy of macrophages by UA through increasing the expression of both LC3B and Beclin-1 led to alter macrophage function. What is more, similar to UA, autophagy inhibitor 3-MA obviously decreased inflammation factors releases hinting the vital role of autophagy in regulating inflammation. In all, above study suggested that UA is a potential anti-inflammatory natural compound for treating AKI by inducing autophagy.

1. Introduction

In the past decade, autophagy has represented an emerging new factor in human diseases as cancer [1], cardiomyopathy [2], and neurodegenerative disorders [3]. Recent studies have demonstrated that autophagy is rapidly induced in kidneys during AKI to protect tubular cells from injury and even death [4]. Macrophage autophagy plays a protective role against atherosclerosis [5] and liver fibrosis [6]. Macrophage autophagy is proposed as a potential strategy for AKI while related mechanisms are remains unclear.

Toll-Like Receptor 4 (TLR4) is one member of TLR family and is activated by bacterial LPS, which leads to the development of AKI [7]. Activated TLR4 signal pathway initiates the immune response and its activation by bacterial endotoxin is responsible for various chronic and acute inflammatory disorders [8]. MyD88 is the downstream of TLR4. Studies on MyD88-deficient mice clearly demonstrated that MyD88 is essential in response to inflammation [9]. Also there are other data showing that MyD88 is indispensable in responses to LPS [10]. As related research continues, the role of MyD88 in the pathological processes of inflammation attracts more and more attentions.

Ursolic acid (UA) is a lipophilic pentacyclic triterpenoid found in a wide variety plants, and possesses a wide range of biological functions, such as anti-inflammation [11], anti-oxidation [12], and anti-fibrosis [13]. These beneficial properties are of apparent value in preventing AKI, although the underlying molecular mechanisms remain largely unknown. This study is to investigate the protective effect of UA on AKI and related mechanisms was also explored both in *vivo* and *in vitro*. Specially, the role of autophagy involved in this process was revealed.

Abbreviations: AKI, acute kidney injury; BUN, blood urea nitrogen; DEX, dexamethasone; H&E, haematoxylin and eosin; LPS, lipopolysaccharide; TLR4, Toll-Like Receptor 4; UA, ursolic acid

* Corresponding authors at: 16 Jiangsu Road, Shinan District, Qingdao 266003, Shandong Province, China (G. Liu).
E-mail address: liuguangw@qduhospital.cn (G. Liu).

[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.cyto.2019.05.013
Received 29 September 2018; Received in revised form 23 April 2019; Accepted 13 May 2019
Available online 11 July 2019

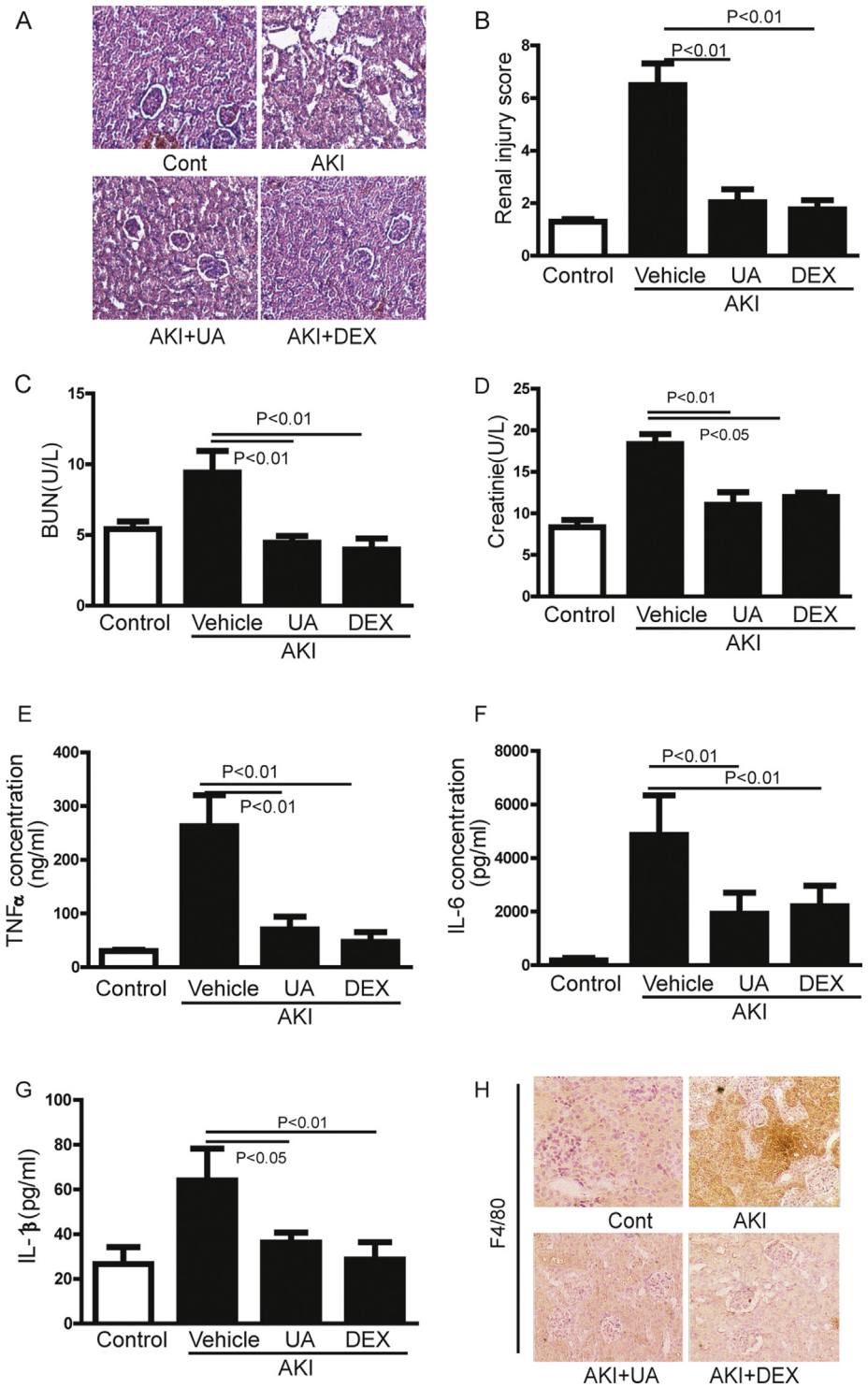

Fig. 1. UA prevented renal injury in LPS-AKI *in vivo*. Mice were pretreated with UA (100 mg kg^{-1}, i.g.) or positive control drug DEX (5 mg kg^{-1}, i.p.) for 1 h before LPS (10 mg kg^{-1}, i.p.) injection. After 24 h, the kidney tissues and the blood samples were collected from mice. Renal function was assessed by HE staining (A and B) and plasma creatinine and BUN level (C and D); Besides, inflammatory cytokines TNF-α (E), IL-6 (F) and IL-1β (G) in serum were determined using ELISA kits; Macrophages infiltration in kidneys tissues was detected by immunohistochemistry with F4/80 antibody (H). UA, ursolic acid; DEX, dexamethasone.

Our findings may offer a new mechanism for UA on treating inflammatory disorder, which benefits the development of ideal drugs.

2. Materials and methods reagents

2.1. Materials and reagents

UA (purity > 98%) was purchased from Chengdu Herbpurify Co. Ltd. (Chendu, China); LPS (Escherichia coli serotype 055:B5, LPS) was purchased from Sigma Aldrich (St. Louis, MO, USA); Antibodies for

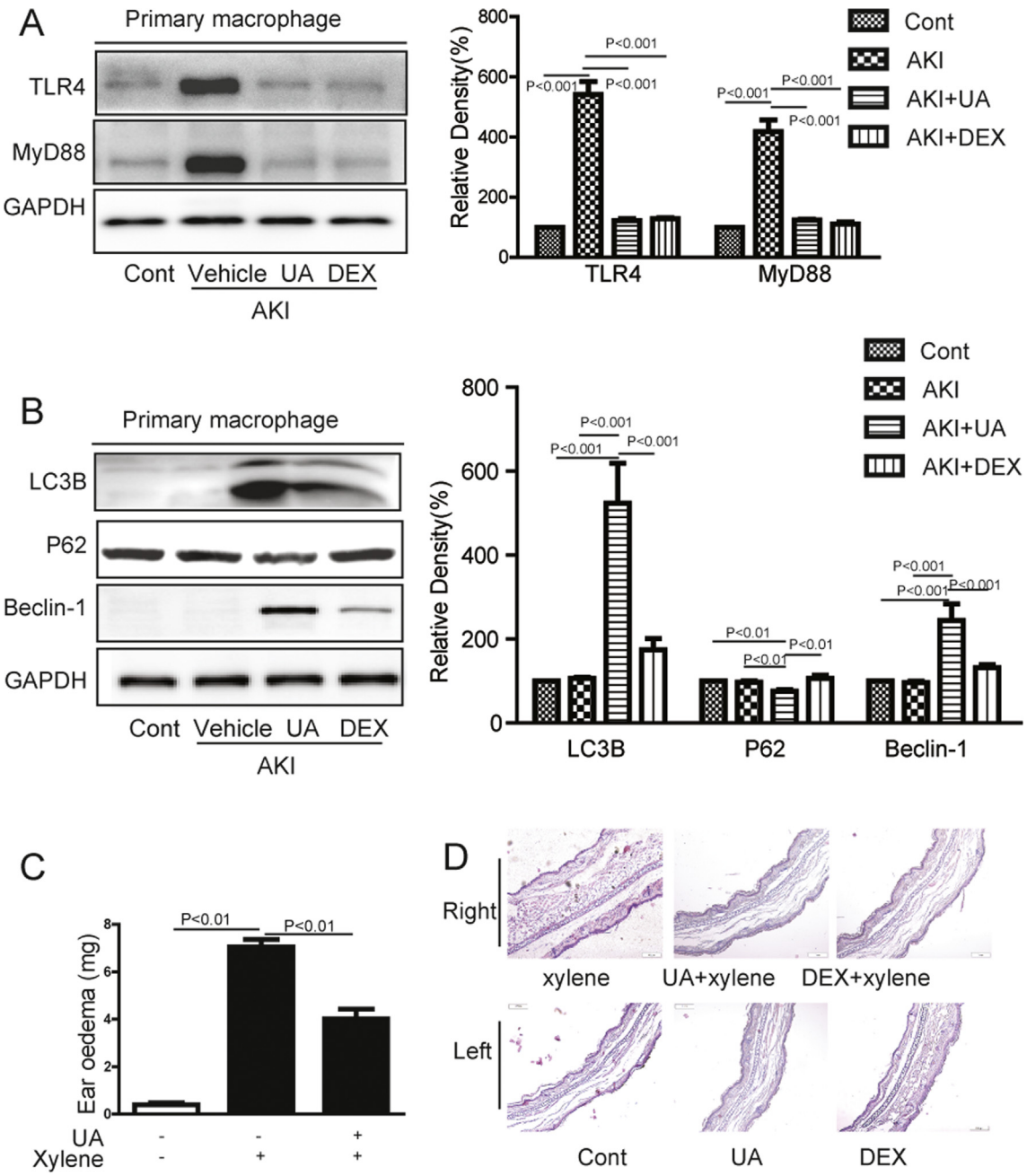

Fig. 2. UA modulated autophagy pathway and suppressed inflammation during AKI. Primary peritoneal macrophages were isolated from control mice, AKI mice, UA-treated mice and DEX-treated mice, TLR4 and MyD88 were detected by western blotting (A); Also LC3B, p62, and Beclin-1 protein expressions were detected by western blotting (B); Additionally, mice were pretreated with UA (100 mg kg^{-1}, i.g.) for 1 h before being administered xylene (30 μL) for 1 h, ear weight was measured (C); The ear tissue samples were stained with H&E (D) (HE, original magnification, 400×). Cont, left ear of mice not treated with xylene; UA, ursolic acid; DEX, dexamethasone.

TLR4 and MyD88 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Antibodies for LC3B, Beclin-1 and GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA); The anti-SQSTM1/p62 antibody was purchased from Abcam (Cambridge, UK); The BCA protein kits were purchased from Thermo Fisher (Suwanee, GA, USA); HE Staining Kit was obtained from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China); F4/80 antibody was purchased from Bio-Rad (Hercules, CA); ELISA kits for TNF-α, IL-1β, and IL-6 were from Neobioscience (Shenzhen, China). SiRNA for TLR4 and MyD88 were purchased from Gene Pharma Company (Shanghai, China). The BCA protein kits were purchased from Thermo Fisher (Suwanee, GA, USA).

2.2. Blood parameters

Before the mice were killed, blood samples were collected from the orbit. Then serum TNF-α, IL-6 and IL-1β were investigated by commercial kits.

2.3. Cell culture

RAW 264.7 line cells (ATCC, Rockville, USA) and isolated primary peritoneal macrophages were cultured in DMEM with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) in a humidified incubator with 5% CO_2 at 37 °C.

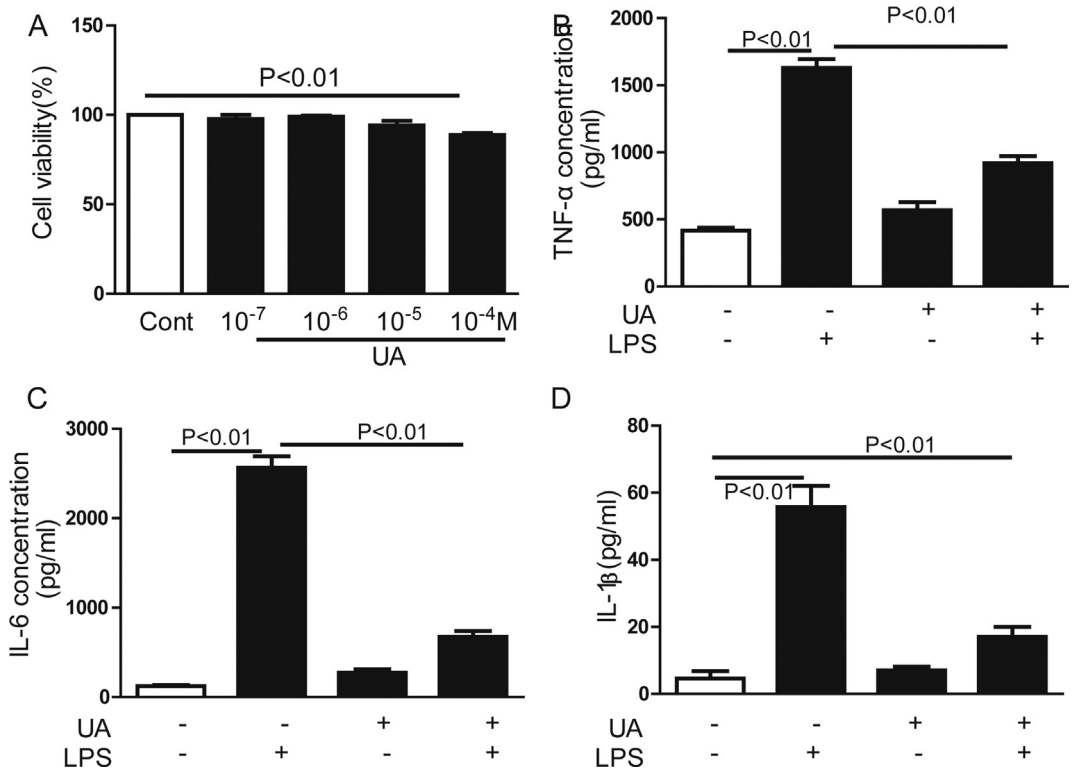

Fig. 3. UA suppressed LPS-induced pro-inflammatory cytokine release *in vitro*. Firstly, RAW264.7 cells were pretreated with different doses of UA (10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} M) for 24 h, cell viability was detected by MTT assay (A); RAW264.7 cells were pretreated with UA (10^{-5} M) for 1 h before being stimulated with LPS (50 μg/ml) for another 24 h, the pro-inflammatory cytokines TNF-α (B), IL-6 (C) and IL-1β (D) were determined using ELISA Kit. UA, ursolic acid.

2.4. MTT assay

RAW264.7 cells were seeded into 96-well plates at a density of 1×10^5 cells per well overnight. Then, cells were treated with UA (10^{-6}, 10^{-5}, 10^{-4} M) for 24 h. The next step is to choose MTT assay to determine the cytotoxicity according to previous report [14].

2.5. SiRNA transfection

SiRNAs targeting both TLR4 and MyD88 genes were used to silence gene expression. Related experiments were performed according to previous report [15].

2.6. Elisa

RAW264.7 cells were treated with UA for 1 h and were then incubated with LPS for 24 h. After the medium was collected, the secretion levels of TNF-α, IL-6 and IL-1β were determined using ELISA kits according to the manufacturer's instructions.

2.7. Animal experiments and ethical statement

BALB/C mice (male, 6–8 weeks old, 18–22 g) were purchased from Changzhou Cavens Laboratory Animal Co., Ltd. (Changzhou, Jiangsu Province, China). All mice were housed in cages (≤8 mice per cage) and given food and water under standard conditions (SPF) with air filtration (22 ± 2 °C, 12 h light/12 h dark). All animal procedures follow the NIH guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 1978).

For the xylene-induced mice ear oedema model [16], UA (100 mg kg^{-1}) was administered orally (by gavage). After 1 h, 30 μL xylene was applied to the anterior and posterior surfaces of the right ears. The left ears served as controls without treatment. An hour later, the mice were sacrificed by cervical dislocation, and two ear punches (7 mm, i.d.) were collected and weighed immediately. The oedema was evaluated by comparing the increase in the weight of the left ear punch with the increase in the weight of the right ear punch. Then ear tissues were collected and fixed in 10% formaldehyde for more than 24 h at room temperature. After being dehydrated in gradient concentrations of alcohol, tissues were embedded in paraffin. Lastly, sections were stained with haematoxylin and eosin (H&E) and immunohistochemistry and then imaged under a light microscope.

For the AKI model [17], mice were randomly divided into four groups: a control group, LPS group (10 mg kg^{-1}, i.p.), UA (100 mg kg^{-1}, i.g.) group and a positive control DEX (5 mg kg^{-1}, i.g.) group. Before the LPS injection, mice were pretreated with UA for 1 h. After 24 h, serum samples were collected by orbital puncture while they were under diethyl-ether anaesthesia, and the cytokines (TNF-α, IL-6, and IL-1β) were examined using ELISA kits. The serum levels of urea nitrogen (BUN) and creatinine were determined on a Roche Module P800 (Roche, Shanghai, China). Furthermore, the kidney tissues were harvested and fixed in 10% formaldehyde and then embedded in paraffin and sliced after been dehydrated in gradient concentrations of alcohol. The sections were stained with H&E and immunohistochemistry and then imaged under a light microscope.

2.8. Western blotting

Treated RAW cells line or primary peritoneal macrophages from experimental mice were washed twice with ice-cold PBS and lysed with RIPA buffer supplemented with a protease cocktail and phosphatase inhibitors. Cell lysates were separated using 8–12% SDS-PAGE and then transferred onto PVDF membranes. After membranes were blocked with 5% non-fat milk in TBST (20 mM Tris-HCl, 500 mM NaCl, and 0.1% Tween 20) at room temperature for 1 h, membranes were incubated with targeted primary antibodies and corresponding secondary antibodies. Lastly, the protein-antibody complexes were detected by ECL Advanced Western Blot detection Kit. Quantity One software (Bio-

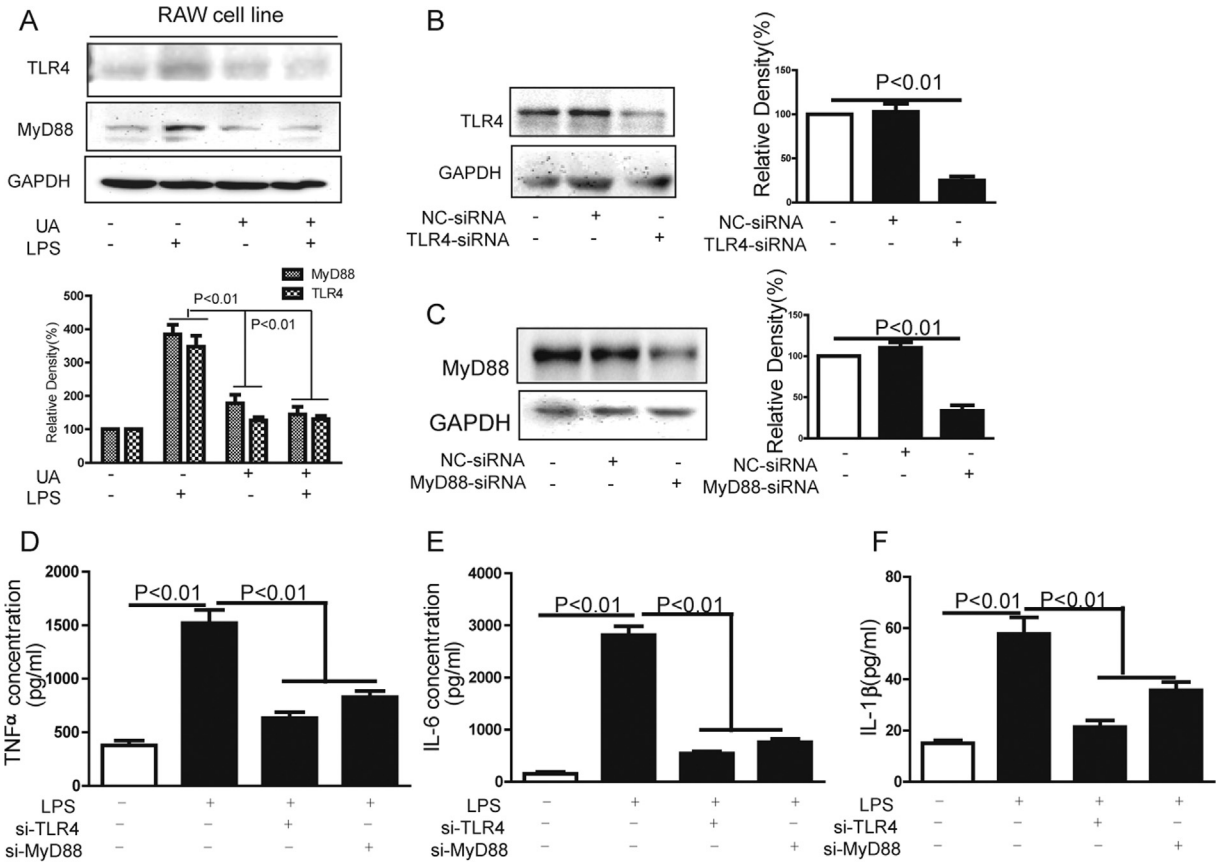

Fig. 4. UA reduced LPS-induced cytokines releases by blocking TLR4/MyD88 signal pathway both in RAW cell line. RAW264.7 cells were pretreated with UA (10^{-5} M) for 1 h before being stimulated with LPS (50 μg/ml) for another 24 h. Protein expressions of TLR4 and MyD88 were detected by western blotting (A); Cells were transfected with siRNAs for TLR4 (B) and MyD88 (C) successfully and then stimulated with LPS for 24 h, cytokines TNF-α (D), IL-6 (E), and IL-1β (F) were determined using ELISA kit. NC-siRNA, negative control siRNA. UA, ursolic acid.

Rad) was used to quantitate the intensity of bands.

2.9. Isolation of mouse peritoneal macrophages

Mouse peritoneal macrophages were prepared as previously described [7] with some modifications. Briefly, after mice from UA, Dex and LPS-treated groups were sacrificed, they were soaked in 75% alcohol for 1–2 min, transferred to the ultra-clean platform and then injected with 3 ml of 0.05% EDTA-PBS in the peritoneum to collect thioglycollate-elicited peritoneal macrophages. The collected cells were centrifuged at 160g, 4 °C for 5 min, and the cell pellet was washed with PBS and centrifuged again. The cell pellet was suspended in 10% FBS-DMEM medium and cultured in a culture dish. After incubation for 3 h, the cells were washed with PBS to remove unattached cells. Finally, the adherent cells were monolayer macrophages.

2.10. Immunohistochemistry

Immunohistochemistry was carried out as described previously in paraffin–embedded tissue sections 5 mm thick [18]. Primary antibody was rat polyclonal anti–F4/80 (1:100). Sections were used for immunohistochemical staining with antibody. Color reaction was developed with diaminobenidine.

2.11. Data analysis

All results are presented as the means ± SEM. For statistical analysis, the significance of the intergroup differences was analysed by one-way analysis of variance (one-way ANOVA) with Dunnett's multiple comparisons test using GraphPad Prism 6.0 software. The significant difference was defined as $P < 0.05$.

3. Results

3.1. UA prevents renal injury in LPS-AKI by regulating TLR4/MyD88 and autophagy in vivo

Tubular injury is an important feature of AKI that may eventually lead to tubulointerstitial fibrosis [19]. In LPS-induced AKI model, H&E staining assay was used to explore the histologic tubular injury. Compared with the normal kidney tubules in the control group, loss brush border or vacuolization could be obviously observed in model group. As expected, both UA and DEX (dexamethasone, positive drug) significantly attenuated these phenomena (Fig. 1A). To better observe the injury level of tubular injury visually, evidence of cell injury specially vacuolization was scored on a semiquantitative zero to three scale, and results were added to yield the tubular injury score (Fig. 1B). Compared with model group, both UA and DEX obviously decreased tubular injury score. Furthermore, renal function was assessed by plasma creatinine and blood urea nitrogen. Consistent with the result from H&E staining assay, both UA and DEX had obviously protect effect on AKI (Fig. 1C and D). Macrophages are one of the innate leukocytes accumulated in the kidney and promote inflammation in acute phase of AKI [20]. F4/80-positive macrophages were observed in kidney tissue in AKI group while both UA and DEX decreased the F4/80-positive macrophages infiltration (Fig. 1H). Consistent with this result, LPS-induced inflammatory cytokines TNF-α, IL-6 and IL-1β were also obviously reduced by UA and DEX pretreatment (Fig. 1E, F and G). Furthermore,

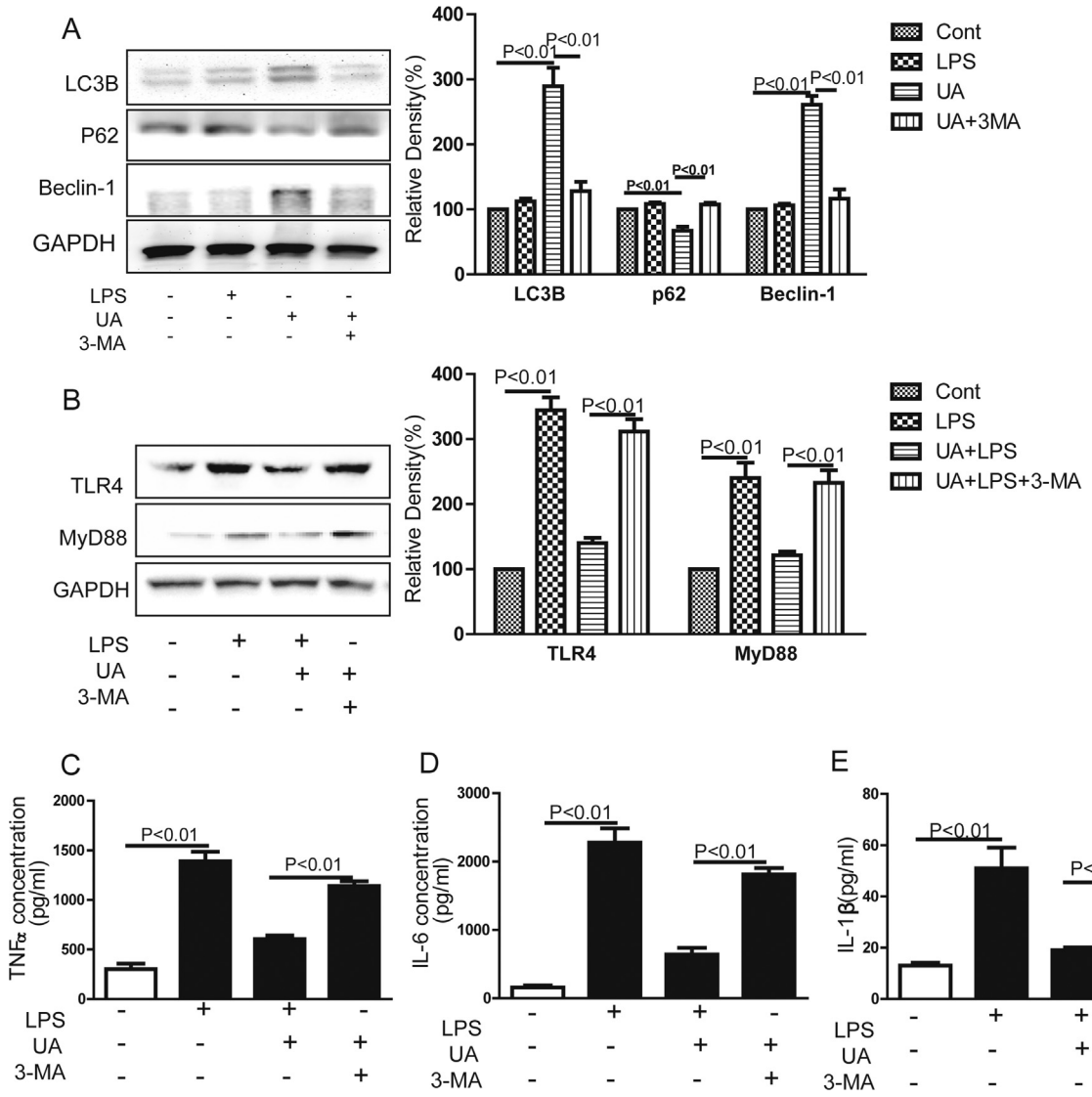

Fig. 5. UA inhibited inflammation by enhancing macrophage autophagy. RAW264.7 cells were pretreated with UA (10^{-5} M) or 3-MA (1 mM) for 1 h before being stimulated with LPS (50 μg/ml) for another 24 h, LC3B, p62, and Beclin-1 protein expressions were detected by western blotting (A); TLR4 and MyD88 were detected by western blotting (B); Cytokines TNF-α (C), IL-6 (D), and IL-1β (E) were determined using ELISA kit. UA, ursolic acid.

primary macrophages were isolated from experimental mice including control mice, AKI mice, UA-treated mice and DEX-treated mice, autophagy characteristic proteins LC3B, p62, Beclin-1 and inflammatory proteins TLR4, MyD88 were detected. As shown in Fig. 2A and B, UA modulated autophagy pathway and suppressed TLR4, MyD88 expression in macrophages during AKI. In addition, in a xylene-induced ear oedema mouse model, a significant inhibitory effect of UA on ear weight was observed (Fig. 2C). Meanwhile, ear section H&E staining data showed that UA significantly suppressed the xylene-induced ear oedema (Fig. 2D).

3.2. UA suppresses LPS-induced TNF-α, IL-6 and IL-1β expression in vitro

According to the results in vivo, related mechanisms were subsequently explored *in vitro*. Firstly, cytotoxic effect of UA on RAW264.7 was tested by MTT assay. As shown in Fig. 3A, UA had no cytotoxicity to cells until its dose was up to 1×10^{-4} M. To minimize the cytotoxicity of UA on cell viability, 1×10^{-5} M was chosen optional dose for the following study. When cells were stimulated by LPS, pro-inflammatory cytokines TNF-α, IL-6, and IL-1β were significantly increased while pretreatment of UA before cells were stimulated by LPS obviously decreased cytokines releases (Fig. 3B–D).

3.3. UA inhibits LPS-activated cytokines releases by TLR4/MyD88 pathway in vitro

Classically speaking, TLR4 stimulated by LPS induces the releases of critical proinflammatory cytokines. There are two pathways located the downstream of TLR4 during the pro-inflammatory process: MyD88-dependent and -independent pathways [21]. The data based on Fig. 4A showed that in RAW264.7 cell line, LPS activated TLR4/MyD88 pathway while UA obviously blocked this pathway. Furthermore, silence of TLR4 and MyD88 (Fig. 4B–C) significantly decreased inflammatory factors releases (Fig. 4D–F). These findings indicated that UA exerted its anti-inflammatory effect mainly through TLR4/MyD88 pathway.

3.4. UA-induced autophagy regulates inflammatory cytokine release

A renoprotective role of autophagy in AKI has been demonstrated by genetic inhibition and pharmacological studies [22]. Autophagy can interact with many vital processes such as inflammation, programmed

cell death, and adaptive immune [23]. As shown in Fig. 5A, no significant difference could be observed between LPS exposure group and control group in the expression ratio of LC3II/I and levels of p62 as well as Beclin-1 while obvious changes could be observed in UA-treated group. Meanwhile, these changed could be observed remarkably reversed in 3-MA co-treatment group indicating the occurance of autophagy. In the following study, autophagy inhibitor 3-MA obviously decreased TLR4 and MyD88 expressions (Fig. 5B) hinting that autophagy induced by UA regulated TLR4/MyD88 pathway. Meanwhile 3-MA reversed inflammation factors TNF-α, IL-6 and IL-1β releases (Fig. 5C–E) implied its regulation on cytokines mediated by blocking TLR4/MyD88 pathway.

4. Discussion

LPS-induced mouse inflammation is a well model of AKI. In this model, LPS significantly induces the release of inflammatory cytokines which promote kidney disease. Increased inflammatory factors are observed in cardiovascular diseases [24], immune-mediated diseases [25], kidney diseases [26], and neurodegenerative diseases [27]. In our study, UA could obviously ameliorate LPS-induced AKI (Fig. 1A–D). Besides, UA significantly decreased the levels of inflammatory mediators TNF-α, IL-6 and IL-1β in serum as well as reduced macrophage infiltration in kidney tissue in LPS-induced AKI model (Fig. 1E–H). To a certain extent, UA has a better effect than DEX, a clinical drug for inflammation treatment. Above data indicated that UA could attenuate acute inflammation by inhibiting inflammatory infiltration. Although the protective effect of UA on the AKI model is corroborated, detailed mechanisms research remains to be explored in the future.

TLR4 is one TLR family members to be identified in 1997 [28]. It is considered as the gene encoding LPS receptor [29]. TLR4/MyD88 is responsible for the physiological recognition of LPS in many different cells that express both proteins, such as lymphoid cells, macrophages, and endothelial, epithelial, as well as vascular smooth-muscle cells [30]. In our study, TLR4/MyD88 pathway was activated by LPS and blocked by UA in both primary macrophages from experimental mice and RAW264.7 cell lines. Previous studies have indicated that blocking LPS-induced TLR4 pathway is an alternative strategy for discovering potential anti-inflammatory drugs. To further explore the significance of TLR4/MyD88 in regulating inflammatory factors, RNAs for both TLR4 and MyD88 were silenced. As expected, silence of TLR4 and MyD88 obviously decreased the releases of TNF-α, IL-6 and IL-1β hinting regulation effect of TLR4/MyD88 on inflammatory factors (Fig. 4).

Autophagy maintains cellular homeostasis and functions as a cytoprotective response to a variety of general stress-response pathways [31]. Macrophage autophagy has been shown protective against inflammation [32]. There are many vital molecules involved in autophagy such as Beclin-1 (involved in vesicle nucleation) [33], LC3B (involved in autophagosome membrane expanision and autophagosome-lysosome fusion) [34] and p62 (an autophagosome cargo protein that targets other proteins that bind to it for selective autophagy). The effect of UA on macrophage autophagy was subsequently studied. UA induced both primary macrophages from experimental mice and RAW264.7 cell lines autophagy occurrence by increasing LC3B and Beclin-1 expression as decreasing p62 expression (Figs. 2B and 5A). Furthermore, the changes in cell lines were obviously reversed by autophagy inhibitor 3-MA. More important, autophagy inhibitor 3-MA blocked TLR4/MyD88 pathway as well as reduced inflammatory factors releases (Fig. 5) hinting that autophagy induced by UA suppressed inflammation responses in macrophages by regulating TLR4/MyD88 pathway.

In conclusion, our study suggest that UA enhanced macrophage autophagy to regulate TLR4/MyD88 pathway and thus reduces inflammatory process. Out study provides evidence that UA might be an optimal anti-inflammation therapy by virtue of its macrophage autophagy-enhancing activity.

Author contributions

J.Z, HY.Z and FB.J designed and performed the experiments; XH.Q and ZG.S wrote the paper; GW.L and WW.Z contributed to paper writing.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Acknowledgement

This study was supported by the National Science Foundation for Distinguished Young Scholars of China (Grant No. 81802473); The Natural Science Foundation of China (81803766); The Natural Science Foundation of Shandong Province (ZR201709240212).

References

[1] Y. Kondo, T. Kanzawa, R. Sawaya, S. Kondo, The role of autophagy in cancer development and response to therapy, Nat. Rev. Cancer 5 (9) (2005) 726–734.
[2] Y. Tanaka, G. Guhde, A. Suter, E.L. Eskelinen, D. Hartmann, R. Lullmann-Rauch, P.M. Janssen, J. Blanz, K. von Figura, P. Saftig, Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice, Nature 406 (6798) (2000) 902–906.
[3] J. Yuan, M. Lipinski, A. Degterev, Diversity in the mechanisms of neuronal cell death, Neuron 40 (2) (2003) 401–413.
[4] M.J. Livingston, Z. Dong, Autophagy in acute kidney injury, Semin. Nephrol. 34 (1) (2014) 17–26.
[5] B.Z. Shao, B.Z. Han, Y.X. Zeng, D.F. Su, C. Liu, The roles of macrophage autophagy in atherosclerosis, Acta Pharmacol. Sin. 37 (2) (2016) 150–156.
[6] J. Lodder, T. Denaes, M.N. Chobert, J. Wan, J. El-Benna, J.M. Pawlotsky, S. Lotersztajn, F. Teixeira-Clerc, Macrophage autophagy protects against liver fibrosis in mice, Autophagy 11 (8) (2015) 1280–1292.
[7] B. Zhang, G. Ramesh, S. Uematsu, S. Akira, W.B. Reeves, TLR4 signaling mediates inflammation and tissue injury in nephrotoxicity, J. Am. Soc. Nephrol. 19 (5) (2008) 923–932.
[8] T.H. Mogensen, Pathogen recognition and inflammatory signaling in innate immune defenses, Clin. Microbiol. Rev. 22 (2) (2009) 240–273. Table of Contents.
[9] O. Adachi, T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, K. Nakanishi, S. Akira, Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function, Immunity 9 (1) (1998) 143–150.
[10] T. Kawai, O. Adachi, T. Ogawa, K. Takeda, S. Akira, Unresponsiveness of MyD88-deficient mice to endotoxin, Immunity 11 (1) (1999) 115–122.
[11] R. Checker, S.K. Sandur, D. Sharma, R.S. Patwardhan, S. Jayakumar, V. Kohli, G. Sethi, B.B. Aggarwal, K.B. Sainis, Potent anti-inflammatory activity of ursolic acid, a triterpenoid antioxidant, is mediated through suppression of NF-kappaB, AP-1 and NF-AT, PLoS One 7 (2) (2012) e31318.
[12] P.G. Do Nascimento, T.L. Lemos, A.M. Bizerra, A.M. Arriaga, D.A. Ferreira, G.M. Santiago, R. Braz-Filho, J.G. Costa, Antibacterial and antioxidant activities of ursolic acid and derivatives, Molecules 19 (1) (2014) 1317–1327.
[13] E. Crosas-Molist, I. Fabregat, Role of NADPH oxidases in the redox biology of liver fibrosis, Redox Biol. 6 (2015) 106–111.
[14] J. van Meerloo, G.J. Kaspers, J. Cloos, Cell sensitivity assays: the MTT assay, Methods Mol. Biol. 731 (2011) 237–245.
[15] B. Dalby, S. Cates, A. Harris, E.C. Ohki, M.L. Tilkins, P.J. Price, V.C. Ciccarone, Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications, Methods 33 (2) (2004) 95–103.
[16] Y. Ma, Y. Li, X. Li, Y. Wu, Anti-inflammatory effects of 4-methylcyclopentadecanone on edema models in mice, Int. J. Mol. Sci. 14 (12) (2013) 23980–23992.
[17] A. Zarjou, A. Agarwal, Sepsis and acute kidney injury, J. Am. Soc. Nephrol. 22 (6) (2011) 999–1006.
[18] X. Chen, D.B. Cho, P.C. Yang, Double staining immunohistochemistry, N Am. J. Med. Sci. 2 (5) (2010) 241–245.
[19] D.P. Basile, M.D. Anderson, T.A. Sutton, Pathophysiology of acute kidney injury, Compr. Physiol. 2 (2) (2012) 1303–1353.
[20] Y.J. Day, L. Huang, H. Ye, J. Linden, M.D. Okusa, Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: role of macrophages, Am. J. Physiol. Renal. Physiol. 288 (4) (2005) F722–F731.
[21] T. Kawai, S. Akira, Toll-like receptor downstream signaling, Arthritis Res. Ther. 7 (1) (2005) 12–19.
[22] L. He, M.J. Livingston, Z. Dong, Autophagy in acute kidney injury and repair, Nephron Clin. Pract 127 (1–4) (2014) 56–60.
[23] A.J. Choi, S.W. Ryter, Autophagy in inflammatory diseases, Int. J. Cell Biol. 2011 (2011) 732798.
[24] J.T. Willerson, P.M. Ridker, Inflammation as a cardiovascular risk factor, Circulation 109 (21 Suppl 1) (2004) II2-10.
[25] T. Lazarevic-Pasti, A. Leskovac, V. Vasic, Myeloperoxidase inhibitors as potential drugs, Curr. Drug Metab. 16 (3) (2015) 168–190.
[26] B.A. Lavin-Gomez, R. Palomar-Fontanet, M. Gago-Fraile, J.A. Quintanar-Lartundo,

E. Gomez-Palomo, D. Gonzalez-Lamuno, M.T. Garcia-Unzueta, M.A. Arias-Rodriguez, J.A. Gomez-Gerique, Inflammation markers, chronic kidney disease, and renal replacement therapy, Adv. Perit. Dial. 27 (2011) 33–37.

[27] S. Amor, F. Puentes, D. Baker, P. van der Valk, Inflammation in neurodegenerative diseases, Immunology 129 (2) (2010) 154–169.

[28] R. Medzhitov, P. Preston-Hurlburt, C.A. Janeway Jr., A human homologue of the Drosophila Toll protein signals activation of adaptive immunity, Nature 388 (6640) (1997) 394–397.

[29] F. Di Lorenzo, L. Kubik, A. Oblak, N.I. Lore, C. Cigana, R. Lanzetta, M. Parrilli, M.A. Hamad, A. De Soyza, A. Silipo, R. Jerala, A. Bragonzi, M.A. Valvano, S. Martin-Santamaria, A. Molinaro, Activation of Human Toll-like Receptor 4 (TLR4). Myeloid Differentiation Factor 2 (MD-2) by Hypoacylated Lipopolysaccharide from a Clinical Isolate of Burkholderia cenocepacia, J. Biol. Chem. 290 (35) (2015) 21305–21319.

[30] Y.I. Miller, S.H. Choi, P. Wiesner, Y.S. Bae, The SYK side of TLR4: signalling mechanisms in response to LPS and minimally oxidized LDL, Br. J. Pharmacol. 167 (5) (2012) 990–999.

[31] Z. Yang, D.J. Klionsky, Mammalian autophagy: core molecular machinery and signaling regulation, Curr. Opin. Cell Biol. 22 (2) (2010) 124–131.

[32] K. Liu, E. Zhao, G. Ilyas, G. Lalazar, Y. Lin, M. Haseeb, K.E. Tanaka, M.J. Czaja, Impaired macrophage autophagy increases the immune response in obese mice by promoting proinflammatory macrophage polarization, Autophagy 11 (2) (2015) 271–284.

[33] C.W. Wang, D.J. Klionsky, The molecular mechanism of autophagy, Mol. Med. 9 (3–4) (2003) 65–76.

[34] J. Fullgrabe, D.J. Klionsky, B. Joseph, The return of the nucleus: transcriptional and epigenetic control of autophagy, Nat. Rev. Mol. Cell Biol. 15 (1) (2014) 65–74.