Blockade of macrophage autophagy ameliorates activated lymphocytes-derived DNA induced murine lupus possibly via inhibition of proinflammatory cytokine production

B. Li[1], Y. Yue[1], C. Dong[1], Y. Shi[2], S. Xiong[1]

[1]Jiangsu Provincial Key Laboratory of Infection and Immunity, Institutes of Biology and Medical Science, Soochow University, Suzhou;
[2]Department of Nephrology, The second Affiliated Hospital of Soochow University, Suzhou, Jiangsu Province, P.R. China.

Abstract

Objective

Systemic lupus erythematosus (SLE) is a typical inflammatory autoimmune disease for its unknown pathogenesis and potential fatality. It has been reported that autophagy has a crosstalk with autoimmunity, but its impact on the pathogenesis of SLE remains unclear. Here, we investigated the role of autophagy in inflammatory response of macrophages under SLE conditions.

Methods

First, we detected the expression of autophagy-related genes (Atg5, Atg12 and Beclin 1) in the macrophages derived from activated lymphocytes-derived DNA (ALD-DNA) induced murine lupus as well as in the PBMC from SLE patients. And then through adoptive transfer of Beclin 1 knockdown macrophages, we further investigated the potential effect of macrophage autophagy on the SLE-associated inflammatory response and disease severity by evaluating serum anti-dsDNA antibodies and proteinuria levels, immune complex deposition as well as renal pathological changes.

Results

We found that autophagy related genes were significantly upregulated in the splenic and renal macrophages of lupus mice and in the PBMC of SLE patients. Adoptive transfer of Beclin 1 knockdown macrophages could significantly decrease the anti-dsDNA antibodies and proteinuria levels, robustly reduce renal immune complex deposition and remit glomerulonephritis, indicating the amelioration of murine lupus. This protective effect was associated with the obviously decreased production of proinflammatory cytokines IL-6 and TNF-α.

Conclusion

Our results suggested that aberrant activated autophagy in macrophages contributed to the pathogenesis of murine lupus possibly via promoting the production of proinflammatory cytokines TNF-α and IL-6, and inhibition of autophagy might represent a novel regulation strategy for excessive activation of proinflammatory macrophages and a new therapeutic regime for SLE.

Key words
SLE, autophagy, TNF-α, IL-6

Baihui Li, MSc
Yan Yue, PhD
Chunsheng Dong, PhD
Yongbing Shi, MD
Sidong Xiong, PhD

Please address correspondence to:
Sidong Xiong, PhD,
Jiangsu Key Laboratory of Infection and Immunity,
Institutes of Biology and Medical Science,
Soochow University,
Suzhou 215123,
Jiangsu Province, P. R. China.
E-mail: sdxiongfd@126.com

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Introduction

Systemic lupus erythematosus (SLE), with the incidence of 0.1% in total population (1), is a complex chronic autoimmune disease of unknown aetiology and characterised with abundant autoantibodies and diffuse inflammation. It is generally considered as a multifactorial disease in which genetic, sex hormones, environment and other factors are involved (2-4). Although numerous studies have been focused on this enigmatic disease, its pathogenesis is not completely elucidated.

Autophagy is a fundamental catabolic procession in which cytoplasmic materials are sequestered by double-membrane autophagosome, and delivered to lysosome for degradation and recycling. Apart from its physiological role in regulating cellular homeostasis and eliminating dead cells, autophagy is also considered as a cellular response for various forms of stress (5), and its complex interactions with immune responses and inflammation have attracted considerable attention (6). Conspicuously, deregulated autophagy has been implicated in ever increasing autoimmune diseases including experimental autoimmune myocarditis (7), inflammatory bowel disease (8), and rheumatoid arthritis (9) and so on.

Currently, autophagy has been identified as a key component in the etiology of SLE (10). In addition to the indirect evidence for the role of autophagy in SLE by genetic analysis studies (11, 12), a more recent study directly explored autophagy in T cells from two distinct lupus-prone mouse models and SLE patients. It was reported that autophagic activity is deregulated in peripheral T cells from both lupus-prone mice and SLE patients, and suggested that autophagy could regulate the survival of autoreactive T cells (13). Alessandri *et al*. (14) demonstrated that no significant differences in spontaneous autophagy between T lymphocytes from SLE patients and from healthy donors apart from $CD4^+$ naive T cells, but T lymphocytes from SLE patients were resistant to autophagic induction and displayed an overexpression of genes negatively regulating autophagy. Besides, autophagy has been proven to be activated in B cells from lupus mice and SLE patients, and autophagy is required for the survival of autoreactive B cell and the development of plasma cell (15). However, hitherto the role of autophagy in macrophages, which represents the primary inflammatory cells and exerts pivotal pathogenic effects in SLE (16), has not been elucidated. Although autophagy genes has been found increased in apoptotic cell-engulfing macrophages from SLE patients (17), its role in macrophages and contribution to the development of SLE remained unclear.

In this study, we investigated the role of macrophage autophagy in the development of murine lupus generated by immunisation with homologous activated lymphocyte-derived DNA (ALD-DNA). It was found that autophagy was dramatically activated in macrophages of lupus mice. Notably, blockade of macrophage autophagy could alleviate lupus manifestation, evidenced by decreased anti-dsDNA antibodies and proteinuria as well as relived glomerulonephritis. This protective effect may be attributed to the decreased production of proinflammatory cytokines TNF-α and IL-6. Our findings indicated that macrophage autophagy contributed greatly to the pathogenesis of SLE possibly via promoting proinflammatory cytokines production, and it may represent a potential therapeutic target for SLE disease.

Materials and methods

Patients and healthy donors

This study was approved by the Ethics Committee of Soochow University. A total of 3 SLE patients and 3 healthy donors were recruited, and 5 ml peripheral blood was obtained from each person. All of the peripheral blood samples were collected after obtaining written informed consents. The diagnosis of SLE was established according to the 1982 revised American College of Rheumatology criteria. All SLE patients with Lupus nephritis (LN) fulfilled the American College of Rheumatology revised classification criteria for SLE. Patients who had other autoimmune diseases were excluded. All patients were positive for plasma anti-

dsDNA antibodies. The healthy donors were matched with the patients for age, sex, and race. There are no symptoms or signs of renal disease in healthy donors according to available information. All samples were obtained from the Second Affiliated Hospital of Soochow University (Suzhou, China).

Mice

Sixty-nine female BALB/c mice (six to eight weeks old) were purchased from the SLRC Company (Shanghai, P. R. China) and maintained in pathogen-free housing condition. All animal procedures were performed according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals (Ministry of Health, People's Republic of China, 1998). The protocol was approved by the Ethics Committee of Soochow University.

ALD-DNA preparation

For generation of ALD-DNA, splenocytes were seeded at 2×10^6 cells/ml in 75 cm^2 cell culture flask and cultured in the presence of ConA (5mg/ml) for 6 days to induce apoptosis. The apoptotic cells were stained with FITC-labeled Annexin V (BD Biosciences, Franklin Lakes, NJ, USA) and propidium iodide (Sigma-Aldrich, St. Louis, MO, USA), and sorted using a FACS Aria (BD Biosciences, Franklin Lakes, NJ, USA). Genomic DNA from apoptotic splenocytes were treated with S1 nuclease (Takara Bio, Shiga, Japan) and proteinase K (Sigma-Aldrich, St. Louis, MO, USA), then purified using the DNeasy Blood & Tissue Kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Unactivated lymphocytes-derived DNA (UnALD-DNA) was prepared from the inactivated (resting) splenocytes and extracted using the same methods. To exclude contaminations with LPS, sterile endotoxin-free plastic ware and reagents were used for DNA preparation. DNA concentration was determined by absorbance (A) measurement at 260 nm. The final A260/A280 for all the DNA preparation was >1.8. Less than 0.01 U/μg endotoxin was present in any of the DNA samples based on a Limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD, USA). The apoptotic DNA ladder of ALD-DNA was confirmed by agarose gel electrophoresis.

Generation of murine lupus model

Female 6- to 8-week-old BALB/c mice were immunised s.c. with ALD-DNA (50 μg/mouse) plus CFA (Sigma-Aldrich, St. Louis, MO, USA) on day 1, followed by s.c. injection of ALD-DNA (50 μg/mouse) emulsified with IFA (Sigma-Aldrich, St. Louis, MO, USA) on days 14 and 28 for total of three times. Mice were bled from retro-orbital sinus before immunisation and at 2 weeks intervals until 3 months after the initial immunisation. 10 weeks later, mice were sacrificed, and spleens and kidneys were collected for further analysis.

Cell culture and transfection of GFP-LC3 plasmid

RAW264.7 cells were cultured in DMEM (GIBCO, Gaithesburger, MD, USA) supplemented with 10% FBS (Hyclone, Thermo Fisher Scientific, Waltham, MA, USA), penicillin (100 units/ml) and streptomycin (100 μg/ml) in a 5% CO_2 incubator at 37°C. RAW264.7 macrophages were transiently transfected with GFP-LC3 plasmid, which was kindly provided by Dr. Guanghui Wang (Soochow University School of Pharmaceutical Science), using Lipofectamine LTX with PLUS Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Twenty-four hours after transfection, cells were stimulated with ALD-DNA for the detection of redistribution of GFP-LC3.

Reagents and pharmacological inhibitor treatment

Autophagy inhibitors 3-methyladenine (3-MA) and bafilomycin A1 (BafA) were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). RAW264.7 and peritoneal macrophages were stimulated with ALD-DNA (50 μg/ml), UnALD-DNA (50 μg/ml) or PBS with 3-MA (10mM) or BafA (100nM) for 12 hours and then cell supernatant was collected and subjected to ELISA assays for proinflammatory cytokines detection.

Real-time PCR analysis

Total RNA of PBMC from lupus mice or SLE patients was extracted with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was synthesised with PrimeScript® RT reagent Kit (Takara Bio, Shiga, Japan). The levels of mRNA encoding ATG5, ATG12, Beclin-1 and LC3B was quantified by real-time PCR using SYBR Green system (Takara Bio, Shiga, Japan) following the manufacturer's protocol. The primers were designed spanning exons to avoid genomic DNA amplification, primer sequences were described in Table I. All gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method. The Ct value of each sample was normalised to that of house-keeping genes (murine hgprt or human gapdh).

Table I. Primer sequences used in real-time PCR.

Gene	Forward	Reverse
mouse hgprt	5'-GTTGGATACAGGCCAGACTTTGTTG-3'	5'-GAGGGTAGGCTGGCCTATAGGCT-3'
mouse atg5	5'-GACAAAGATGTGCTTCGAGATGTG-3'	5'-GTAGCTCAGATGCTCGCTCAG-3'
mouse atg12	5'-GGCCTCGGAACAGTTGTTTA-3'	5'-CAGCACCGAAATGTCTCTGA-3'
mouse beclin 1	5'-GGCCAATAAGATGGGTCTGA-3'	5'-CACTGCCTCCAGTGTCTTCA-3'
human gapdh	5'-ATCCCATCACCATCTTCCAG-3'	5'-GAGTCCTTCCACGATACCAA-3'
human atg5	5'-GGGAAGCAGAACCATACTATTTG-3'	5'-AAATGTACTGTGATGTTCCAAGG-3'
human beclin 1	5'-AGATACCGACTTGTTCCTTACG-3'	5'-GCCTTTCTCCACATCCATCC-3'
human lc3b	5'-CGGTGATAATAGAACGATACAAGG-3'	5'-CTGAGATTGGTGTGGAGACG-3'

Western Blot

Macrophages were washed and harvested in cold PBS, and centrifuged at 5,000 rpm for 5 min. Cells were lysed in RIPA buffer and the lysate was centrifugation at 11,000 rpm for 10 min at 4°C and protein concentration was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The proteins were separated by SDS-PAGE and then transferred to PVDF membrane (Millipore, Billerica, MA, USA). The antibody dilutions were as follows: anti-Beclin1 (1:1000), anti-LC3 (1:1000) and anti-ATG5 (1:1000) (all from Cell Signaling Technology, Danvers, MA, USA); anti-GAPDH (1:10000, Dako, Glostrup, Denmark). Immunoreactivity was either detected with an ECL kit (Amersham Biosciences, Arlington Heights, IL, USA) and expose to film (Kodak, Rochester, NY, USA), or incubated the membrane with fluorescent secondary antibody (IRDye, LI-COR, Lincoln, NE, USA) and scanned with Odyssey® Western Blot Analysis system (LI-COR, Lincoln, NE, USA). The signal intensity of primary antibody binding was quantitatively analysed with Sigma Scan Pro 5 and was normalised to GAPDH.

Plasmid construction and lentivirus production

To construct Beclin 1-shRNA, shRNA oligos were cloned into a pLL3.7 lentiviral vector, the following sequences were used in this study: sense strand, 5'-TAAGAUCCUGGACCGGGUCACCTTCAAGAGAGGTGACCCGGTCCAGGATCTTATTTTTTC-3'; anti-sense strand, 5'-TCGAGAAAAAATAAGAUCCUGGAC CGGGUCACCTCTCTTGAAGGTGACCCGGTCCAGGATCTTA-3'. The pLL3.7-Beclin 1-shRNA and the Lenti-pLL3.7 packaging system were transfected into to 293T cells to produce lentivirus. Meanwhile, the empty pLL3.7 lentiviral vector was used as a negative control. After 72 hours transfection, lentiviral supernatant was collected and centrifuged at 25.000 rpm for 120 min, and then resuspended in PBS followed by the lentivirus titration. For Beclin 1 knockdown, macrophages were infected by lentivirus for 36 hours with 10 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO, USA).

Adoptive transfer of macrophages

Endogenous macrophages were deleted using multilamellar liposomes containing Dichloromethylenediphosphonic acid disodium salt (DMDP) (Sigma-Aldrich, St. Louis, MO, USA) as previously described (18) before adoptive transfer of Beclin 1 knockdown microphages. The depletion efficiency of endogenous macrophages achieved >90% as assessed by flow cytometry for $F4/80^+$ cells. Then, macrophages were infected with lentivirus Beclin1-shRNA or vector for 18 h respectively, and then collected and adoptively transferred i.v. to ALD-DNA, UnALD-DNA or PBS immunised mice (2×10^6 cells/mouse), one time each week for a total of 4 times.

Pathological analysis

For histological analysis, tissues from mice kidneys were prepared and stained with haematoxylin and eosin using standard procedures. Images were acquired with Nikon SCLIPSS TE2000-S microscope (Nikon, Melville, NY, USA) equipped with ACT-1 software (Nikon; original magnification × 200). The glomerulonephritis score was determined by using the ISN/RPS2003 classification.

Anti-dsDNA antibodies and proteinuria examination

Serum samples were collected periodically and determined by ELISA for the presence of anti-dsDNA antibodies using ELISA assay. Proteinuria was measured with a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

ELISA assay

Levels of IL-6 and TNF-α were detected by ELISA assays with cytokines ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. A standard curve was generated using known amounts of the respective purified recombinant mouse cytokines.

Statistical analysis

All data are expressed as means±SD of three independent experiments or from a representative experiment of three independent experiments. Two group comparisons were performed using unpaired Student's *t*-test. Multiple group comparisons were performed using one-way ANOVA followed by Dunnett's *t*-test. Pearson correlations were computed to determine relationships between proinflammatory cytokines production and autophagy related genes. The significance of the differences in the experimental data was described in the legends. The statistical significance level was set as $*p<0.05$; $**p<0.01$; $***p<0.001$.

Results

Autophagy was significantly activated in the macrophages of lupus mice and in the PBMC of SLE patients

A growing number of evidence supported the important role of autophagy in the pathogenesis of SLE (10, 19). To determine whether autophagy activity was altered in lupus mice, mRNA expression of autophagy-related gene proteins (Atg5, Atg12 and Beclin 1) was detected in the PBMC of ALD-DNA induced lupus mice. As shown in Figure 1A, significant increases in Atg5, Atg12 and Beclin 1 were evidenced in lupus mice compared with normal mice. The increased autophagic genes was also observed in the PBMC of SLE patients (Fig. 1B), indicating the potential role of autophagy in immune cells under SLE conditions. Considering the pivotal pathological role of macrophages in ALD-DNA induced lupus mice (20, 21), autophagy activation in splenic and renal macrophages was specially evaluated. As shown in Figures 1C and 1D, a 3.5-fold and a 1.7-fold increases in the level of LC3-II were observed respectively in the splenic and renal macrophages from lupus mice compared with those in control mice. Consistent with data in vivo, experiment in vitro also showed that ALD-DNA stimulation could robustly activate autophagy in murine macrophages cell line RAW264.7, reflected by the increased LC3-II, Beclin 1, ATG5 expressions (Fig. 1E) as well as the redistribution (from diffuse to punctuated states) of GFP-LC3 in RAW264.7 cells (Fig. 1F). These results indicated that ALD-DNA stimulation could directly induce autophagy in macrophages.

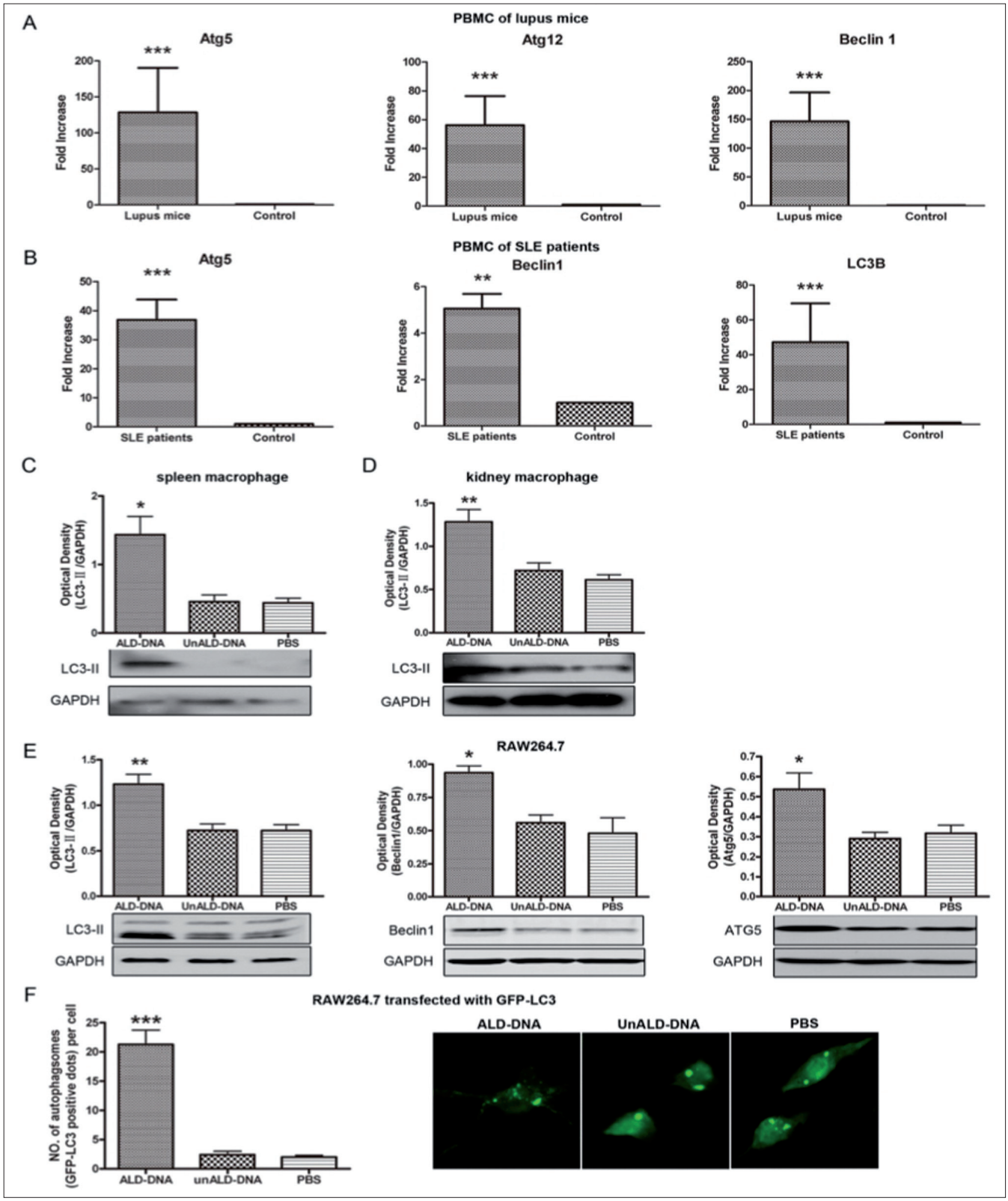

Fig. 1. Autophagy was involved in the pathogenesis of SLE. **A**: Mice were immunised with ALD-DNA, UnALD-DNA or PBS 3 times at 2-week intervals. At week 10, mRNA expression of Atg5, Atg12 and Beclin 1 in PBMC was assessed by real-time PCR. Data are means±SD from 6 mice in each group. **B**: mRNA levels of Beclin 1, Atg5 and LC3B in PBMC of SLE patients was assessed by real-time PCR. Meanwhile, protein expression of LC3 in splenic (**C**) and renal macrophages (**D**) was assessed by Western Blot. Data are representative of results obtained in three independent experiments. **E**: RAW264.7 cells were treated with ALD-DNA, UnALD-DNA or PBS for 12 hours, and then protein levels of LC3, Beclin 1, and Atg5 were assessed by Western Blot. Data are representative of results obtained in three independent experiments. **F**: RAW264.7. Cells were transfected with GFP-LC3 plasmid for 24 hours, and then treated with ALD-DNA, UnALD-DNA or PBS for 12 hours, the expression and location of GPF-LC3 were evidenced by confocal assays (original magnification×600). Data are means±SD of three patients.*p<0.05; **p<0.01; ***p<0.001.

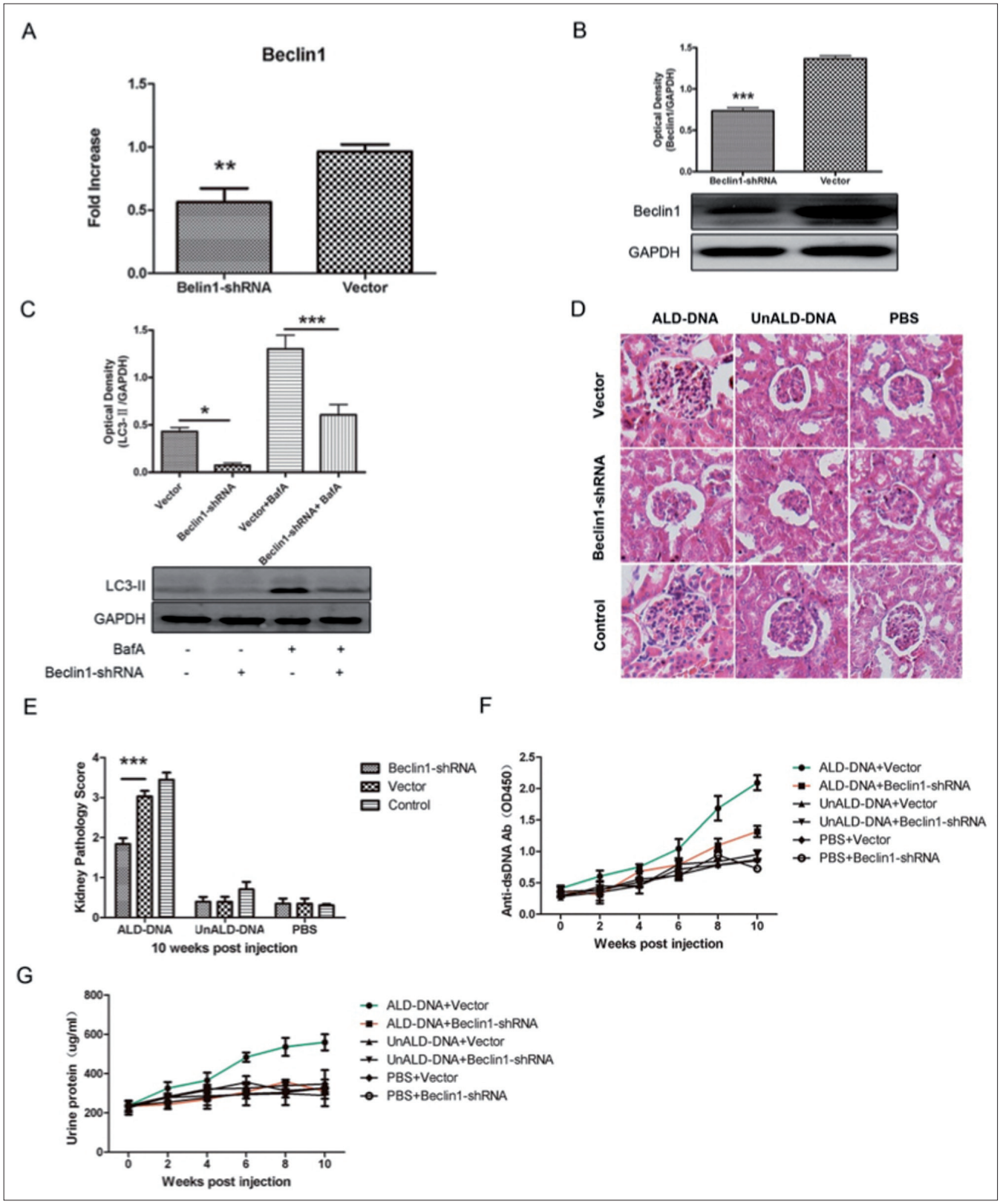

Fig. 2. Knockdown of Beclin 1 in macrophages alleviated ALD-DNA induced murine lupus. Mice were adoptively transferred with Beclin 1-knockdown macrophages or control macrophages following endogenous macrophages depletion with DMDP, and then immunised with ALD-DNA, UnALD-DNA, or PBS 3 times at 2-week intervals. The knockdown efficiency of Beclin 1-shRNA in macrophages was determined by (**A**) real-time PCR and (**B**) Western Blot. The expression of LC3 in Beclin 1 knockdown macrophages were detected by (**C**) Western Blot. Renal pathological changes in murine lupus were monitored by renal pathological observation (**D**) magnification ×200) and evaluated by (**E**) kidney pathology score. (**F**) The level of serum anti-dsDNA antibodies and **G.** proteinuria were monitored every 2 weeks until week 10 following the initial immunisation. Each group contained 5 mice. Experiments were repeated with 3 times with similar results. $^{**}p<0.01$; $^{***}p<0.001$.

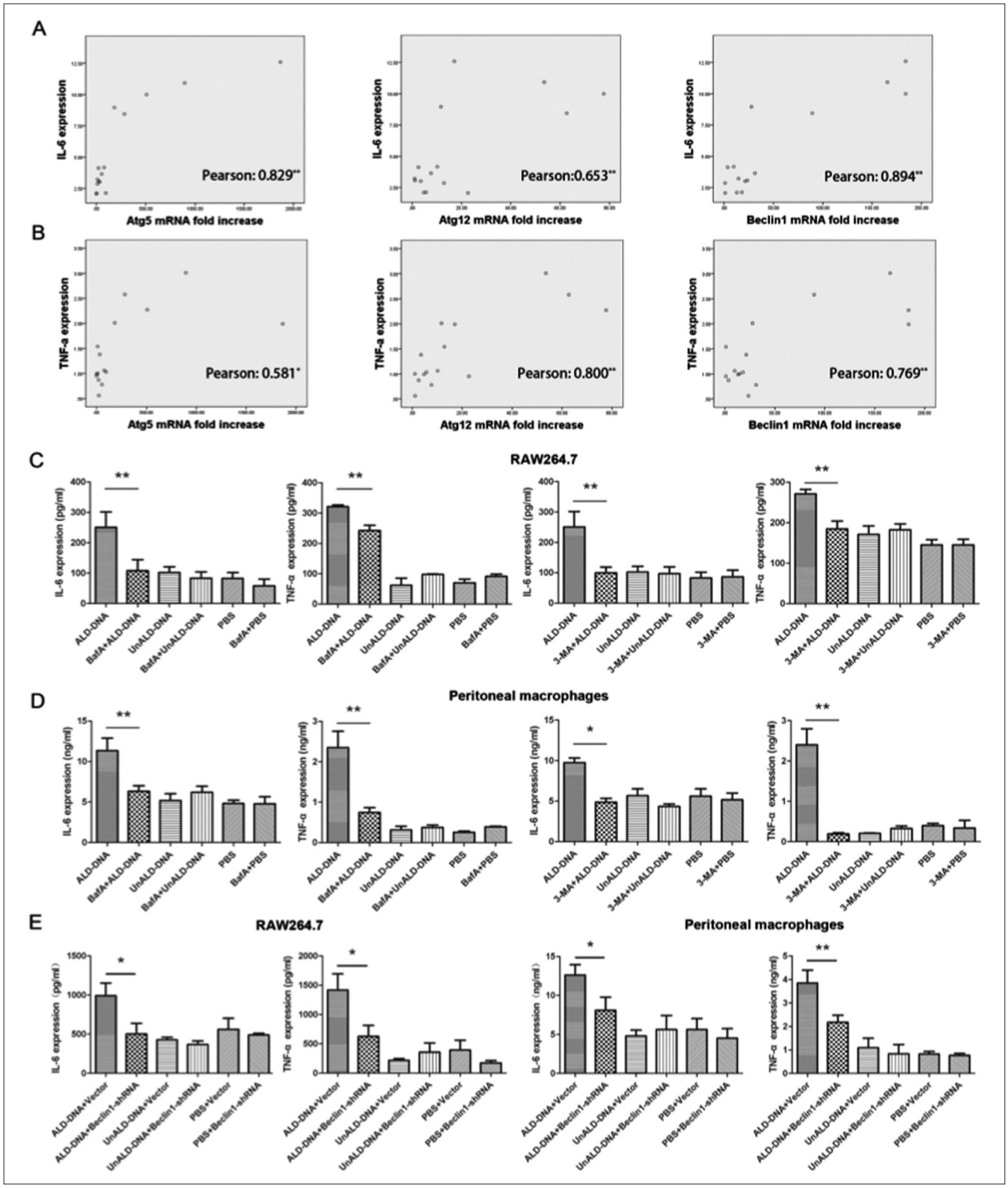

Fig. 3. Autophagy promoted the proinflammatory cytokines production in ALD-DNA stimulated macrophages. **A-B**: The correlation of serum proinflammatory cytokines levels with autophagy related genes in PBMC of lupus mice was tested by Pearson correlations. Murine serum IL-6 and TNF-α (n=15) were analysed by ELISA assay, mRNA expression of Atg5, Atg12 and Beclin 1 in PBMC was assessed by real-time PCR. Pearson correlations were computed to determine relationships between proinflammatory cytokines production and autophagy related genes. **C**: RAW264.7 cells were stimulated with ALD-DNA, UnALD-DNA or PBS for 12 hours with early (3-MA) or late stage (BafA) inhibitor, and then expression of IL-6 and TNF-α in the cell supernatant was detected by ELISA. **D**: Peritoneal macrophages were stimulated with ALD-DNA, UnALD-DNA or PBS for 12 hours with 3-MA or BafA, and then levels of IL-6 and TNF-α in the cell supernatant were detected by ELISA. E. RAW264.7 and peritoneal macrophages were infected with lentivirus expressing Beclin 1-shRNA or vector, expression of IL-6 and TNF-α in the cell supernatant was detected by ELISA. Experiments were repeated with 3 times with similar results. $^{*}p<0.05$; $^{**}p<0.01$.

Inhibition of macrophage autophagy significantly alleviated ALD-DNA induced murine lupus

To investigate the role of macrophage autophagy in the pathogenesis of murine lupus, Beclin 1 knockdown macrophages were generated using a lentiviral shRNA (Fig. 2A-B) and autophagy inhibition was evidenced by the decreased LC3 (Fig. 2C). These macrophages were adoptively transferred to lupus mice by tail intravenous injection following depletion of endogenous macrophages with DMDP. It was found that pathological severity was significantly alleviated after the mice receiving Beclin 1 knockdown macrophages, as evidenced by the remitted glomerulonephritis (Fig. 2D) and the reduced kidney score (Fig. 2E). The levels of anti-dsDNA antibodies (Fig. 2F) and proteinuria (Fig. 2G) were also obviously decreased. These data indicate that downregulation of autophagic activity in macrophage could alleviate the symptoms of ALD-DNA-induced murine lupus and suggest the important pathogenic role of macrophage autophagy in the pathogenesis of murine lupus.

Autophagy promoted the proinflammatory cytokines production in ALD-DNA stimulated macrophages

Excessive proinflammatory response is considered as the primary contribution to SLE pathogenesis (22). Through correlation analysis, we found that the mRNA expression of autophagic gene proteins (Atg5, Atg12, Beclin 1) in PBMC of lupus mice was closely and positively associated with the serum IL-6 and TNF-α levels (Figs. 3A-B), suggesting the impact of autophagy on the proinflammatory response of immune cells. To further decipher whether autophagy could affect the proinflammatory cytokines production by macrophages, RAW264.7 cells were treated with BafA or 3-MA to inhibit autophagy during stimulation with ALD-DNA, UnALD-DNA or PBS. Trypan blue staining showed that treatment of autophagic inhibitors did not significantly affect the cell viability. It was found that autophagy inhibition could robustly decrease the production of TNF-α and IL-6 (Fig. 3C). Consistently, the similar phenomenon was also evidenced in ALD-DNA stimulated peritoneal macrophages (Fig. 3D). No significant change of cytokines was observed in UnALD-DNA or PBS stimulated macrophages after BafA or 3-MA treatment compared with the control counterparts. In addition, we detected the production of TNF-α and IL-6 inBeclin 1 knockdown macrophages, and no significantly cell viability change was observed after Beclin 1 knockdown. It was found that these proinflammatory cytokines were significantly reduced in both Beclin 1 knockdown RAW264.7 cells and Beclin 1 knockdown peritoneal macrophages (Fig. 3E). These data indicated that autophagy could significantly facilitate proinflammatory cytokines production by ALD-DNA stimulated macrophages.

Inhibition of macrophage autophagy could robustly reduce the production of proinflammatory cytokines in lupus mice

To validate the proinflammatory effect of macrophage autophagy in vivo, mice were adaptively transferred with Beclin 1 knockdown macrophages before immunised with ALD-DNA, UnALD-DNA or PBS, and then the levels of serum TNF-α and IL-6 were detected. As shown in Figure 4, compared with control groups, adoptive transfer of Beclin 1 knockdown macrophages significantly blocked the upregulation of TNF-α and IL-6 since week 4 following the initial immunisation and the low levels of these cytokines maintained until week 10, at which time the levels of IL-6 and TNF-α were only 3.4 ng/ml and 1.4 ng/ml, respectively, significantly less than those (9.7 ng/ml and 2 ng/ml) in the group receiving vector transfected macrophages. These data suggested that blockade of macrophage autophagy ameliorated lupus manifestation possibly by inhibiting proinflammatory cytokines production.

Discussion

In this study, we evaluated the role of macrophage autophagy in the pathogenesis of ALD-DNA induced murine lupus. We found that autophagy was abnormally activated in the splenic and renal macrophages and contributed significantly to the production of proinflammatory cytokines IL-6 and TNF-α. Consequently, inhibition of macrophage autophagy could robustly decrease the anti-dsDNA antibodies level, remit the glomerulonephritis and reduce the proteinuria, which may be attributed to the significantly reduced IL-6 and TNF-α production.

Except for the contribution to the physiological recycling of protein and disposal of superfluous or damaged, autophagy has been proven essential for the survival, homeostasis and function of immune cells, and plays a pivotal role in shaping and regulating innate and adaptive immune responses. Actually, aberrant autophagy activation has been evidenced in a growing number of autoimmune diseases (19), among which SLE is perhaps a recently noticed one (23). It has been reported that peripheral T lymphocytes of SLE patients and lupus-prone mouse exhibit increased autophagy vacuoles compared with those in control groups (13). In consistent with these results, here we observed the increased autophagy associated genes in the PBMC from lupus mice and SLE patients, which may be ascribed to the increased autophagy in the lymphocytes of SLE patient, or to the different composition of cell populations between control and SLE patients PBMC (24). Since the mRNA levels of autophagy related genes do not always coincident with autophagic activity, the increase of autophagy associated genes might be insufficient to evaluate autophagy. Moreover, Atg5, Atg12 and Beclin 1 could also participate in other physiological and pathological process such as apoptosis (25), experiments like electron microscopy are needed to further confirm the activation of autophagy in the SLE patient PBMC.

Autophagy might influence the pathogenesis of SLE in different aspects. Atg5 mutation is linked to SLE susceptibility and contributes to SLE pathogenesis by leading to high IL-10 production (26), and deletion of Atg5 contributes to renal injury in aging mice (27). In the macrophages of SLE patients, an upregulation of autophagy genes may be involved in the clearance of apoptotic

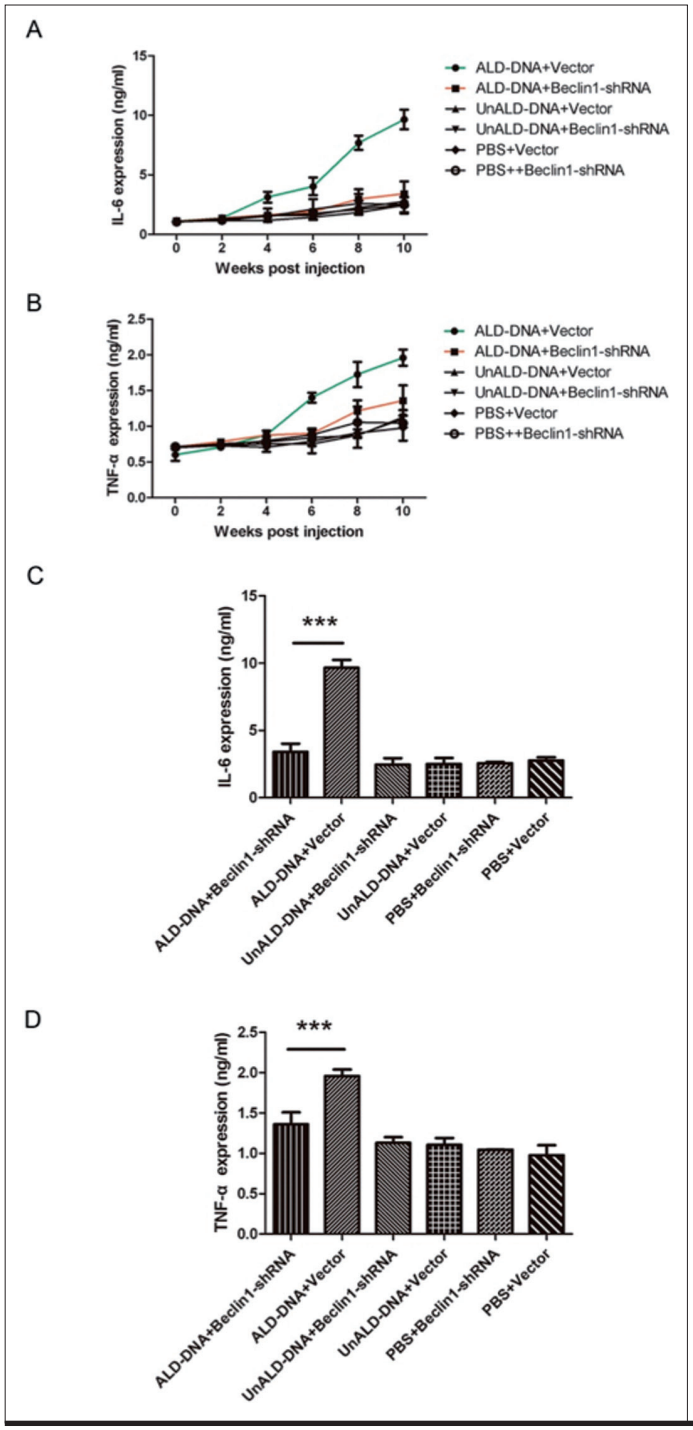

Fig. 4. Blockade of autophagy ameliorated ALD-DNA induced murine lupus by inhibiting proinflammatory cytokines production. Mice were adoptively transferred with Beclin 1-knockdown macrophages or control macrophages following endogenous macrophages depletion with DMDP, and then immunised with ALD-DNA, UnALD-DNA, or PBS 3 times at 2-week intervals.
A: The dynamic of serum IL-6 level in immunised mice was determined by ELISA assay every 2 weeks.
B: The dynamic of serum TNF-α level in immunised mice was determined by ELISA assay every 2 weeks.
C: Serum IL-6 level in mice was tested by ELISA at week 10 after initial immunisation.
D: Serum TNF-α level in mice was tested by ELISA at week 10. Each group contained 5 mice. Experiments were repeated with 3 times with similar results. ***$p<0.001$.

cells (17). Besides, autophagy could directly regulate the survival of autoreactive T cells and B cells in lupus mice and SLE patients, and may also take part in the regulation of inflammation (28) and antigen presentation (29).

Except for the autoreactive T and B cells, macrophages have been identified as the primary proinflammatory innate cells and contribute greatly to the onset and development of SLE macrophages (21, 30). However, hitherto the impact of autophagy on the proinflammatory response of lupus macrophages remained unclear, although it has been proved that phagocytic uptake dying cells through autophagy by macrophages leads to a pro-inflammatory response (31). In this study, we found that autophagy associated protein LC3 expression was significantly increased in splenic and renal macrophages of ALD-DNA induced lupus mice, indicating the upregulation of autophagy. Although previous study has demonstrated that serum factors, like autoantibodies, of SLE patients could induce cell autophagy (32), here we reported that ALD-DNA stimulation could be an important initiator for autophagy in macrophages. As shown by the in vitro assays, increased LC3-II, Beclin 1, Atg5 as well as the redistribution of LC3, which reflected the recruitment of LC3 to autophagic vesicles, have been observed in ALD-DNA stimulated RAW264.7 cells. Of course, we could not exclude the possible effect of autoantibodies on the activation of macrophage autophagy *in vivo*. Considering that LC3-II would accumulate as a consequence of increased upstream autophagosomes or decreased downstream autophagosome-lysosome degradation, so further study using the lysosomal proteases E64d and pepstatin A, which block LC3-II/autophagosome degradation, should be performed to decipher the precise reason for the increased expression and redistribution of LC3 in ALD-DNA stimulated macrophages.

Consistent with our results that autophagy related genes were upregulated in the macrophages of lupus mice, Majai *et al*. (17) have demonstrated that autophagy related genes were upregulated in apoptotic cells-engulfed macrophages of SLE patients. However, the precise mechanism of macrophage autophagy in SLE pathogenesis is unknown, as versatile impacts of autophagy on macrophages have been reported including regulating the differentiation (33), activation (34) as well as cell fate and survival (35-37).

Recent studies have shown that autophagy regulates proinflammatory cytokines production. Autophagy induction drives the degradation of pro-IL-1β and inhibits the secretion of mature IL-1β in murine macrophages (38); pDC treated with inhibitors of autophagy failed to produce IFN-α in response to HIV-1 (39); the treatment of SIRT1 inhibitor increased IL-6 and TNF-α depended on autophagy dysfunction (40). However, whether autophagy participates in the proinflammatory cytokines regulation in SLE still needs further research.

Interestingly, in our study we found that blockade of autophagy using inhibitor 3-MA or BafA as well as knockdown

of Beclin 1 prominently decreased the proinflammatory cytokines TNF-α and IL-6 production. Consistent with our results, Zhang *et al*. (33) reported that blockade of autophagy using 3-MA or chloroquine (CQ) robustly decreased the level of proinflammatory cytokines TNF-α and IL-12. However, it has been reported that sorafenib, a cancer-targeted therapeutic agent, induced autophagy in human macrophages and reduced the secretion of IL-10, but not IL-6, TNF-α nor TGF-β (34). This seemingly conflicting phenomenon may be attributed to the different autophagy inducers, cell lines, or other factors in the experiment systems. In this study, we did not detect the impact of autophagy in the production of IL-6, TNF-α and other proinflammatory cytokines like INF-α in PBMC from SLE patients and health donors, further study is needed to validate our notion that autophagy promoted the proinflammatory cytokines production in SLE patients.

Collectively, we evaluated the role of macrophage autophagy in the pathogenesis of murine lupus, and found that abnormally activated autophagy in macrophages contribute to the development of lupus disease by promoting the production of proinflammatory cytokines TNF-α and IL-6. And blockade of macrophage autophagy significantly alleviated the lupus severity in ALD-DNA immunised mice possibly by inhibiting proinflammatory cytokines production. This finding revealed a novel molecular mechanism of macrophage autophagy in SLE pathogenesis and suggested that macrophage autophagy might be a potential candidate target for SLE treatment.

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