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ORIGINAL ARTICLE

Alpha7 Nicotinic Acetylcholine Receptor Alleviates Inflammatory Bowel Disease Through Induction of AMPK-mTOR-p70S6K-Mediated Autophagy

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Abstract—Alpha7 nicotinic acetylcholine receptor (α7nAChR) has been reported to be protective in several kinds of disorders through inflammatory suppression. Here, we investigated the role of α7nAChR in inflammatory bowel disease (IBD) on α7nAChR deficient mice ($\alpha7nAChR^{-/-}$) and the wild-type mice ($\alpha7nAChR^{+/+}$). Three percent dextran sulfate sodium (DSS) was used for the creation of IBD mice model and lipopolysaccharides (LPS)/DSS as an inflammatory stressor in murine bone marrow-derived macrophages (BMDMs). The severity of IBD was determined and HE staining as well as enzyme-linked immunosorbent assay (ELISA) and real-time PCR were used to detect the level of inflammatory activation. Western blot was used to determine the levels of autophagy-related proteins. Transmission electron microscopy and mRFP-GFP-LC3 plasmid were applied to determine the levels of autophagy. We demonstrated that deficiency in α7nAChR produced a detrimental effect on IBD severity and inflammatory reaction in DSS-induced colitis models. Those effects were led to *via* autophagy dysfunction. α7nAChR deficiency attenuated the protective and anti-inflammatory effect of autophagy inducer in IBD mice and BMDMs challenged with LPS/DSS. The alleviative effect of activating α7nAChR was attenuated through inhibiting adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK)-mediated signaling. In conclusion, α7nAChR contributes to alleviate IBD through the induction of AMPK-mammalian target of rapamycin rabbit (mTOR)-p70 ribosomal protein S6 kinase (p70S6K)-mediated autophagy, thus providing a novel target for the treatment of IBD.

KEY WORDS: alpha7 nicotinic acetylcholine receptor; inflammatory bowel disease; autophagy; lipopolysaccharides; dextran sulfate sodium.

INTRODUCTION

Inflammatory bowel disease (IBD) is recognized as a group of intestinal disorders characterized with chronic and recurrent process [1–3]. IBD is comprised by two members, including ulcerative colitis (UC) and Crohn's disease (CD) [4, 5]. The pathogenesis of UC is derived from rectum, and expanded to the proximal colon and even the whole length of colon, while CD mainly affects the small intestine and large intestine [6, 7]. Although so far, the etiology of IBD remains unclarified due to its complication, several factors, including eating habits, genetic mutation, disturbance of intestinal microbiota homeostasis, and over-triggering of intestinal inflammatory and immune reaction, have been

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considered to contribute to the pathogenesis and progression of IBD [8–10]. Macrophages have been demonstrated to be involved in IBD *via* the triggering of inflammatory and immune reaction [11, 12]. As a result, suppressing the over-triggered inflammatory and immune reaction mediated by macrophages might provide a potential role in the treatment of IBD.

Alpha7 nicotinic acetylcholine receptor (α7nAChR) is regarded as a subtype of nAChRs, which belongs to a superfamily of cys-loop cationic ligand-gated channels [13, 14]. It has been previously demonstrated by us and other researchers that activating α7nAChR contributes to the alleviation of several kinds of inflammation- and immune-related diseases *via* the triggering to the "cholinergic anti-inflammatory pathway" [15–19]. Recent evidence has demonstrated the alleviative effect of α7nAChR in IBD [20]. However, the underlying mechanisms have not been fully illustrated.

Autophagy is defined as a self-eating catabolic pathway, functioning in degrading some long-lived proteins, misfolded proteins, and useless organelles for recycling [21, 22]. It has been previously reviewed by us that intestinal autophagy contributes to the alleviation of IBD through down-regulation of inflammatory and immune responses as well as maintenance of gut microbiota homeostasis [23, 24]. In addition, activating α7nAChR has been reported by us and other researchers to induce autophagy process in macrophages [15, 25]. Activating α7nAChR has been further shown to play an alleviative role in several kinds of disorders through the modulation of AMPK-mTOR-p70S6K signaling pathway [15, 26]. However, whether autophagy is involved in α7nAChR-mediated influence on IBD remains unclear.

Here in this study, we raised the hypothesis that activating α7nAChR-alleviated IBD *via* the induction of autophagy in macrophage. We aimed to explore the mechanism on the pathogenesis of IBD and thus provide a novel insight in the development of novel strategies in the treatment of IBD.

MATERIALS AND METHODS

Animal Care and Use

Mice deficient in α7nAChR (α7nAChR$^{-/-}$) and the wild-type mice (α7nAChR$^{+/+}$) were purchased from Jackson laboratory (Bar Harbor, MA) (B6.129P2-Cnr2^{tm1Dgen}/J, stock number: 005786). All animals were kept at 22 °C under a 12-h light/dark cycle with unlimited access to water and standard rodent diet. Animal experiments were approved and conducted in accordance with the guidelines for the Animal Care Committee of the General Hospital of PLA and guidelines for Care and Use of Laboratory Animals published by the National Institutes of Health, USA.

Induction of Colitis

Colitis was induced in α7nAChR$^{+/+}$ and α7nAChR$^{-/-}$ mice with 3% DSS (mol. wt. 36,000 to 50,000 kDa, MP Biomedicals LLC, Santa Ana, CA, USA) dissolved in drinking water given *ad libitum* for 7 days as previously described [27–29]. Rapamycin (1.25 mg/kg body weight; Selleck Chemicals, Houston, TX) was intraperitoneally given daily from days 1 to 7.

Clinical Score and Histological Analysis

Body weight, the presence of occult or gross blood per rectum, and stool consistency were determined by two investigators blinded to the treatment groups. A scoring system was used to assess diarrhea and the presence of occult or overt blood in the stool. Changes of body weight were indicated as loss of baseline body weight as a percentage. Postmortem, the colon was removed and pieces of colonic tissue were used for *ex vivo* analysis. For histology, rings of the transverse part of the colon were fixed in 4% buffered formalin and embedded in paraffin. Sections were stained with HE according to standard protocols. Histological scoring was performed in a blinded way by a pathologist. Focally increased the number of inflammatory cells in the lamina propria was scored as 1, confluence of inflammatory cells extending into the submucosa as 2 and transmural extension of the infiltrate as 3. For tissue damage, discrete lymphoepithelial lesions were scored as 1, mucosal erosions as 2, and extensive mucosal damage and/or extension through deeper structures of the bowel wall as 3. The two equally weighted subscores (cell infiltration and tissue damage) were added and the combined histological colitis severity score ranged from 0 to 6.

Cell Culture and Treatment

Bone marrow-derived macrophages (BMDMs) were obtained through the incubation of bone marrow cells as previously described [30, 31]. In brief, bone marrow was flushed out from femurs and tibias, cultured and differentiated in bone marrow growth medium comprised of Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 30% L929 cell-conditioned

media (source of macrophage-colony stimulating factor, M-CSF) and penicillin/streptomycin at 37 °C in a humidified incubator with 5% CO_2. Bone marrow growth medium was renewed every 2 days. After 7-day cultivation, fresh medium was replaced and BMDMs were primed with 10 ng/ml lipopolysaccharides (LPS, Sigma, Louis, MO, USA) for 1 h, and then were stimulated with 3% DSS (mol. wt. 36,000 to 50,000 kDa, MP Biomedicals LLC, Santa Ana, CA, USA) for 24 h with or without the administration of an autophagy inducer, rapamycin (1 μg/L; Selleckchem, Houston, TX, USA), α7nAChR agnonist, PNU-282987 (10 μM; Sigma-Aldrich, St. Louis, MO, USA) and/or adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) inhibitor, compound C (10 μM; Sigma-Aldrich) at 10 min in advance as previously described [27].

Western Blot

Murine BMDMs or left colons isolated from mice were washed with PBS for one time and lysed in lysis buffer on ice for 15 min. Protein concentration was detected by the bicinchoninic acid (BCA) method (Thermo Scientific, Pittsburgh, PA, USA). Samples were loaded in 10% or 12% Tris/Gly gels, subjected to SDS-PAGE, and transferred on NC membranes (Millipore, Billerica, MA, USA). Immunoblot was conducted using the rabbit anti-Beclin-1 monoclonal antibody (1:500; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-light chain 3 (LC3) polyclonal antibody (1:500; Novus Biologicals, Littleton, CO, USA), rabbit anti-p62 antibody (1:500; Cell Signaling Technology, Danvers, MA, USA) and mouse anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:1000; Beyotime Biotechnology, Shanghai, China). The membranes were then incubated with a Donkey anti-Rabbit or Donkey anti-Mouse secondary antibody (1:5000, LI-COR Biosciences, Lincoln, NE, USA) accordingly. Images were obtained and analyzed using the Odyssey infrared imaging system (LI-COR Bioscience).

Real-time PCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used for the extraction of total RNA from Murine BMDMs or left colons. Reverse transcription was conducted for the extracted RNA to obtain the cDNA with PrimeScript™ RT Master Mix (Takara, Otsu, Shiga, Japan). Real-time PCR was then conducted in the LightCycler quantitative PCR apparatus (Stratagene, Santa Clara, CA, USA) using the FastStart Unitversal SYBR Green Master (Roche, Konzern-Hauptsitz, Grenzacherstrasse, Switzerland). Expression value was normalized to GAPDH in the same sample and then normalized to the control. The sequences of the primer pairs are listed as follows: interleukin (IL)-1β: sense, 5′-CTCGTGCTGTCGGACCCCAT-3′ and antisense, 5′-AGTGTTCGTCTCGTGTTCGGAC-3′; IL-6: sense, 5′-TAGTCCTTCCTACCCCAATTTCC-3′ and antisense, 5′-TTGGTCCTTAGCCACTCCTTC-3′; IL-18: sense, 5′- CAGGCCTGACATCTTCTGCAA-3′ and antisense, 5′-CTCCAGCATCAGGACAAAGAAAGCCG-3′; tumor necrosis factor-α (TNF-α): sense, 5′-AAGC CTGTAGCCCACGTCGTA-3′ and antisense, 5′-GGCA CCACTAGTTGGTTGTCTTTG-3′; GAPDH: sense, 5′-GTATGACTCCACTCACGGCAAA-3′ and antisense, 5′-GGTCTCGCTCCTGGAAGATG-3′.

Enzyme-Linked Immunosorbent Assay

Blood samples were collected from mice and centrifuged at 3000 rpm (soft) for 30 min at 4 °C. The serum in the upper layer was obtained and collected. For experiments on BMDMs, cultural medium was collected after stimulation for the analysis of the levels of cytokines in the supernatant. The levels of proinflammatory cytokines including IL-1β, IL-6, IL-18, and TNF-α in serum were analysis using commercial available ELISA kits (R&D system, New York, NY, USA) according to the manufacturer's instructions.

Transmission Electron Microscopy

Murine BMDMs were cultured at 37 °C on glass coverslips overnight, followed by the treatments mentioned above. Murine BMDMs or sections of left colons were harvested and fixed overnight at 4 °C in 2.5% glutaraldehyde in 0.1 M PBS, and then post-fixed in 1% buffered osmium tetroxide for 2 h. Specimens were processed in routine procedure and examined under an electron microscope (H-700; Hitachi, Tokyo, Japan).

Autophagy Flux Assessment

Murine BMDMs were isolated and cultured on the slides. Tandem fluorescent mRFP-GFP-LC3 plasmid (HanBio, Shanghai, China) were transfected when the confluence reached to 50–70% [15]. In brief, after the culture in DMEM supplemented with 10% FBS for 24 h, cells were incubated with plasmids for 6 h and then changed back to fresh DMEM supplemented with 10%

FBS for the cultivation of another 36 h to ensure the expression of genes. After transfection, cells were treated as mentioned above. Cellular autophagosomes (G^+R^+) and autolysosomes (G^-R^+) were detected by confocal microscopy (Leica TCS SP8, Leica, Biberach, Germany). Total number of puncta (> 1 μm) per cell was counted.

Statistical Analysis

Data were presented as means ± SEM. A two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test for repeated measures was used for the analysis of the statistical significance of DAI scores and percentage of initial weight between treatments. For other analysis, a Kruskal-Wallis test followed by Dunn's post-hoc test and one-way

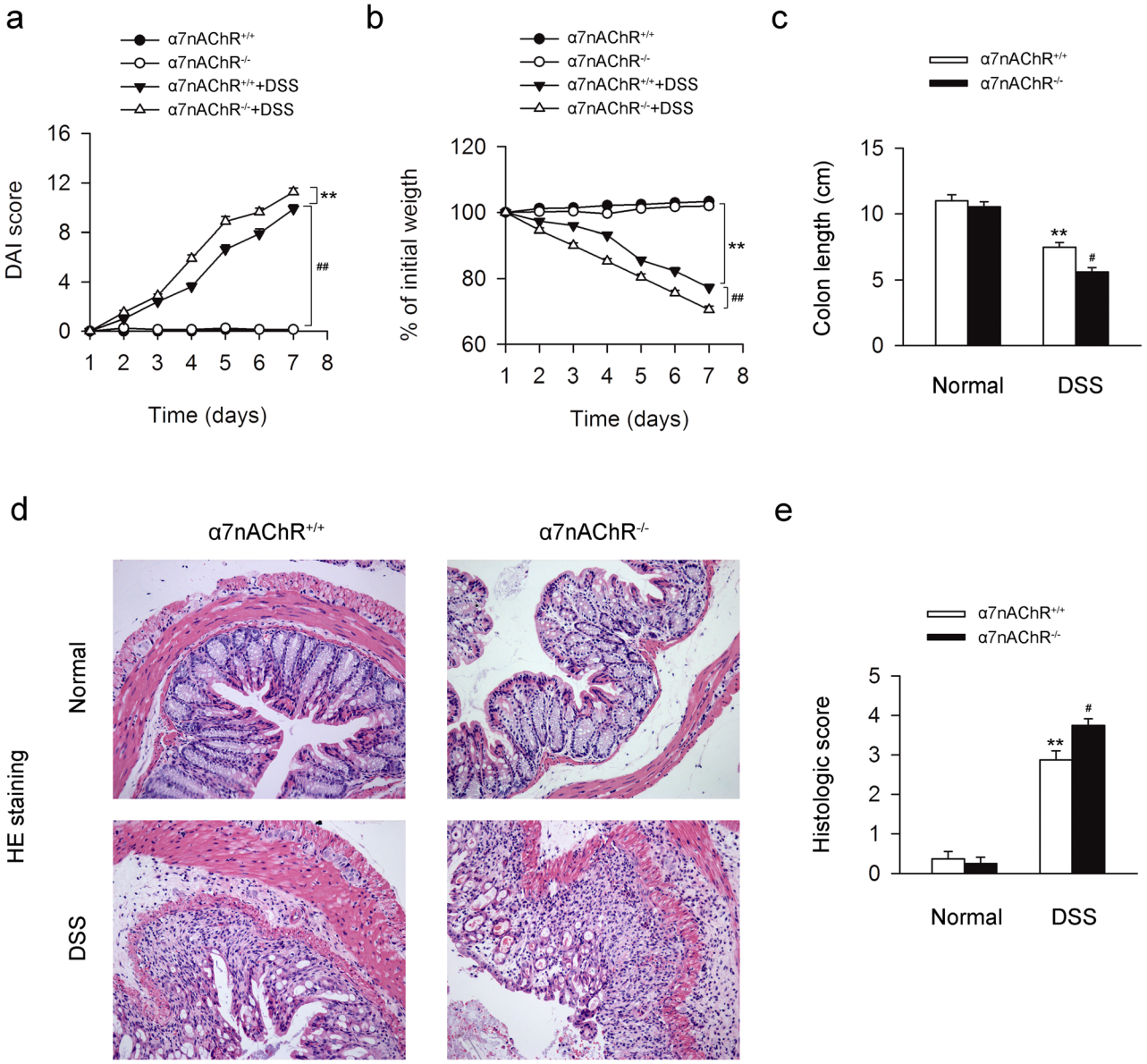

Fig. 1. Knocking out α7nAChR deteriorates DSS-induced colitis in IBD symptoms. $\alpha7nAChR^{+/+}$ and $\alpha7nAChR^{-/-}$ mice received DSS (3%) for 7 days and the control mice were given tap water. DAI score, body weight loss and colon length were detected and inflammatory infiltration of left colon was detected by HE staining. Compared to $\alpha7nAChR^{+/+}$ mice in the DSS-treated group, $\alpha7nAChR^{-/-}$ mice exhibited significant aggravation in DAI score (**a**), body weight loss (**b**), colon length shortening (**c**), and the enhancement of colon inflammation induced by DSS (**d**, **e**) ($n = 8$ per group). $^{**}P < 0.01$ *vs.* $\alpha7nAChR^{+/+}$ group; $^{\#}P < 0.05$ *vs.* $\alpha7nAChR^{+/+}$ DSS group, $^{\#\#}P < 0.01$ *vs.* $\alpha7nAChR^{+/+}$ DSS group; data are presented as mean ± SEM.

ANOVA followed by Bonferroni post-hoc test were used to determine nonparametric data and continuous variables, respectively. A P value < 0.05 was considered statistically significant. Data were analyzed with SPSS 21.0K for Windows (SPSS, Chicago, IL, USA).

RESULTS

Knocking out α7nAChR Deteriorates DSS-Induced Colitis

To determine the effect of α7nAChR on IBD, we gave α7nAChR$^{+/+}$ and α7nAChR$^{-/-}$ mice with 3% DSS for 7 days for the development of a severe illness characterized by the presence of bloody diarrhea (Fig. 1a), sustained weight loss (Fig. 1b) and shortened colon length (Fig. 1c). We found that α7nAChR knockout in DSS-treated mice further deteriorated the severity of IBD in DAI score, weight loss, and colon length (Fig. 1a–c). In addition, α7nAChR deficiency led to the aggravation of inflammatory infiltration of the left colon in DSS-induced colitis mice (Fig. 1d, e). We further found that α7nAChR deficiency largely enhanced the levels of several pro-inflammatory cytokines including IL-1β, IL-6, IL-18, and TNF-α in serum as well as left colon from DSS-induced mice (Fig. 2a, b). Taken together, these data indicate that α7nAChR deficiency aggravates the severity of IBD symptoms and inflammatory reaction in DSS-induced colitis mice.

α7nAChR Deficiency Decreased the Level of Autophagy in the Left Colon from DSS-Induced Colitis Mice

We then detected the influence of α7nAChR deficiency on autophagy *in vivo*. We found that the level of autophagy-related Beclin-1 and LC3-II/I ratio were increased and the levels of p62 was decreased in the left colon from α7nAChR$^{+/+}$ colitis mice compared to those in the normal group. However, knocking out α7nAChR largely attenuated those effects on Beclin-1, LC3-II/I, and p62 (Fig. 3a, b). Trends of changes similar to Beclin-1 and LC3-II/I ratio were found in the analysis of autophagosome level in macrophages from the left colon (Fig. 3c, d). Taken together, these data indicate that α7nAChR produces a positive effect on autophagy in the left colon from DSS-induced colitis mice.

α7nAChR Deficiency Decreased the Level of Autophagy in BMDMs Under the Challenge of LPS/DSS

Since macrophages were vital in the pathogenesis and progression of IBD through the triggering of inflammatory

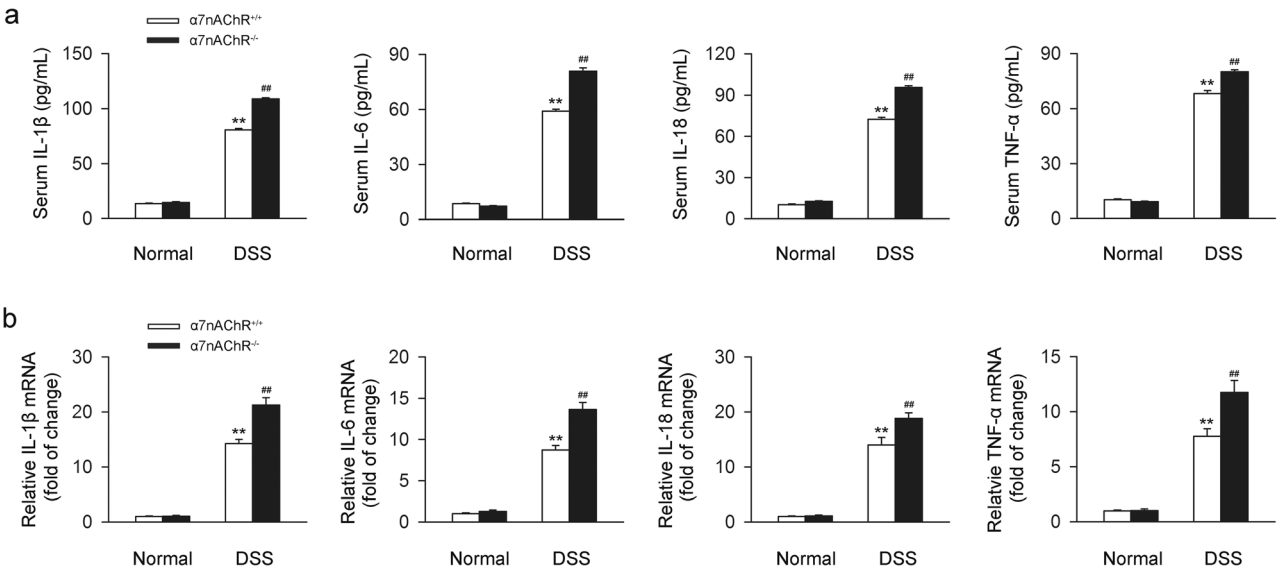

Fig. 2. α7nAChR deletion aggravates the level of inflammatory reaction in DSS-induced mice. α7nAChR$^{+/+}$ and α7nAChR$^{-/-}$ mice received DSS (3%) for 7 days and the control mice were given tap water. After 7-day treatment with DSS, the serum and left colon were obtained at the 8th day since the beginning of DSS treatment. The level of IL-1β, IL-6, IL-18, and TNF-α in serum was detected by ELISA and those in the left colon were analyzed by real-time PCR. Compared to α7nAChR$^{+/+}$ mice in the DSS-treated group, α7nAChR$^{-/-}$ mice exhibited significant enhancement of the level of IL-1β, IL-6, IL-18, and TNF-α in serum (**a**) and left colon (**b**) from DSS-induced mice ($n = 6$ per group). $^{**}P < 0.01$ *vs.* α7nAChR$^{+/+}$ group; $^{\#\#}P < 0.01$ *vs.* α7nAChR$^{+/+}$ DSS group; data are presented as mean ± SEM.

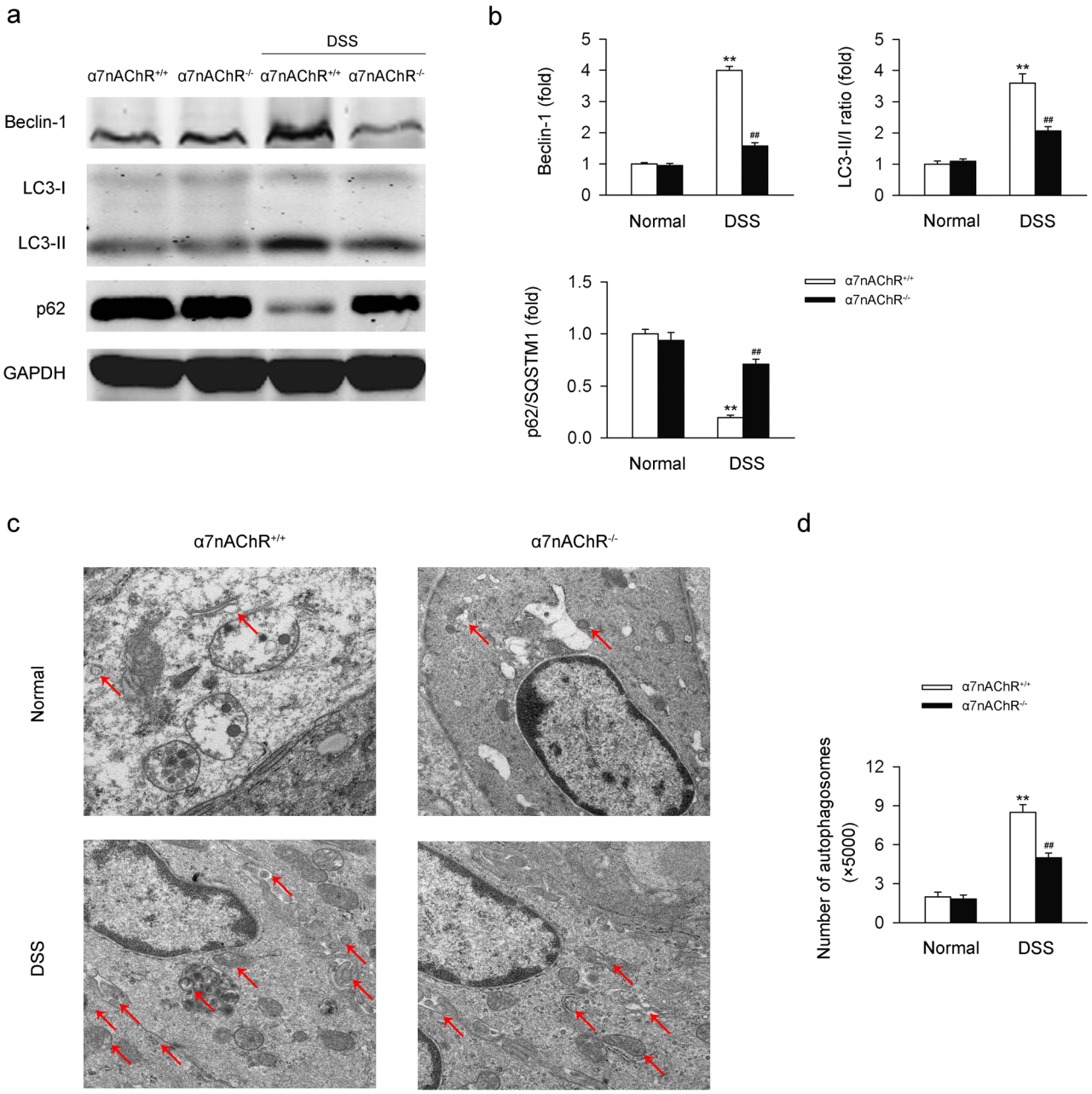

Fig. 3. α7nAChR deletion decreased the level of autophagy in the left colon from DSS-induced colitis mice. α7nAChR$^{+/+}$ and α7nAChR$^{-/-}$ mice received DSS (3%) for 7 days and the control mice were given tap water. Western blot was applied for the detection of the level of Beclin-1, LC3, and p62 and transmission electron microscopy was used for the analysis of autophagosome number. Compared to α7nAChR$^{+/+}$ mice in the DSS-treated group, α7nAChR$^{-/-}$ mice exhibited significant decrease of the level of Beclin-1 and LC3-II/I ratio and increase of p62 (**a**, **b**) ($n = 5$ per group) as well as a decrease of autophagosome number (**c**, **d**) in the left colon from DSS-induced mice ($n = 6$ per group). $^{**}P < 0.01$ *vs.* α7nAChR$^{+/+}$ group; $^{\#\#}P < 0.01$ *vs.* α7nAChR$^{+/+}$ DSS group; data are presented as mean ± SEM.

reaction, we further investigated the effect of α7nAChR on autophagy in BMDMs *in vitro*. LPS in combination of DSS was used for inflammatory loading. We found that the level of autophagy-related Beclin-1 and LC3-II/I ratio were increased and the level of p62 was decreased in BMDMs isolated from α7nAChR$^{+/+}$ mice compared to those in the normal group. However, knocking out α7nAChR largely attenuated those effects on Beclin-1, LC3-II/I, and p62 (Fig. 4a, b). Trends of change similar to Beclin-1 and LC3-II/I ratio were found in the analysis of

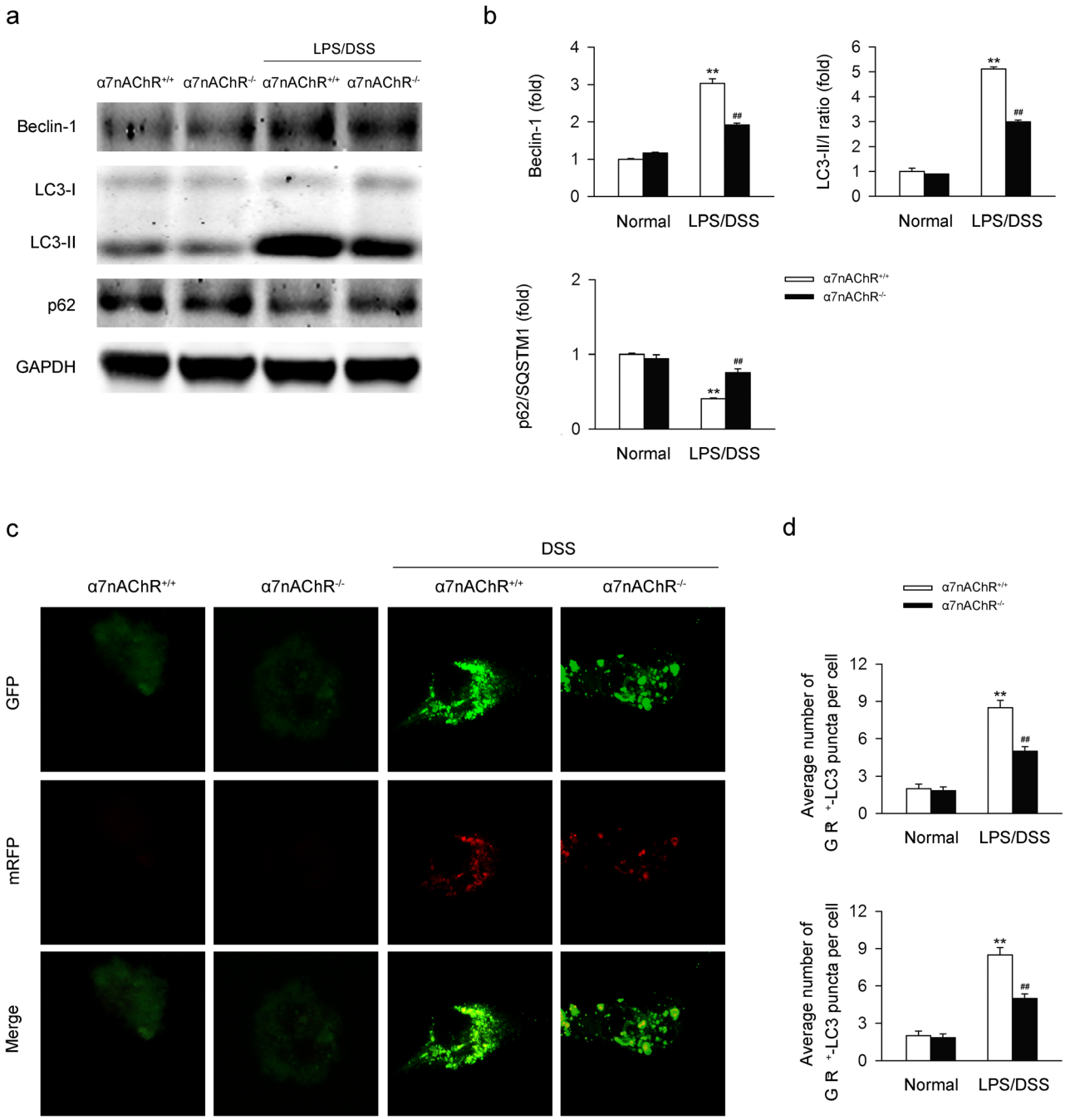

Fig. 4. α7nAChR deletion decreased the level of autophagy in BMDMs under the challenge of LPS/DSS. BMDMs from α7nAChR$^{+/+}$ and α7nAChR$^{-/-}$ mice were isolated and treated with/without LPS/DSS for 24 h. Western blot was used for the detection of the level of Beclin-1, LC3, and p62. The mRFP-GFP-LC3 plasmid was transfected into BMDMs for the analysis of autophagy flux. Compared to BMDMs isolated from α7nAChR$^{+/+}$ mice under the challenge of LPS/DSS, those isolated from α7nAChR$^{-/-}$ mice exhibited significant decrease of the level of Beclin-1 and LC3-II/I ratio and increase of p62 (**a**, **b**) ($n = 5$ per group) and a decrease of autophagy flux (**c**, **d**) under the challenge of LPS/DSS ($n = 6$ per group). $^{**}P < 0.01$ *vs.* α7nAChR$^{+/+}$ group; $^{\#\#}P < 0.01$ *vs.* α7nAChR$^{+/+}$ DSS group; data are presented as mean ± SEM.

autophagy flux in BMDMs (Fig. 4c, d). Taken together, these data indicate that α7nAChR produces a positive effect on autophagy in BMDMs under the challenge of LPS/DSS.

Knocking out α7nAChR Ameliorates the Alleviative Effect of Rapamycin on DSS-Induced Colitis

We further investigated whether autophagy was involved in the protective effect of α7nAChR on IBD. Rapamycin was used for the induction of autophagy in DSS-treated α7nAChR$^{+/+}$ and α7nAChR$^{-/-}$ mice from days 1 to 7. We found that for α7nAChR$^{+/+}$ mice, the administration of rapamycin largely alleviated the severity of IBD symptoms in DAI score, weight loss and colon length, while knocking out α7nAChR significantly attenuated those protective effects of rapamycin (Fig. 5a–c). Similar trends of changes were

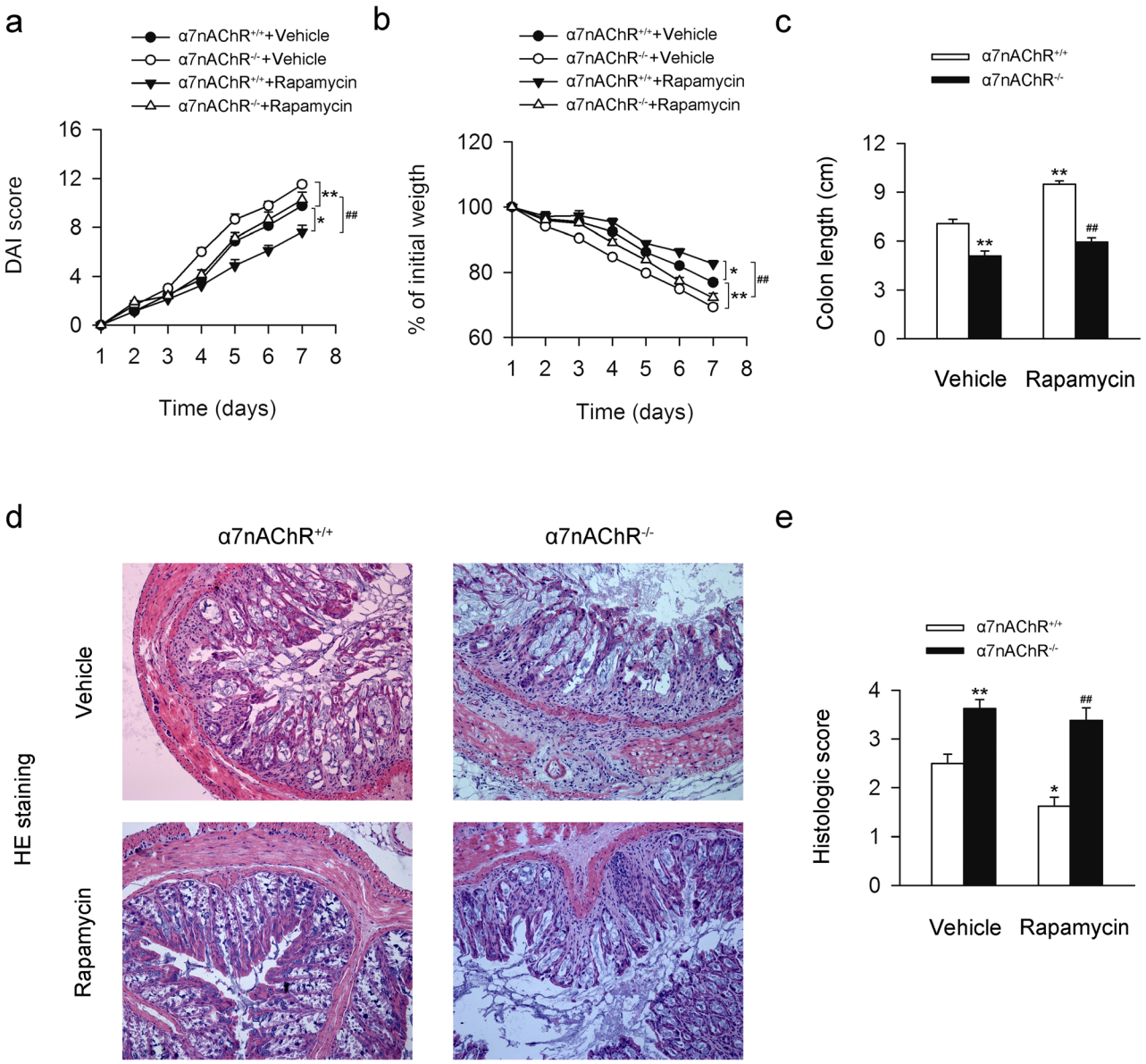

Fig. 5. α7nAChR deletion ameliorates the alleviative effect of rapamycin on DSS-induced colitis in IBD symptoms. α7nAChR$^{+/+}$ and α7nAChR$^{-/-}$ mice received DSS (3%) for 7 days. For certain groups, rapamycin was intraperitoneally given to mice from day 1–7. DAI score, body weight loss, and colon length were detected and inflammatory infiltration of left colon was detected by HE staining. Compared to α7nAChR$^{+/+}$ mice in vehicle group, those with the administration of rapamycin exhibited significant alleviation in DAI score (**a**), body weight loss (**b**), colon length shortening (**c**), and the enhancement of colon inflammation induced by DSS (**d**, **e**) ($n = 8$ per group). However, knocking out α7nAChR largely attenuated those effects ($n = 8$ per group) (**a**–**e**). $^{*}P < 0.05$ *vs.* α7nAChR$^{+/+}$+vehicle group, $^{**}P < 0.01$ *vs.* α7nAChR$^{+/+}$+vehicle group; $^{\#\#}P < 0.01$ *vs.* α7nAChR$^{+/+}$+rapamycin group; data are presented as mean ± SEM.

observed in the levels of inflammatory infiltration in the left colon from DSS-induced colitis mice (Fig. 5d, e). In addition, rapamycin largely decreased the levels of pro-inflammatory cytokines including IL-1β, IL-6, IL-18, and TNF-α in serum as well as left colon from DSS-induced mice, which were largely attenuated by knocking out α7nAChR (Fig. 6a, b). Taken together, these data indicate that autophagy deficiency was involved in the aggravated effect of α7nAChR deletion on DSS-induced colitis in the severity of IBD symptoms as well as inflammatory reaction.

Knocking out α7nAChR Ameliorates the Anti-inflammatory Effect of Rapamycin in BMDMs under the Challenge of LPS/DSS

We then detected the association between autophagy and α7nAChR-mediated protection of IBD in BMDMs under inflammatory loading *in vitro*. We found that in BMDMs isolated from α7nAChR$^{+/+}$ mice under the challenge of LPS/DSS, the administration of rapamycin largely decreased the levels of pro-inflammatory cytokines including IL-1β, IL-6, IL-18, and TNF-α. However, the anti-inflammatory effects of rapamycin were attenuated by the knockout of α7nAChR (Fig. 7a–d). Similar trends of changes were detected in the levels of IL-1β, IL-6, IL-18, and TNF-α secreted in the supernatant (Fig. 7e–h). Those results indicate that autophagy is involved in the anti-inflammatory effect of α7nAChR in BMDMs under the challenge of LPS/DSS.

AMPK-mTOR-p70S6K Signaling Pathway Is Involved in the Autophagy-Inductive and Anti-inflammatory Effects of α7nAChR in BMDMs Under the Challenge of LPS/DSS

We finally investigated whether AMPK-mTOR-p70S6K signaling, a classic pathway of autophagy, was involved in the α7nAChR-mediated process. Compound C was used for the blockage of AMPK-mTOR-p70S6K signaling pathway and PNU-282987 was used for the activation of α7nAChR. We found that the administration of PNU-282987 largely enhanced the number of autophagosome in BMDMs under the challenge of LPS/DSS, while this effect was attenuated by the treatment of compound C (Fig. 8a, b). In addition, we tested the levels of several pro-inflammatory cytokines including IL-1β, IL-6, IL-18, and TNF-α in BMDMs as well as the supernatant.

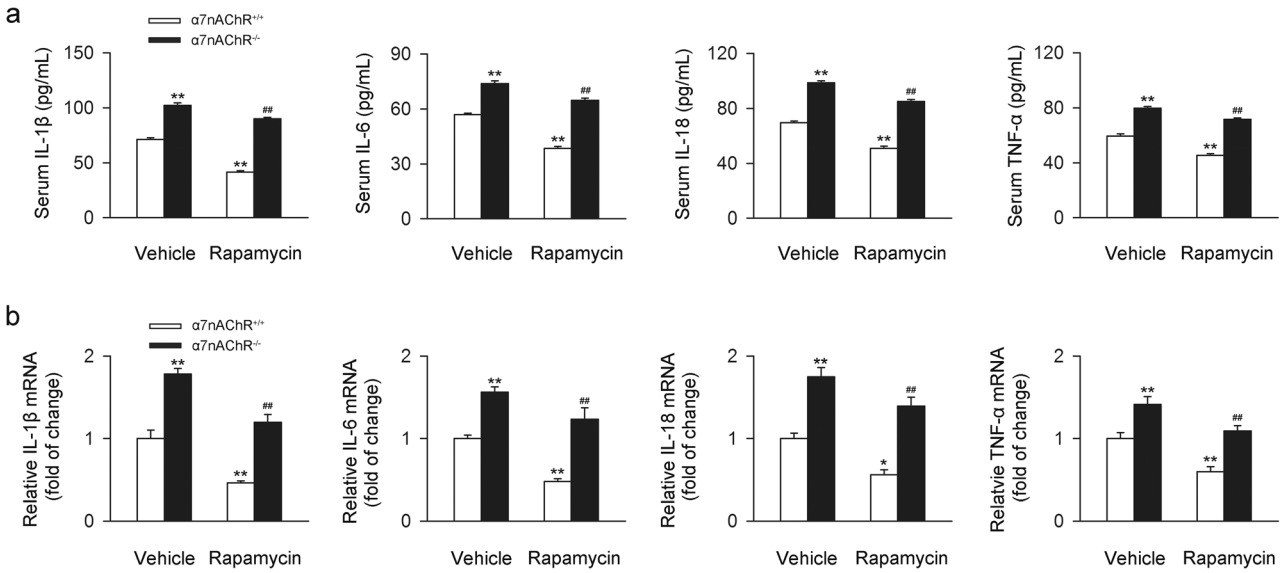

Fig. 6. α7nAChR deletion ameliorates the anti-inflammatory effect of rapamycin on DSS-induced colitis. α7nAChR$^{+/+}$ and α7nAChR$^{-/-}$ mice received DSS (3%) for 7 days. For certain groups, rapamycin was intraperitoneally given to mice from days 1 to 7. After 7-day treatment with DSS, the serum and left colon were obtained at the 8th day since the beginning of DSS treatment. The level of IL-1β, IL-6, IL-18, and TNF-α in serum was detected by ELISA and those in the left colon were analyzed by real-time PCR. Compared to α7nAChR$^{+/+}$ mice in vehicle group, those with the administration of rapamycin-exhibited significant suppression in the level of IL-1β, IL-6, IL-18, and TNF-α in serum (**a**) and left colon (**b**) ($n = 6$ per group). However, knocking out α7nAChR largely attenuated those effects ($n = 6$ per group) (**a**, **b**). $^{*}P < 0.05$ *vs.* α7nAChR$^{+/+}$+vehicle group, $^{**}P < 0.01$ *vs.* α7nAChR$^{+/+}$+vehicle group; $^{\#\#}P < 0.01$ *vs.* α7nAChR$^{+/+}$+rapamycin group; data are presented as mean ± SEM.

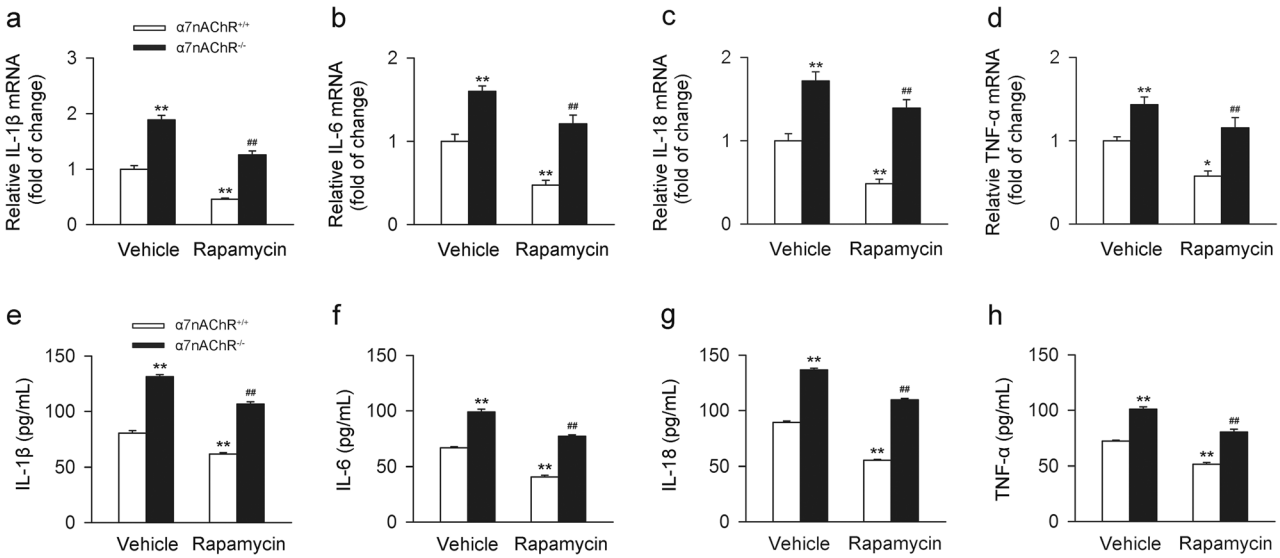

Fig. 7. α7nAChR deletion ameliorates the anti-inflammatory effect of rapamycin in BMDMs under the challenge of LPS/DSS. BMDMs from $\alpha7nAChR^{+/+}$ and $\alpha7nAChR^{-/-}$ mice were isolated and treated with LPS/DSS for 24 h. For certain groups, rapamycin was given at 10 min in advance and PBS in the same volume of rapamycin was given as vehicle. The levels of IL-1β, IL-6, IL-18, and TNF-α in BMDMs were detected by real-time PCR. Compared to BMDMs isolated from $\alpha7nAChR^{+/+}$ mice in vehicle group, those with the administration of rapamycin exhibited significant suppression in the levels of IL-1β (**a**), IL-6 (**b**), IL-18 (**c**), and TNF-α (**d**) in mRNA ($n = 6$ per group). However, knocking out α7nAChR largely attenuated those effects ($n = 6$ per group) (**a**–**d**). The levels of IL-1β, IL-6, IL-18, and TNF-α in the supernatant were detected by ELISA. Compared to BMDMs isolated from $\alpha7nAChR^{+/+}$ mice in the vehicle group, those with the administration of rapamycin exhibited significant suppression in the levels of IL-1β (**e**), IL-6 (**f**), IL-18 (**g**), and TNF-α (**h**) in the supernatant ($n = 6$ per group). However, knocking out α7nAChR largely attenuated those effects ($n = 6$ per group) (**e**–**h**). $^{*}P < 0.05$ *vs.* $\alpha7nAChR^{+/+}$+vehicle group, $^{**}P < 0.01$ *vs.* $\alpha7nAChR^{+/+}$+vehicle group; $^{\#\#}P < 0.01$ *vs.* $\alpha7nAChR^{+/+}$+rapamycin group; data are presented as mean ± SEM.

We found that PNU-282987 largely decreased the levels of IL-1β, IL-6, IL-18, and TNF-α in mRNA in BMDMs as well as secreted in the supernatant, which were significantly attenuated by compound C (Fig. 8c, d). Taken together, these data indicate that AMPK-mTOR-p70S6K signaling was involved in the α7nAChR-mediated effects of autophagy induction as well as inflammatory suppression.

DISCUSSION

Previous studies have demonstrated the alleviative effect of activating α7nAChR in IBD [20, 32, 33]. For instance, it was reported by selective activation of α7nAChR significantly attenuated several local markers of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis through the decrease of pro-inflammatory cytokines and reversing the rise in splenic T-cells [20]. Similar results were found by another group of researchers, who demonstrated that selective activation of α7nAChR by PNU-282987 largely alleviated the severity of DSS-induced colitis *via* the decrease of macrophage infiltration and suppression of the levels of pro-inflammatory cytokines [32]. Consistent with those findings, here in our current study, we demonstrated that knocking out α7nAChR largely aggravated the severity of DSS-induced colitis in DAI score, body weight loss, colon length shortening, histologic score, and the production and secretion of several pro-inflammatory cytokines. We further ran a series of tests on murine BMDMs and got similar trends of changes in the production of pro-inflammatory cytokines with the deficiency in α7nAChR. Collectively, those data indicate the detrimental effect of α7nAChR deletion in DSS-induced colitis.

Although previous studies have reported the alleviative effect of α7nAChR in IBD, few studies were available in the exploration of its specific mechanism. Here in this study, we focus on autophagy process. As we mentioned above, autophagy is recognized as a self-eating catabolic process in organisms, which is closely involved in various life processes. According to previous studies conducted by us and other groups of researchers, autophagy plays a protective role in several kinds of disorders, including multiple sclerosis, ischemic stroke,

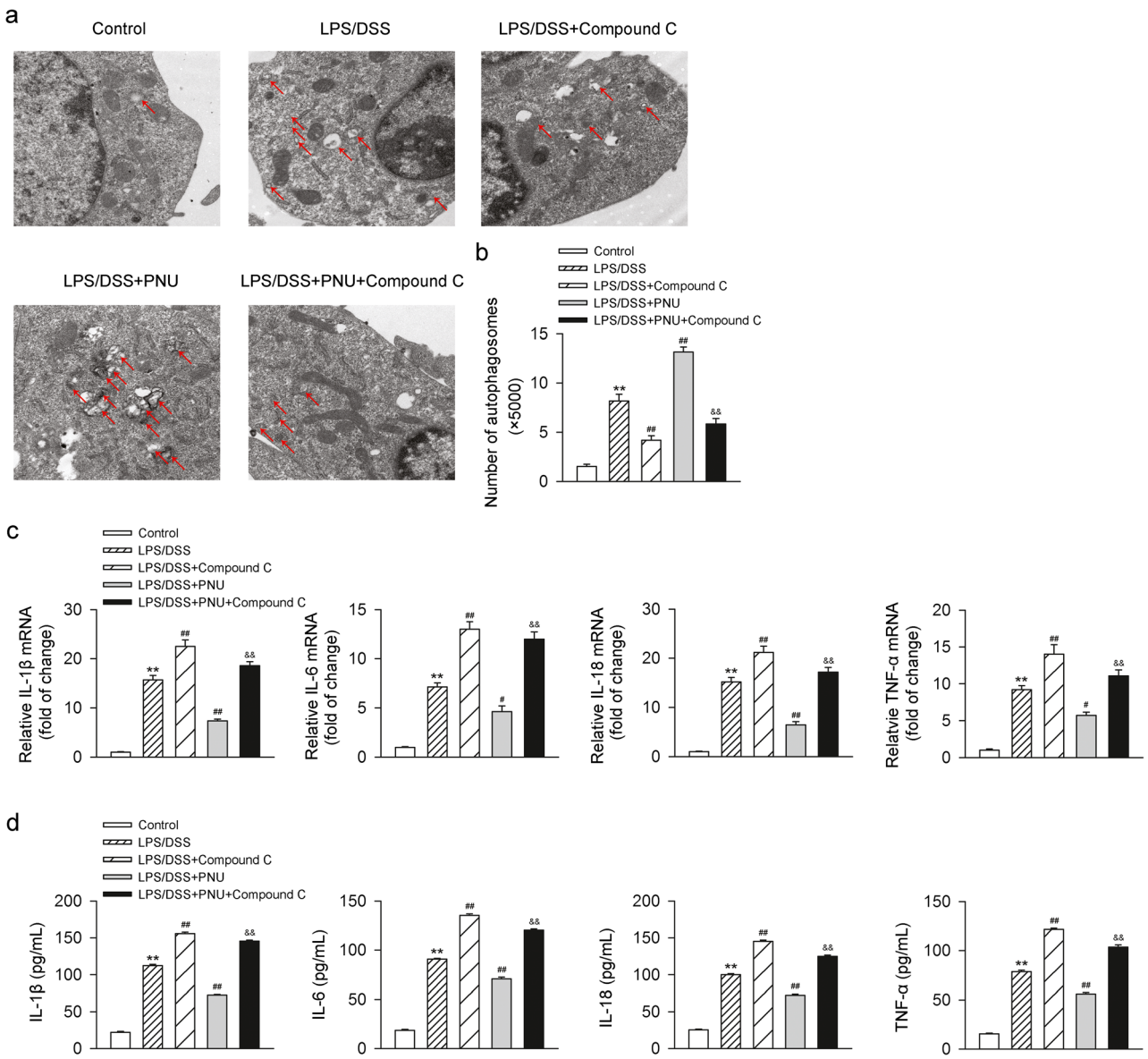

Fig. 8. Involvement of AMPK-mTOR-p70S6K in the autophagy-inductive and anti-inflammatory effects of α7nAChR in BMDMs under the challenge of LPS/DSS. BMDMs from α7nAChR$^{+/+}$ and α7nAChR$^{-/-}$ mice were isolated and treated with LPS/DSS for 24 h. For certain groups, compound C and/or PNU-282987 were given at 10 min in advance. PBS in the same volume was given as vehicle. Transmission electron microscopy was used for the analysis of autophagosome number. Real-time PCR and ELISA were used for the detection of the levels of IL-1β, IL-6, IL-18, and TNF-α in BMDMs and the supernatant, respectively. Compared to BMDMs in LPS/DSS group, those with the administration of PNU-282987 exhibited significant enhancement of autophagosome number ($n = 6$ per group) (**a**, **b**) and suppression of the levels of IL-1β, IL-6, IL-18, and TNF-α in mRNA (**c**) and the supernatant (**d**) ($n = 6$ per group). However, the administration of compound C largely attenuated those effects of PNU-282987 (**a**–**d**). $^{**}P < 0.01$ *vs*. control group; $^{\#}P < 0.05$ *vs*. LPS/DSS group, $^{\#\#}P < 0.01$ *vs*. LPS/DSS group; $^{\&\&}P < 0.01$ *vs*. LPS/DSS + PNU-282987 group. PNU, PNU-282987; data are presented as mean ± SEM.

myocardial infarction, and atherosclerosis, *via* the suppression of many forms of inflammatory reaction [34–40]. In IBD, a previous study conducted by us revealed that selective activation of cannabinoid receptor 2 (CB2R) significantly alleviated the severity of DSS-induced colitis through the induction of autophagy, thus suppressing the active level of the NLRP3 inflammasome [27]. In consistent with those findings, in our current study, we found that the administration of rapamycin for the induction of autophagy largely attenuated the severity of IBD in DSS-

induced colitis and suppressed the level of inflammatory reaction in both colon from DSS-induced colitis *in vivo* and murine BMDMs under inflammatory loading *in vitro*.

We then detected whether autophagy was involved in the α7nAChR-mediated protective effect on IBD. It was previously reported that activating α7nAChR could enhance the level of autophagy in several kinds of cells, thus playing a positive role in fighting against diseases [25, 41]. In addition, a previous study conducted by us revealed that selective activating α7nAChR by PNU-282987 significantly up-regulated the level of autophagy in microglial cells in the central nervous system, thus contributing to the alleviation of multiple sclerosis in mice models [15]. Here in this study, we for the first time demonstrated that autophagy process was involved in the alleviative effect of activating α7nAChR effect on IBD. We showed that on the occurrence of DSS-induced colitis, knocking out α7nAChR largely decreased the level of autophagy in colon as well as BMDMs. We then used rapamycin for the induction of autophagy and found that knocking out α7nAChR largely attenuated the alleviative effect of rapamycin in IBD. Those results indicate the involvement of autophagy in α7nAChR-mediated effect on IBD.

We finally investigated the molecular mechanisms underlying this process. It has been previously reported that AMPK-mTOR-p70S6K signaling serves as a classic pathway in the induction of autophagy [21, 22]. AMPK-mTOR-p70S6K signaling has been shown to play a regulatory role in many kinds of diseases [42–45]. Here in our current study, we used compound C for the blockage of AMPK-mTOR-p70S6K signaling. We found that the administration of compound C largely attenuated the effects of activating α7nAChR in the up-regulation of autophagy and suppression of pro-inflammatory cytokines in BMDMs under the challenge of LPS/DSS. Those results indicate the involvement of AMPK-mTOR-p70S6K signaling in the α7nAChR-mediated process in IBD.

In conclusion, here in our current study, we demonstrated that α7nAChR deficiency aggravated the severity of IBD in DSS-induced colitis mice as well as murine BMDMs under the challenge of LPS/DSS. We further reported that AMPK-mTOR-p70S6K signaling pathway was involved in the α7nAChR-mediated alleviative effect on IBD. We believe that this study might provide a potential and effective target in the development of anti-IBD drugs and effective strategy in the treatment of IBD.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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