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Androgen receptor inhibition alleviated inflammation in experimental autoimmune myocarditis by increasing autophagy in macrophages

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Abstract. – OBJECTIVE: Experimental autoimmune myocarditis (EAM) is characterized by pronounced macrophage infiltration, cardiac necrosis, and cardiac fibrosis. Our previous studies have demonstrated that suppressed androgen receptor (AR) enables anti-inflammation to promote tissue repair by decreasing M1 macrophages and increasing M2 macrophages in an EAM model. Given that autophagy mediates inflammatory response in macrophages, we investigated whether AR inhibition executes its protective role in inflammation through the autophagy pathway in EAM.

MATERIALS AND METHODS: To determine whether AR inhibition can perform its anti-inflammatory effects by upregulating autophagy, we pre-treated mice with 3-methyl adenine (3-MA), a pharmacological inhibitor of autophagy. Immunofluorescence assay and Western blot were used to detect autophagy levels and autophagy activity in five different groups. Immunofluorescence marked F4/80 and LC3 to illustrate the autophagy level in macrophages. TUNEL assays were used to detect the apoptosis level in heart tissue of five different groups.

RESULTS: We demonstrated that AR inhibition resolves injury with sustained inhibition of inflammatory cytokines associated with enhanced autophagy, especially in macrophages. Increased LC3II/I expression corroborated complete autolysosome formation detected by electron microscopy and correlated with degradation of SQSTM1/p62 in the AR inhibition group by Western blot. These effects could be reversed within 3-MA, a pharmacological inhibitor of autophagy. Specifically, pharmacological inhibition of autophagy increased apoptosis and inflammation, which could be attenuated by AR inhibition.

CONCLUSIONS: AR inhibition alleviates the inflammatory response and tissue apoptosis by enhancing autophagy, especially in macrophages.

Key Words:
Myocarditis, Androgen receptor, Macrophage, Autophagy, Inflammation.

Introduction

Myocarditis is characterized by inflammation of the cardiac muscle. The development of severe myocardial injury or the persistence of the immune response may lead to dilated cardiomyopathy or heart failure. Cardiotropic infections or tissue damage followed by activation of heart-specific autoimmunity are common causes of myocarditis in humans[1]. Animal models represent useful tools to study the pathogenesis of myocarditis, and mouse models of experimental autoimmune myocarditis (EAM) reflect key aspects of the human disease[2]. Several lines of evidence have suggested that monocytes/macrophages represent the majority of inflammatory cells, thereby subserving a critical role in myocarditis. We previously demonstrated in EAM mice that intervention with the androgen receptor degreaser ASC-J9 attenuates heart inflammation by decreasing M1 macrophages and improving M2 macrophages. However, the mechanism by which AR mediates protection by dampening inflammation remains unknown[3,4].

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The androgen receptor is a hormone receptor and a prototypical nuclear receptor that has been reported to contribute to autophagy[5,6]. Autophagy is a cellular protein derivative pathway that mediates the turnover of organelles and damaged proteins to maintain homeostasis and is integral in expanding the lifespan of nematodes with congruent effects in mice[7]. Recently, increasing evidence suggests that autophagy plays a critical role in the development and pathogenesis of inflammation and the immune response[8]. Especially in macrophages, autophagy plays an important role in the regulation of inflammatory mediators and affects cytokine production[9,10].

Therefore, we sought to understand whether AR inhibition regulates autophagy to mediate anti-inflammatory effects in an EAM model. In this study, we identified that inhibition of AR with ASC-J9 suppressed heart inflammation and apoptosis by upregulating autophagy, especially within macrophages.

Materials and Methods

Animals and Treatment

Male BALB/c mice aged 6-7 weeks were fed in the experimental animal center of the Second Hospital of Shandong University. All animals were treated according to the guidelines of the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH) and supported by the Animal Care and Utilization Committee of Shandong University. Mice were randomly assigned to five groups: (1) normal; (2) EAM; (3) EAM+ASC-J9; (4) EAM+3-methyladenine (3-MA; autophagy inhibitor); and (5) EAM+ASC-J9+3-MA, with 5 mice in each group. Mice in the normal group were injected with phosphate-buffered saline (PBS), and all other groups were infected subcutaneously with the murine α-MyHC sequence (MyHC-α614-629: acetyl SLKLMATLFSTYAS) (GLbiochem, Shanghai, China) emulsified 1:1 with complete Freund's adjuvant (CFA, Sigma-Aldrich, St. Louis, MO, USA) on days 0 and 7 (200 µg in 0.2 ml for each mouse). ASC-J9® was purchased from MedChemExpress (Princeton, NJ, USA). The mice were intraperitoneally injected with ASC-J9 (75 mg/kg of body weight) every other day between days 8 and 20 of EAM. 3-MA was purchased from Sigma-Aldrich (St. Louis, MO, USA). And mice were injected intraperitoneal with 3-MA (30 mg /kg of body weight) at the same time point as the ASC-J9 injection.

Quantitative RT-PCR (RT-qPCR)

Total RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). The isolated RNA (1ug) was reverse transcribed into cDNA with an RT-PCR kit (TAKARA, Dalian, China), according to the manufacturer's instructions. QRT-PCR was performed by using a SYBR Green RT-PCR kit (TAKARA, Dalian, China) in a Bio-Rad CFX86. The reaction conditions were: pre-denaturation at 95°C for 2 min; 95°C 5 s, 60°C 15 s, 40 cycles. Details of the primer sequence are shown in Table I. $2^{\Delta\Delta ct}$ was used to analyze the data. The relative mRNA expression was normalized to the GAPDH mRNA levels.

Histopathology

Mouse hearts were fixed, paraffin-embedded, sectioned at 4 µm, and heated at 60°C overnight. Following dewaxing and dehydration, the sections were stained with hematoxylin and eosin (H&E) stain (ServiceBio, Wuhan, China). The Image-Pro Plus IPP (Mediaplayer, New York, NY, USA) was used to measure the severity scores of the myocardial inflammation. The grades of inflammatory severity scores were measured as previously described[11].

Immunofluorescence Microscopy

Paraffin-embedded tissues were sequentially sliced into sections of 4 µm and heated at 60°C overnight. Following dewaxing and dehydration, antigen retrieval and blocking with serum were performed. Then, the primary antibodies against F4/80 (BD Bioscience, San Jose, CA, USA) and LC3 (ad51520, 1:2000, Abcam, Cambridge, MA, USA) were added into the sections for incubation at 4°C overnight. The next day, sections were rin-

Table I. Primer sequences.

Factors	Upstream primers	Downstream primers
GAPDH	5'-TGTGTCCGTCGTGGATCTGA-3'	5'-TGCTGTTGAAGTCGCAGGAG-3'
iNOS	5'-GAAAAGTCCAGCCGCACCAC-3'	5'-GCACAATCCACAACTCGCTCC-3'
TNF-α	5'-CATCTTCTCAAAATTCGAGTGACAA-3'	5'-TGGGAGTAGACAAGGTACAACCC-3'

sed, followed by the addition of secondary antibody, and then placed under the fluorescent microscope for observation after being sealed using the anti-cancellation mounting medium containing DAPI (4,6-diamidino-2-phenylindole).

Western Blot Analysis

Heart tissues were lysed in RIPA extraction solution (ServiceBio, Wuhan, China) containing a protein inhibitor cocktail. Protein concentration was assessed using the BCA assay (ServiceBio, Wuhan, China). Total protein lysates (120 µg/lane) were subjected to 12% SDS-PAGE (ServiceBio, Wuhan, China) and transferred onto PVDF membranes (Millipore, Billerica, MA, USA) after ionization. After then, the membranes were washed in Phosphate-Buffered Saline and Tween (PBST) for 10 min and blocked with 5% skim-milk for 2 h. The antibodies GAPDH (1:1000, Antigene, Wuhan, China), LC3 (ab51520, 1:1000, Abcam, Cambridge, MA, USA), and p62 (ab109012, 1:3000, Abcam, Cambridge, MA, USA) were used for incubation at 4°C overnight. The membranes were incubated with HRP-labelled secondary antibody (1:3000, Antigene, Wuhan, China) at room temperature for 2 h. After washed with PBST for 10 min, the protein bands were scanned and analyzed in AlphaView Q software (Alpha Innotech, Jan Jose, CA, USA) using ECL illustration (Millipore, Billerica, MA, USA). The relative expression level of the target protein was normalized to the gray value of the GAPDH protein band.

Transmission Electron Microscopy

The myocardial tissues were placed in cold 6% glutaraldehyde (pH 7.3) for 1 h. The samples were refrigerated for 24 h, washed in PBS (12 h), placed in cold veronal acetate (pH 7.3) containing 1% osmium tetroxide (1 h), and stained with phosphotungstic acid (10 min). Then, the samples were embedded in Ciba 502 by polymerization (35°C, 12 h; 45°C, 8 h; 60°C, 12 h). The sections (60-70 nm) were stained with toluidine blue and placed on carbon-coated 200 mesh grids. An RCA EMU-3F electron microscope at 50 kV was used to examine the tissues.

TUNEL Assay

The TUNEL assay was performed on slide-mounted mouse cardiac tissue sections using the *in-situ* Cell Death Detection kit (Roche Applied Science, Basel, Switzerland) according to the manufacturer's directions and visualized using a Leica DMI 6000B inverted fluorescence microscope (Leica Microsystems Wetzlar, Germany) using a Retiga Aqua blue camera (Q-imaging Vancouver, BC, USA).

Statistical Analysis

Statistical analysis was performed using unpaired Student's *t*-test for 2 groups and One-way ANOVA with a post-hoc Student-Newmann-Keuls test or Dunnett test for multiple comparisons. All analyses were performed by GraphPad Prism 6.0 (La Jolla, CA, Canada). All quantitative data are presented as mean± SEM or percentages in the figures. *p*-values <0.05 were considered statistically significant.

Results

Autophagic Flux Was Augmented in ASC-J9-Treated EAM Mice

Autophagy plays a critical role in the development and pathogenesis of inflammation and the immune response. In our previous study, we demonstrated that inhibiting AR mediates the inflammatory response in an EAM model. Therefore, we examined whether inhibition of AR with ASC-J9 alters the autophagy level in EAM mice. We used TEM to observe myocardial microstructure and autophagosomes. Myocardial cells in the normal group exhibited integrated membranes and cytoplasm; however, the ultrastructure of the EAM group was broken. In contrast, this condition was ameliorated in the ASC-J9 group. Additionally, the ASC-j9 group exhibited an increased number of autophagosomes compared with the EAM group (Figure 1A). To evaluate the level of autophagy after ASC-J9 administration, immunohistochemistry was used to detect the autophagosomal marker LC3 in the affected cells in each group. Treatment with ASC-J9 enhanced LC3 expression (Figure 1C). To further evaluate autophagy processing, EAM mice were treated with ASC-J9. We detected that a single treatment with ASC-J9 increased LC3II/I expression compared to the EAM groups (p=0.0225). This increase was accompanied by a dramatic decrease in p62 expression (p=0.039). However, the LC3II/I expression was completely abrogated when animals were pre-treated i.p. with 3-MA, a pharmacological inhibitor of autophagy (p=0.041). In addition, reduced LC3II/I expression (p=0.04) and increased p62 protein levels (p=0.05) in 3-MA group could be partially reversed after treatment with ASC-J9 (Figure 1B).

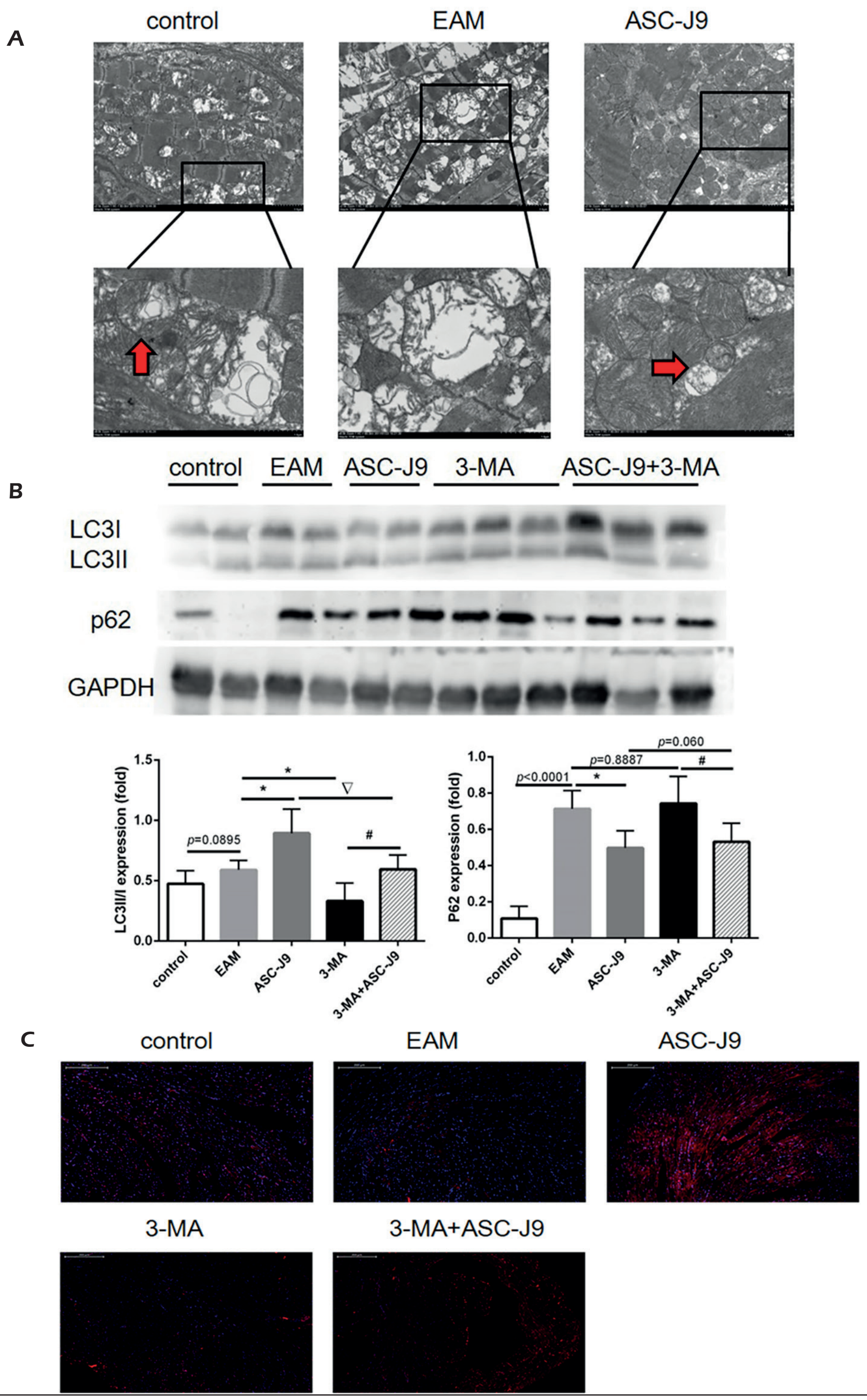

Figure 1. AR inhibition augments autophagic flux in the EAM model. Balb/c mice were randomly divided into control, EAM, EAM+ASC-J9 (ASC-J9), EAM+3-MA (3-MA), and EAM+3-MA+ASC-J9(3-MA+ASC-J9) groups. N=5 in each group. Heart samples were obtained from mice 21 days after immunization. **A,** Transmission electron microscopy images of heart tissues that were fixed and sectioned for the detection of autophagy activity. The autophagosomes were marked as arrows. **B,** Western blot bands and gray value analysis of LC3I, LC3II, p62 and GAPDH. **C,** Immunofluorescence images represent positivity of LC3+ puncta (red) of each group. Scale bar: 200 μm. **p*<0.05 ***p*<0.01, ASC-J9/3-MA group compared with EAM group; #*p*<0.05, 3-MA+ASC-J9 compared with 3-MA group; $^{\triangledown}$*p*<0.05, 3-MA+ASC-J9 compared with ASC-J9 group.

Inhibiting AR Attenuated Inflammation in an Autophagy-Dependent Manner

Our previous study demonstrated that AR inhibition with ASC-J9 alleviated the inflammatory heart response in an EAM model. Next, we sought to determine whether the anti-inflammatory response of ASC-J9 was affected by autophagy levels. Mice immunized with the murine α-MyHC sequence to establish an EAM model showed marked inflammatory infiltration in the mouse myocardium[11]. Histopathological analysis of samples revealed obvious inflammatory cell infiltration in EAM mice, which were dramatically alleviated by ASC-J9 administration (p=0.004). Pre-treatment with 3-MA disrupted the effect of ASC-J9, resulting in a significant increase in inflammatory cell infiltration compared to EAM group (p=0.035). Furthermore, the increased inflammatory cell infiltration in 3-MA group could be reversed by ASC-J9 administration (p=0.011) (Figure 2A). Functionally, we evaluated the dependence of ASC-J9 and autophagy on the expression of inflammatory cytokines. Consistent with our previous results, ASC-J9 significantly decreased the pro-inflammatory cytokines TNF-α (p=0.0038) and iNOS (p=0.004). 3-MA treatment dramatically increased the expression of TNF-α (p=0.0035) and iNOS (p=0.011). The suppressive effect of ASC-J9 on TNF-α (p=0.0051) and iNOS (p=0.0026) was reversed after pre-treatment with 3-MA (Figure 2B).

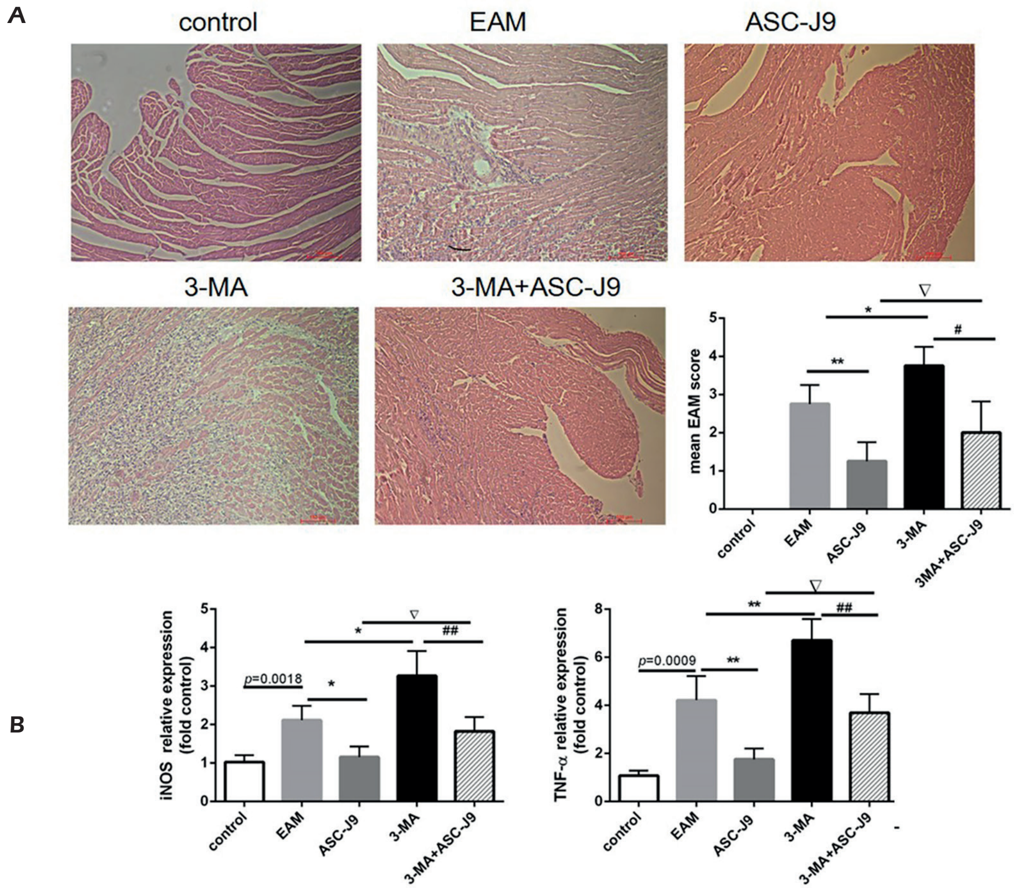

Figure 2. Inhibiting AR attenuates the inflammatory response through the autophagy pathway. Heart samples from each group were obtained on day21 after immunization (n=5 in each group). **A,** H&E stain of heart tissue to detect inflammatory cell infiltration and the analysis of the inflammatory score. Scale bar: 100 µm. **B,** Gene expression of pro-inflammatory cytokines (TNF-α and iNOS) was quantified by using qRT-PCR. *$p<0.05$ **$p<0.001$, ASC-J9/3-MA group compared with EAM group; $^{\#}p<0.05$ $^{\#\#}p<0.001$, 3-MA+ASC-J9 compared with 3-MA group; $^{\nabla}p<0.05$, 3-MA+ASC-J9 compared with ASC-J9 group.

Autophagy Levels in Macrophages with Myocarditis Are Augmented by AR Inhibition

Abrogation of macrophage-localized autophagy has been reported to promote inflammation[10,12,13]. Therefore, we next examined whether ASC-J9 upregulates autophagy in myocardium-infiltrating macrophages. Immunofluorescence microscopy for the detection of the cellular autophagy marker LC3 revealed that treatment with ASC-J9 enhanced LC3 expression, especially in myocardial infiltrating F4/80+ macrophages compared to EAM mice. As noted in the figures, inhibiting autophagy with 3-MA further increased macrophage infiltration, but treatment with ASC-J9 following the 3-MA exposure decreased macrophage infiltration. Moreover, ASC-J9 treatment enhanced macrophage autophagy in the experimental treatment conditions when animals were pre-treated i.p. with 3-MA (Figure 3).

Protection from Apoptosis in an Autophagy-Dependent Manner by AR Inhibition

To determine and quantify the extent of cutaneous tissue damage sustained by animals in the EAM model, the myocardium of each group was subjected to a TUNEL assay to evaluate cell apoptosis. EAM models (p=0.003) showed massive cellular apoptosis that was dramatically curbed by ASC-J9 (p=0.047). Inhibiting autophagy by 3-MA markedly exacerbated tissue damage as illustrated by massive cell apoptosis in the myocardium (p=0.031), which could be minimally reversed when the mice were treated with ASC-J9 (p=0.018) (Figure 4).

Discussion

Autophagy is a cellular process involved in the disposal of damaged organelles, denatured proteins, and invading pathogens through the lysosomal degradation pathway, which has been implicated in a number of fundamental biological processes, including ageing, immunity, development, and differentiation[14-16]. Autophagy has been demonstrated to have a protective role in cardiovascular diseases such as ischemic heart diseases, atherosclerosis, diabetic cardiomyopathy, and myocarditis[17-20]. Recently, AR has been reported to regulate autophagy[21,22], and our previous stu-

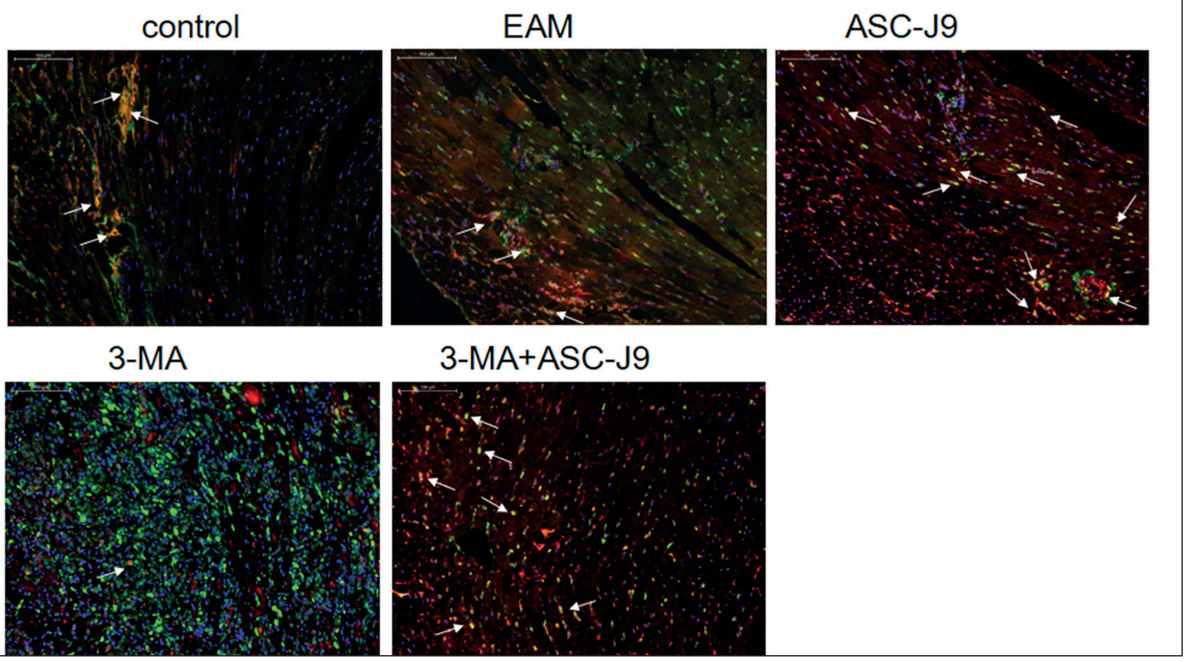

Figure 3. ASC-J9 enhances macrophage-specific autophagy in EAM. Heart tissues were harvested from all treatment groups. The tissue sections were stained for F4/80 (macrophage) and LC3 (autophagy). Representative immunofluorescence images of heart tissue to detect the co-localization of F4/80 (green), LC3 (*red*), (yellow, indicative of co-localization, is marked as *arrows*), and DAPI (*blue*). Scale bar: 100 µm.

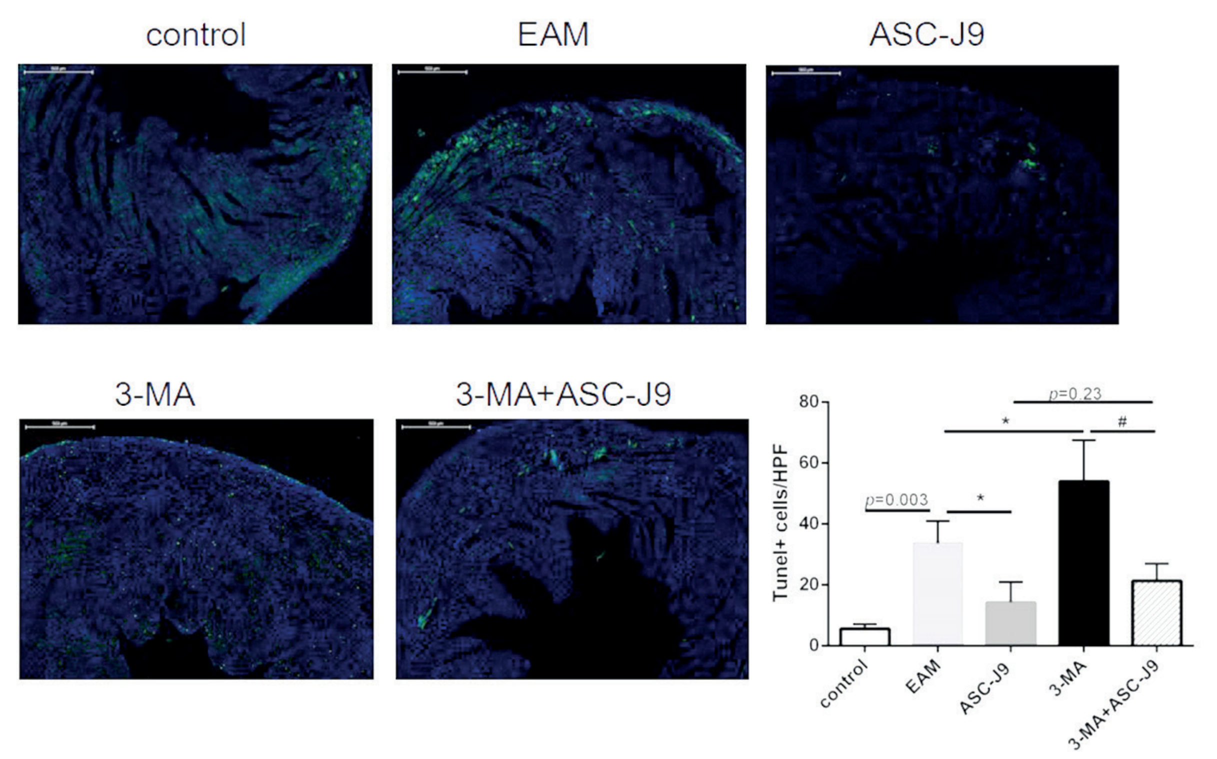

Figure 4. ASC-J9 protects EAM mice from apoptosis via autophagy. Heart tissues were subjected to TUNEL staining to evaluate cellular apoptosis. Representative images of TUNEL+ cells in five different groups (n=5 in each group). Scale bar: 100 µm. *$p<0.05$, ASC-J9/3-MA group compared with EAM group; $^{\#}p<0.05$, 3-MA+ASC-J9 compared with 3-MA group.

dy has proved that inhibiting AR has a protective role in EAM. In this study, we observed that inhibiting AR with ASC-J9 attenuates cardiac inflammation with enhanced autophagy in the myocardium. 3-MA aggravated heart injury and cardiac inflammation in EAM model, which could be reversed by ASC-J9. This indicated that inhibiting AR protect EAM through enhancing autophagy.

Autophagy and autophagy-related proteins can protect cells from pathogen infection by engulfing and killing the pathogen and play an important role in regulating immunity, restricting the activation of inflammasomes, and reducing the secretion of inflammatory cytokines, such as IL-6, IL-8, IL-1β, and TNF-α[23,24]. Myocarditis is an inflammatory disease, and compelling evidence has emphasized the important role of macrophages in myocarditis[25]. Previously, we demonstrated that inhibiting AR with ASC-J9 alleviated heart inflammation by reducing inflammatory cell infiltration and diminishing the expression of pro-inflammatory cytokines[3]. Therefore, we proposed that the inhibitory effect of AR inhibition on inflammatory response could be through the regulation of autophagy. Consistent with our former study, ASC-J9 administration decreased inflammatory cell infiltration and reduced the expression of pro-inflammatory cytokines. In addition, these effects of AR inhibition can be completely reversed by autophagy inhibitors, indicating that inhibition of AR reduces the inflammatory response by regulating the autophagy pathway.

Autophagy and apoptosis are directly related, and the balance between autophagy and apoptosis is crucial for therapeutic interventions. Even though, some studies also figured that excessive autophagic activity can destroy a large fraction of the cytoplasm and organelles, especially mitochondria and the endoplasmic reticulum, resulting in abnormal cell morphology, apoptosis, necrosis, and cellular dysfunction[26]. Generally, induction of autophagy may have a general apoptosis-inhibitory effect[27]. Frequently, autophagy occurs before apoptosis. Recent data indicated that autophagy is required for the maintenance of high ATP levels, which, in turn, may serve to ensure the efficient clearance of apoptotic cells[28]. Some studies[20,29,30] support the idea that induced autophagy decreased apoptosis of cardiomyocytes which play a protective role in cardiovascular diseases. In this study, we found that AR inhibition enhanced autophagy with reduced apoptosis in EAM, and 3-MA further increased the cell apoptosis. Pre-treatment with ASC-J9 alleviated cell apoptosis which was increased by autophagy inhibitor. This indicated that impaired autophagy may accelerate apoptosis, and AR inhibiting reduced apoptosis by upregulating autophagy. Thus, as a possibility, autophagy-dependent optimal clearance of apoptotic cells could prevent a detrimental inflammatory response after exposure to pathological stimuli.

In addition, autophagy has emerged as a potent immune regulator[31,32], especially in macrophages. Augmenting autophagy in macrophages has been shown to protect against acute and chronic organ injury through attenuation of inflammation, promotion of cell survival, and support of tissue remodelling[33-35]. Inhibiting autophagy in macrophages enhanced pro-inflammatory cytokines in response to Toll-like receptor signalling[36]. Our study reveals that inhibiting AR with ASC-J9 enhanced autophagy, especially in macrophages. The ASC-J9-mediated inflammatory response could be reversed by autophagy inhibitors, especially the macrophage-related inflammatory response, which showed dramatic alterations in iNOS expression. The AMPK–mTORC1 autophagy signaling pathway acts as a “master switch” of macrophage functional polarization[37,38], and activation of the AMPK-mTORC1-autophagy pathway facilitates the M1 switch to M2[39]. Moreover, recent studies indicate that impaired autophagy flux contributes to M1 polarization[10,40]. Findings from recent reports[3,41] demonstrated that reducing M1 macrophages could alleviate EAM and that increasing M2 macrophages may protect against EAM. We showed that inhibiting AR with ASC-J9 decreased iNOS, a marker of M1 macrophages, which could be fully reversed by 3-MA, an autophagy inhibitor. This indicated that inhibition of AR with ASC-J9 may reduce M1 macrophages through autophagy.

Conclusions

These findings indicated that AR inhibition could alleviate the inflammatory response and tissue apoptosis by enhancing autophagy, especially in macrophages. Therefore, suppressed AR expression may represent a therapeutic method in myocarditis, especially in males. ASC-J9 may be considered a complementary therapeutic agent to protect against cardiac damage in inflammatory cardiomyopathy.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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