AR Deficiency Inhibits LPS-induced M1 Response in Macrophages by Activating Autophagy

Peng Cheng (✉ chengpengfmmu@126.com)

Institute of Neurosciences, Fourth Military Medical University, Xi'an 710032, China. 2Department of Neurology, 425th Hospital of the People's Liberation Army, Sanya 572000, China. <https://orcid.org/0000-0001-5043-6425>

Jianwei Xie

Department of Neurology, 425th Hospital of PLA

Zhiyong Liu

Department of Neurology, 425th Hospital of PLA

Jian Wang

Institute of Neurosciences, Fourth Military Medical University

---

Research

Keywords: Aldose reductase, Bone marrow-derived macrophages, Macrophage polarization, Autophagy, Ubiquitination, Nuclear factor κ B

Posted Date: December 16th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-127244/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Version of Record: A version of this preprint was published on March 26th, 2021. See the published version at <https://doi.org/10.1186/s13578-021-00576-7>.

Abstract

Macrophage M1 polarization mediates inflammatory responses and tissue damage. Recently, aldose reductase (AR) has been shown to play a critical role in M1 polarization in macrophages. However, the underlying mechanisms are unknown. Here, we demonstrated, for the first time, that AR deficiency repressed the induction of inducible nitric oxide synthase in lipopolysaccharide (LPS)-stimulated macrophages via activation of autophagy. This suppression was related to a defect in the inhibitor of nuclear factor κ B (NF- κ B) kinase (IKK) complex in the classical NF- κ B pathway. However, the mRNA levels of the IKK β and IKK γ were not reduced in LPS-treated AR knockout (KO) macrophages, indicating that their proteins were downregulated at the post-transcriptional level. We discovered that LPS stimuli induced the recruitment of more beclin1 and increased autophagosome formation in AR-deficient macrophages. Blocking autophagy by 3-methyladenine and ammonium chloride treatment restored IKK β and IKK γ protein levels and increased nitric oxide synthase production in LPS-stimulated AR-deficient macrophages. More assembled IKK β and IKK γ undergo ubiquitination and recruit the autophagic adaptor p62 in LPS-induced AR KO macrophages, promoting their delivery to autophagosomes and lysosomes. Collectively, these findings suggest that AR deficiency involves in the regulation of NF- κ B signaling, and extends the role of selective autophagy in fine-tuned M1 macrophage polarization.

Introduction

Macrophages demonstrate significant plasticity and can modify their phenotype and function in response to their microenvironment [1]. Macrophages are roughly categorized into two different subsets, namely, inflammatory M1 and anti-inflammatory M2 macrophages [2]. In response to lipopolysaccharide (LPS) treatment either alone or in combination with proinflammatory cytokines, such as interferon- γ , macrophages undergo M1 polarization, characterized by the expression of pro-inflammatory cytokines and cytotoxic mediators (reactive oxygen and nitrogen species), as well as increased phagocytic and antigen-presenting capacity [3].

Aldose reductase (AR), a rate-limiting enzyme in the polyol pathway that catalyzes the reduction of glucose to sorbitol in the presence of reduced nicotinamide adenine dinucleotide phosphate, has emerged as a molecular target in multiple inflammatory diseases [4]. Ravindranath et al. demonstrated that transgenic mice overexpressing AR show a more pronounced inflammatory response in a cecal ligation and puncture model [5]. In addition, AR inhibition suppresses inflammatory disorders or immune responses in several other models [6–10]. It was reported that M1-polarized human monocyte-derived macrophages expressed significantly higher levels of AR mRNA and AR protein compared with M2-polarized macrophages in vitro [11]. Accumulating evidence implicates the classical nuclear factor- κ B (NF- κ B) signaling pathway in the modulation of M1 macrophage polarization [12–15]. Previously, Zhang et al. reported that the expression of AR is upregulated after spinal cord injury in wild type (WT) mice, while phosphorylated NF- κ B is downregulated, and the number of M1-like macrophages is decreased after spinal cord injury in the AR knockout (KO) mice [16]. However, the exact mechanisms involved are unknown.

Recently, it has been reported that AR deficiency or inhibition enhances autophagy in mouse cardiac myocytes under pathological cardiac hypertrophy or fasting conditions [17, 18]. Autophagy is an essential cell-intrinsic mechanism that affords protection against starvation; moreover, it represents a quality-control system that can deliver damaged organelles, worn-out or misfolded proteins, and invading microorganisms from the cytoplasm to the lysosomes for degradation [19]. Defects in autophagy are linked to many human diseases and to the function of cells of the immune response [20]. Experimentally, Toll-like receptor 4 (TLR4) is a sensor for autophagy associated with innate immunity in macrophages [21].

Herein, we demonstrate that LPS treatment leads to a defect at the IKK complex level and the production of more autophagosomes in AR-deficient macrophages. These findings prompted us to investigate the inherent relationships between these processes.

Materials And Methods

Isolation and culture of BMMs

WT and AR KO C57BL/6J mice were bred at the Laboratory Animal Center of Fourth Military Medical University. Bone marrow cells were isolated from adult mice and processed as described previously [22]. Then the cells were cultured in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 0.001% β -mercaptoethanol, 1% penicillin/streptomycin, 1% HEPES, 10% FBS (Gibco, Carlsbad), and 20% sL929 supernatant from sL929 cells, which secrete macrophage colony-stimulating factor (M-CSF) required for the promotion of hematopoietic stem cells differentiation into macrophages. After 7 days of incubation with conditioned medium, the floating cells were removed, and the viable cells attached to flat-bottomed 6- or 24-well plastic culture plates or 75 cm² culture flasks (Nunc, Roskilde, Denmark) were used to obtain BMMs. Next, the culture media were changed to complete medium, without sL929 cell culture supernatant; the cells were further cultured for 3 days to restore BMMs to their resting state. Flow cytometry analysis of surface antigen showed that 98% of the cultured cells expressed F4/80, a specific marker of macrophages (Fig. 1a).

Reagents

Recombinant mouse LPS was purchased from R&D Systems (Minneapolis, MN, USA). Ammonium chloride (NH₄Cl) and 3-methyladenine (3-MA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The reagents were used at the indicated concentrations.

Flow cytometry

BMMs were incubated in blocking solution (rat serum, 20 min at 4°C) and were subsequently stained with FITC-conjugated anti-F4/80 (1:100, AbD Serotec, Hercules, CA, USA) in the dark for 20 min at 4°C. Acquisitions were performed on a Millipore flow cytometer (Guava 6HT). Subsequent data analyses were

completed using FlowJo software version 7.6.2 (Tree Star, Ashland, OR, USA). Results are expressed as % of positive cells.

Immunofluorescence

Cells were cultured to ~70–80% confluence using glass coverslips in 24-well plates. After treatment, primary BMMs were fixed with 4% paraformaldehyde in phosphate buffer saline (PBS, pH = 7.4) for 30 min and then washed thrice with PBS. Non-specific antibody binding was blocked by incubating the cells for 1 h at room temperature (20–25°C) in PBS containing 5% bovine serum albumin and 0.3% Triton-100, following which the cells were stained with the following primary antibodies overnight at 4°C: mouse anti-AR (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-beclin1 (1:200, Santa Cruz Biotechnology), goat anti-microtubule-associated protein 1 light chain 3 β (LC3 β , 1:200, Santa Cruz Biotechnology), mouse anti-lysosome-associated membrane protein 1 (LAMP1, 1:200, Santa Cruz Biotechnology), rabbit anti-IKK β (1:100, Epitomics, Burlingame, CA, USA), rabbit anti-IKK γ (1:200, Santa Cruz Biotechnology), goat anti-p62 (1:200, Santa Cruz Biotechnology), and mouse anti-ubiquitin specific for K63 (PloyUb, 1:400, Abcam, Cambridge, UK). After washing with PBS, the cells were incubated (for 1 h at room temperature in the dark) with their respective secondary antibodies: fluorescein isothiocyanate-, DyLight 488-, and DyLight 594-conjugated secondary antibodies (1:500~800, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Diamidinophenylindole (DAPI, 1 μ g/ml, Sigma-Aldrich) was applied to visualize the nuclei. Rhodamine-phalloidin (1:250, Life Technologies-Invitrogen, Carlsbad, USA) was used to stain the actin cytoskeleton of the cells. The cells were observed using a laser confocal microscope (FluoView FV1000 MPE, Olympus Corporation, Tokyo, Japan). We applied the JACoP plugin of Image J (National Institutes Health, Bethesda, MD, USA) to perform colocalization analysis of any image pair, and the colocalization rate of the green and red signals was evaluated using Manders' overlap coefficient [23].

Western blotting

The extraction of cellular proteins and their detection by western blotting were performed as previously described [21, 24]. Western blotting was performed using the following primary antibodies: mouse anti-AR, mouse anti-beclin1, goat anti-LC3 β , mouse anti-LAMP1 and rabbit anti-IKK γ antibodies (1:500, Santa Cruz Biotechnology); mouse anti-TLR4, rabbit anti-iNOS and rabbit anti-IKK α (1:1000, Abcam); rabbit anti-IKK β and rabbit anti-phospho-IkBa antibodies (1:1000, Epitomics); rabbit anti-IkBa, rabbit anti-p65, and rabbit anti-phospho-p65 antibodies (1:1000, Cell Signaling Technology, Danvers, MA, USA); mouse anti- β -actin antibodies (1:8000, Sigma-Aldrich). Then blots were incubated with their respective secondary antibodies: horseradish peroxidase-conjugated goat anti-rabbit IgG, goat anti-mouse IgG (both 1:8000, Abcam) and donkey anti-goat IgG (1:5000, Santa Cruz Biotechnology). β -actin was used as the loading control. The immunoreactive bands were scanned using the Bio-Rad ChemiDoc™ XRS⁺ imager with Image Lab™ Software (Bio-Rad Laboratories, CA, USA). Band intensity was quantified using Quantity One software (Bio-Rad Laboratories).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA from BMMs was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. PCR was performed on a Bio-Rad CFX 96™ Real-Time system (Bio-Rad Laboratories) employing a SYBR Green qPCR core reagent kit (DRR081A, Takara Bio Inc., Otsu, Japan). qRT-PCR was performed using the following primers, specific for mouse AR: forward primer, 5'-ACGGCTATGGAACAACATA-3' and reverse primer, 5'-TGTGGCAGTATTCAATCAG-3'; mouse IKK β : forward primer, 5'-GGAGCCTGGGAAATGAAAGAA-3' and reverse primer, 5'-GCCAGAGCCCTACCTGATTG-3'; mouse IKK γ : forward primer, 5'-AAGCACCCCTGGAAGAACC-3' and reverse primer, 5'-CCTGCTCTGAAGGCAGATGTA-3'; and mouse β -actin: forward primer, 5'-CGTGCGTGACATCAAAGAGAA-3' and reverse primer, 5'-GCTCGTTGCCAATAGTGATGA-3'. Gene expression was normalized using β -actin as an internal control, and fold changes were calculated.

Transmission electron microscopy

Cells were grown to ~80% confluence in 75 cm² culture flasks. After stimulation, BMMs were harvested and centrifuged at 1,500 g for 10 min. The pellets were fixed in a mixture of 4% paraformaldehyde and 0.05% glutaraldehyde, post-fixed in 0.5% osmium tetroxide, dehydrated in graded ethanol series and propylene oxide, and embedded in epoxy resin. Ultrathin sections were obtained using an ultramicrotome (EM UC6, Leica Microsystems, Baden-Württemberg, Germany); they were then mounted on mesh grids (6–8 sections/grid) and counter-stained with uranyl acetate and lead citrate. The sections were viewed using a transmission electron microscope (JEM-1230, JEOL, Tokyo, Japan) equipped with a Gatan digital camera.

Statistical analysis

All data are expressed as the mean $\pm$ SD. Statistically significant differences between the mean values were determined by two-tailed Student's t-test. Differences at $P < 0.05$ were considered statistically significant. * $P < 0.05$; ** $P < 0.01$.

Results

LPS stimulation upregulates AR expression in BMMs

It has been reported that AR can regulate inflammatory signals and immune responses in several animal disease models [5–8]. Since inflammatory signals and immune responses are tightly correlated with macrophage polarization, we investigated changes in the expression of AR upon LPS stimulation in BMMs from WT C57BL/6 mice.

We used the immunofluorescence staining to label AR in BMMs. The number of AR-immunoreactive profiles in BMMs was significantly increased after treatment with LPS for 24 h (Fig. 1b). We next used qRT-PCR and western blotting to detect the changes of AR mRNA and protein in LPS-stimulated BMMs.

The transcription and expression of AR were both significantly increased following treatment for 24 h (Fig. 1c, d). Collectively, these results indicate that AR expression is triggered upon stimulation of BMMs with LPS.

AR deficiency suppresses the M1 response and NF- κ B activation at the IKK complex level in LPS-stimulated BMMs

In response to LPS treatment, macrophages undergo M1 polarization, characterized by the expression of inducible nitric oxide synthase (iNOS) [25]. Activation of the canonical NF- κ B signaling pathway in response to LPS was demonstrated by monitoring iNOS expression, through immunoblot analysis, and nitric oxide (NO) production—important downstream products of LPS signaling in macrophages [26].

We hypothesized the existence of a relationship between AR and the NF- κ B p65 signaling pathway. To test this hypothesis, we carried out experiments on BMMs from WT or AR KO mice. Stimulation of BMMs with 500 ng/ml LPS increased the levels of iNOS in a time-dependent manner during the first 32 h (Fig. 2a). Expression of iNOS was peaked at 16 h after LPS treatment (Fig. 2a). The induction of iNOS upon LPS stimulation decreased markedly when the AR gene was knocked out (Fig. 2a). These results suggested that the LPS-stimulated induction of iNOS was closely associated with AR.

For most inducers of the classical NF- κ B signaling pathway, IKK α is not required for the phosphorylation of I κ B α ; however, genetic studies have shown that NF- κ B p65 signaling requires IKK β and IKK γ to phosphorylate I κ B α [27]. The canonical NF- κ B pathway uses several important components, including TLR4, IKK β , IKK γ , I κ B α , and p65 [28–30]. Following 16 h of LPS exposure, we detected lower levels of IKK β , IKK γ , phospho-I κ B α (p-I κ B α), phospho-p65 (p-p65), and iNOS proteins in AR KO BMMs than in control cells (Fig. 2b). We used qRT-PCR to measure the mRNA levels of IKK β and IKK γ in the total RNA obtained from the BMMs after 16 h of treatment with or without LPS. The results showed that their mRNA levels were not affected in the LPS-treated AR KO BMMs (Fig. 2c). The above data indicated that the suppression of the M1 response in AR-deficient macrophages after LPS stimulation was due to the post-transcriptional degradation of IKK β and IKK γ . To determine the temporal patterns of change in the IKK β and IKK γ protein levels, we stimulated BMMs from WT or AR KO mice with LPS for 32 h and immunoblotted the lysates for IKK β and IKK γ . The time-course experiments revealed that LPS induced a gradual reduction of IKK β and IKK γ proteins in BMMs from AR KO mice (Fig. 2d).

AR deficiency enhances autophagosome formation and maturation in LPS-stimulated BMMs

To determine whether autophagosome formation occurred because of AR deficiency, we treated BMMs from WT or AR KO mice with or without LPS. Beclin1 is a key component of the class III phosphatidylinositol 3-kinase (PI3K) complex, which initiates autophagosome formation [31]. Incubation of BMMs with 500 ng/ml LPS for 16 h led to the recruitment of beclin1, as shown in the change in beclin1 staining from a diffuse to punctate pattern (Fig. 3a). We then conducted a detailed quantitative analysis of LPS-stimulated beclin1 aggregation. LPS induced a significant increase in the number of beclin1⁺

profiles in AR KO cells (Fig. 3a). The increase in beclin1 levels was also confirmed by immunoblotting (Fig. 3b).

In macroautophagy, cells form double-membraned vesicles, known as autophagosomes, around a portion of the cytoplasm [32]. Two forms of LC3, LC3^I and LC3^{II}, are produced post-transcriptionally [33]. LC3^I is cytosolic, whereas LC3^{II} is membrane-bound and mainly enriched in the autophagosomal vacuoles [34]. The autophagic vacuoles can be imaged using confocal microscopy by virtue of immunofluorescence staining for LC3, which delineates them in the cytoplasm [35]. We then conducted a detailed quantitative analysis of LPS-stimulated autophagy. Without LPS treatment, AR KO macrophages exhibited more LC3⁺ vacuoles compared to WT cells (Fig. 3c). After LPS induction, there was a significant increase in the number of autophagosomes in AR KO BMMs (Fig. 3c). The increase in LC3^{II} was confirmed by immunoblotting with an LC3 antibody (Fig. 3d). To observe the temporal pattern of autophagy within 32 h, we immunoblotted lysates from LPS-stimulated WT or AR KO BMMs for LC3. The time-course experiment showed that LPS treatment significantly increased LC3^{II} protein abundance in AR KO cells compared with WT controls within 32 h (Fig. 3e).

As their maturation proceeds, autophagosomes fuse with lysosomes, resulting in the degradation of their contents [36]. LAMP1 is widely used as a lysosome marker. The merging of LC3 with LAMP1 indicates the presence of mature autophagosomes (autolysosomes). Immunoblotting showed that LAMP1 was significantly upregulated in LPS-stimulated AR KO cells (Fig. 3f). In AR KO BMMs, LPS promoted the maturation of autophagosomes as evidenced by an increase in the colocalization of LC3 with LAMP1 (Fig. 3g).

Transmission electron microscope was also used to examine the effects of LPS on autophagy in BMMs from WT or AR KO mice. The number of double-membrane vacuoles, a typical feature of autophagosomes, was markedly increased in non-stimulated AR KO macrophages compared with that in WT cells; however, the fusion of primary lysosome and autophagosome, a typical feature of autolysosomes, was rare in AR KO and WT macrophages without LPS stimulation (Fig. 3h). Furthermore, it was observed that LPS treatment induced more autophagosomes and autolysosomes in AR-deficient cells compared to controls (Fig. 3h).

Collectively, these results indicate that AR deficiency promotes autophagosome biogenesis in non-stimulated macrophages, and further strengthens biogenesis and maturation in LPS-activated BMMs.

AR deficiency mediates autophagic degradation of IKK β and IKK γ in LPS-treated BMMs

The addition of the PI3K inhibitor 3-MA has been shown to block autophagosome formation [37]. Our results showed that 3-MA (3 mM) was able to block LPS-stimulated autophagy as detected by LC3 immunofluorescence (Fig. 4a) and immunoblotting (Fig. 4b). NH₄Cl is a commonly used lysosome inhibitor. It localizes to acidic vesicles and impairs lysosomal acidification and protease activity, resulting in the accumulation of autophagosomes by impairing their fusion with lysosomes [38]. Incubation of the

cells with 10 mM NH₄Cl for 16 h resulted in the accumulation of LC3⁺ vacuoles (Fig. 4c) and a large increase in LC3 levels (Fig. 4d) in LPS-stimulated macrophages.

To investigate the mechanisms underlying the observed downregulation of key components of the IKK complex and enhanced autophagy, we analyzed the protein levels of IKK β and IKK γ by immunoblotting in LPS-stimulated BMMs after inhibiting autophagy using 3-MA. Our results revealed that the protein levels of IKK β and IKK γ were rescued following 3-MA administration in LPS-treated AR-deficient cells (Fig. 4c). The induction of iNOS in LPS-treated AR KO macrophages was also significantly increased after 3-MA treatment (Fig. 4c). This was further supported by our data showing that the protein levels of IKK β and IKK γ were restored as reflected by the increased levels of iNOS in LPS-stimulated AR-deficient cells upon addition of NH₄Cl (Fig. 4d). These results suggest that IKK β and IKK γ were loaded into autophagosomes and subsequently degraded in lysosomes, which limited iNOS induction and M1 polarization in LPS-activated AR-deficient BMMs.

AR deficiency facilitates IKK β and IKK γ to colocalize with autophagosomes and lysosomes in LPS-stimulated BMMs

In the initial phase of autophagy, beclin1 serves as a platform to recruit autophagy activators and inhibitors [39]. We immunostained BMMs for IKK β , IKK γ and beclin1 after 1 h of LPS incubation. We showed that more intracellular structures were labeled by IKK β and IKK γ colocalized with beclin1⁺ foci in AR KO BMMs after exposure to LPS (Fig. 5a, b). These results indicate that in the initial induction of autophagy, IKK β and IKK γ are recruited by beclin1.

To verify that IKK β entered the autophagosomes, we used immunocytochemistry to label IKK β and autophagic vacuoles. We observed autophagosome formation 1 h after treatment with LPS. Autophagosome induction was poor and few overlapping IKK β -labeled structures and LC3-labeled puncta were observed in resting macrophages (Fig. 5c). Upon LPS stimulation, a higher number of IKK β ⁺ structures colocalized with LC3⁺ vacuoles in AR KO macrophages compared to WT macrophages (Fig. 5c). Similarly, AR knockout increased LC3 staining and its colocalization with IKK γ in LPS-treated BMMs (Fig. 5d).

Next, we detected higher levels of colocalization between the lysosome marker LAMP1 and IKK β and IKK γ in LPS-induced AR KO BMMs (Fig. 5e, f).

These results indicate that after recruitment by Beclin1, IKK β and IKK γ can be engulfed by autophagosomes and probably be degraded after fusion with lysosomes.

AR deficiency, polyubiquitination and p62 co-mediate the autophagic degradation of IKK β and IKK γ in LPS-stimulated BMMs

Autophagic adaptors represent a mechanism through which intracellular targets are delivered to autophagosomes [40]. An adaptor protein, p62, recognizes polyubiquitinated targets and binds to the

ubiquitin-like autophagosome membrane LC3 in the autophagic degradation pathway [41]. It has been shown that TLR4-mediated autophagy is a p62-dependent type of selective autophagy in macrophages [42]. K63-linked polyubiquitination has also been associated with the formation and autophagic degradation of protein inclusions [43]. Interestingly, the preferential binding of p62 to K63-linked chains constitute a further level of regulation during selective autophagy [44].

Confocal microscopy of BMMs immunostained for PolyUb (linkage-specific K63) and IKK β showed that in the absence of LPS stimulation, ubiquitin resided mainly in the cytosol where it appeared as small PolyUb⁺ dots, whereas IKK β showed an occasional granular pattern (Fig. 6a). Treatment of BMMs for 1 h with LPS resulted in the formation of large IKK β aggregates colocalized with PolyUb (Fig. 6a). Confocal microscopy experiments further suggested that in stimulated WT cells, only a small fraction of IKK β was colocalized with PolyUb⁺; contrastingly, in LPS-treated AR KO cells, the overlaps between IKK β and PolyUb⁺ dots were markedly increased (Fig. 6a). Similarly, we found that LPS stimulation induced the recruitment of IKK γ to PolyUb⁺ structures, and the colocalization of IKK γ and PolyUb was markedly increased in AR KO cells (Fig. 6b).

Next, we treated primary macrophages with or without LPS and immunostained the cells for p62 and IKK β . We noted that only a small fraction of p62 was associated with IKK β in non-stimulated and LPS-treated WT cells, but this association was greatly increased in LPS-stimulated AR-deficient BMMs (Fig. 6c). Consistently, LPS-induced interaction between IKK γ and p62 was promoted by the knockout of AR in BMMs (Fig. 6d). These data indicated that IKK β and IKK γ aggregates were polyubiquitinated and they could be targeted by p62 to the autophagy pathway in LPS-induced AR-deficient macrophages.

Discussion

Our results showed that AR deficiency led to the suppression of the M1 phenotype in macrophages, limiting the pro-inflammatory activity through the degradation of key component of the IKK complex. Knockout of AR triggered autophagosome formation; exposure of AR KO macrophages to LPS markedly enhanced autophagosome biogenesis and maturation. Autophagy was able to capture and degrade IKK β and IKK γ through polyubiquitination, leading to the recruitment of p62 and LC3. Autophagosome formation in the AR knockout background was functionally important; pharmacological blockade of autophagy pathway increased the protein levels of IKK β and IKK γ and enhanced the production of iNOS. These data suggested a close relationship among AR, the M1 response, and autophagy.

There are two main NF- κ B pathways in the cell. The noncanonical NF- κ B signaling pathway depends on IKK α and plays a critical role in the development of lymphoid organs responsible for the generation of B and T lymphocytes [45]. Neither IKK β nor IKK γ deficiency affects this pathway [45]. However, the canonical pathway, induced by most physiological NF- κ B stimuli, depends on IKK β and IKK γ ; the activation of this pathway mainly leads to the phosphorylation of I κ B α and nuclear translocation of mostly p65-containing heterodimers [28, 46]. However, IKK γ deficiency appears to have a greater impact on the classical NF- κ B signaling pathway than IKK β deficiency [45]. It has been reported that cells lacking

IKK γ have no detectable NF- κ B response to almost any pro-inflammatory or immune regulatory stimuli [47]. Our results showed that the exposure of AR KO macrophages to LPS impaired IKK β and IKK γ . This may be attributed to the suppression of the TLR4/NF- κ B signaling pathway activation, iNOS induction and M1 polarization in AR-deficient BMMs.

We investigated the mechanism through which certain signals downregulated IKK β and IKK γ proteins without affecting their mRNA levels in AR KO macrophages after LPS treatment. We speculated that IKK β and IKK γ protein levels were decreased at the post-transcriptional level in AR KO macrophages upon LPS stimulation. We found that the exposure of AR KO macrophages to LPS markedly enhanced autophagosome formation and maturation. Furthermore, we investigated the relationship between the impairment of the IKK complex and autophagosome formation. We observed massive colocalization of IKK β , IKK γ , LC3⁺ autophagic vacuoles, and LAMP1⁺ lysosomes in BMMs from AR KO mice after LPS stimulation. Furthermore, our finding that AR deficiency led to the polyubiquitination of IKK β and IKK γ provides a possible answer. The K63-ubiquitinated IKK β and IKK γ can recruit p62, which can deliver them to the autophagy pathway through its LC3-binding domain. Conversely, pharmacological suppression of autophagy partially restored IKK β and IKK γ protein levels and iNOS induction.

Thus, manipulation of AR expression or activity may have a therapeutic potential by regulating macrophage polarization for inflammatory diseases.

Declarations

Acknowledgment

We thank Professor Sookja Kim Chung for providing us the AR KO mice. We would like to thank Editage (www.editage.cn) for English language editing.

Funding

This study was supported by Key Research and Development Project of Hainan Province [ZDYF2019168].

Author contributions

PC and JW conceived and designed the experiments. PC, JWX and ZYL performed the experiments. PC and JW wrote and edited the manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent participate

The animal studies were approved by the Animal Care and Use Committee of Fourth Military Medical University and were conducted in accordance with the National Institutes of Health guidelines for the care

and use of laboratory animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest*. 2012;122(3):787–95.
2. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol*. 2011;11(11):723–37.
3. Funes SC, Rios M, Escobar-Vera J, Kalergis AM. Implications of macrophage polarization in autoimmunity. *Immunology*. 2018;154(2):186–95.
4. Ramana KV, Srivastava SK. Aldose reductase: a novel therapeutic target for inflammatory pathologies. *Int J Biochem Cell Biol*. 2010;42(1):17–20.
5. Ravindranath TM, Mong PY, Ananthakrishnan R, Li Q, Quadri N, Schmidt AM, Ramasamy R, et al. Novel role for aldose reductase in mediating acute inflammatory responses in the lung. *J Immunol*. 2009;183(12):8128–37.
6. Shoeb M, Yadav UC, Srivastava SK, Ramana KV. Inhibition of aldose reductase prevents endotoxin-induced inflammation by regulating the arachidonic acid pathway in murine macrophages. *Free Radic Biol Med*. 2011;51(9):1686–96.
7. Yadav UC, Ramana KV, Aguilera-Aguirre L, Boldogh I, Boulares HA, Srivastava SK. Inhibition of aldose reductase prevents experimental allergic airway inflammation in mice. *PLoS One*. 2009;4(8):e6535.
8. Ramana KV, Srivastava SK. Mediation of aldose reductase in lipopolysaccharide-induced inflammatory signals in mouse peritoneal macrophages. *Cytokine*. 2006;36(3–4):115–22.
9. Ballekova J, Soltesova-Prnova M, Majekova M, Stefek M. Does inhibition of aldose reductase contribute to the anti-inflammatory action of setipiprant? *Physiol Res*. 2017;66(4):687–93.
10. Yadav UC, Shoeb M, Srivastava SK, Ramana KV. Aldose reductase deficiency protects from autoimmune- and endotoxin-induced uveitis in mice. *Invest Ophthalmol Vis Sci*. 2011;52(11):8076–85.
11. Erbel C, Rupp G, Domschke G, Linden F, Akhavanpoor M, Doesch AO, Katus HA, et al. Differential regulation of aldose reductase expression during macrophage polarization depends on hyperglycemia. *Innate Immun*. 2016;22(3):230–7.
12. Luo D, Guo Y, Cheng Y, Zhao J, Wang Y, Rong J. Natural product celastrol suppressed macrophage M1 polarization against inflammation in diet-induced obese mice via regulating Nrf2/HO-1, MAP

- kinase and NF-kappaB pathways. *Aging*. 2017;9(10):2069–82.
13. Chiang CF, Chao TT, Su YF, Hsu CC, Chien CY, Chiu KC, Shiah SG, et al. Metformin-treated cancer cells modulate macrophage polarization through AMPK-NF-kappaB signaling. *Oncotarget*. 2017;8(13):20706–18.
  14. Tian L, Li W, Yang L, Chang N, Fan X, Ji X, Xie J, et al. Cannabinoid Receptor 1 Participates in Liver Inflammation by Promoting M1 Macrophage Polarization via RhoA/NF-kappaB p65 and ERK1/2 Pathways, Respectively, in Mouse Liver Fibrogenesis. *Front Immunol*. 2017;8:1214.
  15. He Y, Ma X, Li D, Hao J. Thiamet G mediates neuroprotection in experimental stroke by modulating microglia/macrophage polarization and inhibiting NF-kappaB p65 signaling. *J Cereb Blood Flow Metab*. 2017;37(8):2938–51.
  16. Zhang Q, Bian G, Chen P, Liu L, Yu C, Liu F, Xue Q, et al. Aldose Reductase Regulates Microglia/Macrophages Polarization Through the cAMP Response Element-Binding Protein After Spinal Cord Injury in Mice. *Mol Neurobiol*. 2016;53(1):662–76.
  17. Baba SP, Zhang D, Singh M, Dassanayaka S, Xie Z, Jagatheesan G, Zhao J, et al. Deficiency of aldose reductase exacerbates early pressure overload-induced cardiac dysfunction and autophagy in mice. *J Mol Cell Cardiol*. 2018;118:183–92.
  18. Zhang D, Bhatnagar A, Baba SP. Inhibition of aldose reductase activity stimulates starvation induced autophagy and clears aldehyde protein adducts. *Chem Biol Interact*. 2019;306:104–09.
  19. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. *J Pathol*. 2010;221(1):3–12.
  20. Shi CS, Shenderov K, Huang NN, Kabat J, Abu-Asab M, Fitzgerald KA, Sher A, et al. Activation of autophagy by inflammatory signals limits IL-1beta production by targeting ubiquitinated inflammasomes for destruction. *Nat Immunol*. 2012;13(3):255–63.
  21. Xu Y, Jagannath C, Liu XD, Sharafkhaneh A, Kolodziejska KE, Eissa NT. Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. *Immunity*. 2007;27(1):135–44.
  22. Tateyama H, Murase Y, Higuchi H, Inasaka Y, Kaneoka H, Iijima S, Nishijima KI. Siglec-F is induced by granulocyte-macrophage colony-stimulating factor and enhances interleukin-4-induced expression of arginase-1 in mouse macrophages. *Immunology*. 2019;158(4):340–52.
  23. Bolte S, Cordelieres FP. A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc*. 2006;224(Pt 3):213–32.
  24. Cheng P, Kuang F, Ju G. Aescin reduces oxidative stress and provides neuroprotection in experimental traumatic spinal cord injury. *Free Radic Biol Med*. 2016;99:405–17.
  25. McWhorter FY, Davis CT, Liu WF. Physical and mechanical regulation of macrophage phenotype and function. *Cell Mol Life Sci*. 2015;72(7):1303–16.
  26. Musial A, Eissa NT. Inducible nitric-oxide synthase is regulated by the proteasome degradation pathway. *J Biol Chem*. 2001;276(26):24268–73.

27. Gray CM, Remouchamps C, McCorkell KA, Solt LA, Dejardin E, Orange JS, May MJ. Noncanonical NF-kappaB signaling is limited by classical NF-kappaB activity. *Sci Signal*. 2014;7(311):ra13.
28. Bonizzi G, Karin M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol*. 2004;25(6):280–8.
29. Adli M, Merkhofer E, Cogswell P, Baldwin AS. IKKalpha and IKKbeta each function to regulate NF-kappaB activation in the TNF-induced/canonical pathway. *PLoS One*. 2010;5(2):e9428.
30. Rothwarf DM, Zandi E, Natoli G, Karin M. IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex. *Nature*. 1998;395(6699):297–300.
31. Shi CS, Kehrl JH. MyD88 and Trif target Beclin 1 to trigger autophagy in macrophages. *J Biol Chem*. 2008;283(48):33175–82.
32. Tanida I. Autophagosome formation and molecular mechanism of autophagy. *Antioxid Redox Signal*. 2011;14(11):2201–14.
33. Tanida I, Ueno T, Kominami E. LC3 conjugation system in mammalian autophagy. *Int J Biochem Cell Biol*. 2004;36(12):2503–18.
34. Tanida I, Minematsu-Ikeguchi N, Ueno T, Kominami E. Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. *Autophagy*. 2005;1(2):84–91.
35. Tanida I, Waguri S. Measurement of autophagy in cells and tissues. *Methods Mol Biol*. 2010;648:193–214.
36. Zhao YG, Zhang H. Autophagosome maturation: An epic journey from the ER to lysosomes. *J Cell Biol*. 2019;218(3):757–70.
37. Pasquier B. Autophagy inhibitors. *Cell Mol Life Sci*. 2016;73(5):985–1001.
38. Calvo-Garrido J, Carilla-Latorre S, Mesquita A, Escalante R. A proteolytic cleavage assay to monitor autophagy in *Dictyostelium discoideum*. *Autophagy*. 2011;7(9):1063–8.
39. Kihara A, Noda T, Ishihara N, Ohsumi Y. Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in *Saccharomyces cerevisiae*. *J Cell Biol*. 2001;152(3):519–30.
40. Lamark T, Svenning S, Johansen T. Regulation of selective autophagy: the p62/SQSTM1 paradigm. *Essays Biochem*. 2017;61(6):609–24.
41. Katsuragi Y, Ichimura Y, Komatsu M. p62/SQSTM1 functions as a signaling hub and an autophagy adaptor. *FEBS J*. 2015;282(24):4672–8.
42. Fujita K, Srinivasula SM. TLR4-mediated autophagy in macrophages is a p62-dependent type of selective autophagy of aggresome-like induced structures (ALIS). *Autophagy*. 2011;7(5):552–4.
43. Tan JM, Wong ES, Kirkpatrick DS, Pletnikova O, Ko HS, Tay SP, Ho MW, et al. Lysine 63-linked ubiquitination promotes the formation and autophagic clearance of protein inclusions associated with neurodegenerative diseases. *Hum Mol Genet*. 2008;17(3):431–9.
44. Zaffagnini G, Martens S. Mechanisms of Selective Autophagy. *J Mol Biol*. 2016;428(9 Pt A):1714–24.

45. Liu F, Xia Y, Parker AS, Verma IM. IKK biology. *Immunol Rev.* 2012;246(1):239–53.
46. Oeckinghaus A, Hayden MS, Ghosh S. Crosstalk in NF-kappaB signaling pathways. *Nat Immunol.* 2011;12(8):695–708.
47. Yamaoka S, Courtois G, Bessia C, Whiteside ST, Weil R, Agou F, Kirk HE, et al. Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. *Cell.* 1998;93(7):1231–40.

Figures

Figure 1

LPS stimulation upregulates AR expression in BMMs. **a** Following induction of BMMs from the bone marrow, approximately 98% of the cells were F4/80-positive. **b** Confocal microscopy of BMMs from WT mice left untreated or treated for 24 h with LPS (500 ng/ml), and then immunostained for AR. Scale bars =10 μ m. Right graph shows quantification analysis of the number of AR⁺ foci per cell. **c**, **d** AR mRNA and protein levels of BMMs from WT mice left untreated or treated for 24 h with 500 ng/ml LPS. The

transcription is expressed as fold of control, and densitometric AR/ β -actin ratios are shown below the blot. Data are expressed as the mean $\pm$ SD from three independent experiments, *P < 0.05, **P < 0.01. Data are representative of three independent experiments.

Figure 1

LPS stimulation upregulates AR expression in BMMs. a Following induction of BMMs from the bone marrow, approximately 98% of the cells were F4/80-positive. b Confocal microscopy of BMMs from WT

mice left untreated or treated for 24 h with LPS (500 ng/ml), and then immunostained for AR. Scale bars =10 μ m. Right graph shows quantification analysis of the number of AR+ foci per cell. c, d AR mRNA and protein levels of BMMs from WT mice left untreated or treated for 24 h with 500 ng/ml LPS. The transcription is expressed as fold of control, and densitometric AR/ β -actin ratios are shown below the blot. Data are expressed as the mean $\pm$ SD from three independent experiments, *P < 0.05, **P < 0.01. Data are representative of three independent experiments.

Figure 2

Stimulation of AR-deficient macrophages with LPS decreases the protein levels of IKK β and IKK γ . a BMMs from WT or AR KO mice were treated with 500 ng/ml LPS for the indicated time periods, and the production of iNOS was assayed. b Effect of AR deficiency on several important components of the TLR4/NF- κ B signaling pathway. Immunoblot analysis for TLR4, IKK α , IKK β , IKK γ , I κ B α , p-I κ B α , p65, p-p65, and iNOS in BMMs from WT or AR KO mice treated with or without 500 ng/ml LPS for 16 h. c qRT-PCR of IKK β and IKK γ mRNA in BMMs from WT or AR KO mice treated with or without 500 ng/ml LPS for 16 h. The transcription is expressed as fold of control. d BMMs from WT or AR KO mice were treated with 500 ng/ml LPS for the indicated time periods. Data represent the mean $\pm$ SD of three different experiments. Data are representative of three independent experiments.

Figure 2

Stimulation of AR-deficient macrophages with LPS decreases the protein levels of IKK β and IKK γ . a BMMs from WT or AR KO mice were treated with 500 ng/ml LPS for the indicated time periods, and the production of iNOS was assayed. b Effect of AR deficiency on several important components of the TLR4/NF- κ B signaling pathway. Immunoblot analysis for TLR4, IKK α , IKK β , IKK γ , I κ B α , p-I κ B α , p65, p-p65, and iNOS in BMMs from WT or AR KO mice treated with or without 500 ng/ml LPS for 16 h. c qRT-PCR of

IKK β and IKK γ mRNA in BMMs from WT or AR KO mice treated with or without 500 ng/ml LPS for 16 h. The transcription is expressed as fold of control. d BMMs from WT or AR KO mice were treated with 500 ng/ml LPS for the indicated time periods. Data represent the mean $\pm$ SD of three different experiments. Data are representative of three independent experiments.

Figure 3

AR deficiency enhances autophagosome formation and maturation in LPS-stimulated macrophages. a BMMs from WT or AR KO mice were treated with or without 500 ng/ml LPS for 16 h and then immunostained for beclin1. Right graph represents quantification analysis of the number of beclin1+ foci per cell. Scale bars = 50 μ m. b Immunoblot analysis of beclin1 in BMMs from WT or AR KO mice treated with or without 500 ng/ml LPS for 16 h. c Induction of LC3+ vacuoles in BMMs from WT or AR KO mice treated with or without 500 ng/ml LPS for 16 h and then immunostained for LC3. Right graph represents quantification analysis of the number of LC3+ puncta per cell. Scale bars = 20 μ m. d Immunoblot analysis of LC3 β in BMMs from WT or AR KO mice treated with or without 500 ng/ml LPS for 16 h. e Time-course analysis of autophagy in macrophages during the 32 h of LPS stimulation. BMMs from WT or AR KO mice were treated with 500 ng/ml LPS for the indicated durations. f Immunoblot analysis of LAMP1 in BMMs from WT or AR KO mice treated with or without LPS for 16 h. g Confocal microscopy of BMMs from WT or AR KO mice treated with or without 500 ng/ml LPS 16 h and immunostained for LC3 and LAMP1. Scale bars = 20 μ m. Right graphs represent colocalization of LAMP1 and LC3 signals evaluated using Manders' overlap coefficient. h Ultrastructural analysis of autophagy by transmission electron microscopy in BMMs. WT or AR KO BMMs were treated with or without 500 ng/ml LPS for 16 h. The panel on the right is the enlargement of the outlined area on the left. Scale bars = 1 μ m. Right graphs represent quantification of the number of autophagosomes and autolysosomes per cross-sectioned cell. Data are expressed as the mean $\pm$ SD from three independent experiments, *P < 0.05, **P < 0.01. Data are representative of three independent experiments.

Figure 3

AR deficiency enhances autophagosome formation and maturation in LPS-stimulated macrophages. a BMMs from WT or AR KO mice were treated with or without 500 ng/ml LPS for 16 h and then immunostained for beclin1. Right graph represents quantification analysis of the number of beclin1+ foci per cell. Scale bars = 50 μm. b Immunoblot analysis of beclin1 in BMMs from WT or AR KO mice treated with or without 500 ng/ml LPS for 16 h. c Induction of LC3+ vacuoles in BMMs from WT or AR KO mice

treated with or without 500 ng/ml LPS for 16 h and then immunostained for LC3. Right graph represents quantification analysis of the number of LC3+ puncta per cell. Scale bars = 20 μ m. d Immunoblot analysis of LC3 β in BMMs from WT or AR KO mice treated with or without 500 ng/ml LPS for 16 h. e Time-course analysis of autophagy in macrophages during the 32 h of LPS stimulation. BMMs from WT or AR KO mice were treated with 500 ng/ml LPS for the indicated durations. f Immunoblot analysis of LAMP1 in BMMs from WT or AR KO mice treated with or without LPS for 16 h. g Confocal microscopy of BMMs from WT or AR KO mice treated with or without 500 ng/ml LPS 16 h and immunostained for LC3 and LAMP1. Scale bars = 20 μ m. Right graphs represent colocalization of LAMP1 and LC3 signals evaluated using Manders' overlap coefficient. h Ultrastructural analysis of autophagy by transmission electron microscopy in BMMs. WT or AR KO BMMs were treated with or without 500 ng/ml LPS for 16 h. The panel on the right is the enlargement of the outlined area on the left. Scale bars = 1 μ m. Right graphs represent quantification of the number of autophagosomes and autolysosomes per cross-sectioned cell. Data are expressed as the mean $\pm$ SD from three independent experiments, *P < 0.05, **P < 0.01. Data are representative of three independent experiments.

Figure 4

AR deficiency mediates the autophagic degradation of IKKβ and IKKγ in LPS-stimulated macrophages. a, c WT or AR KO BMMs were incubated for 16 h in the presence of LPS (500 ng/ml), LPS+3-MA (3 mM), or LPS+NH₄Cl (10 mM), followed by immunostaining for LC3. Lower graphs represent quantification of the number of autophagosomes per cell. Scale bars = 20 μm. b, d Immunoblot analysis for LC3, IKKβ, IKKγ, and iNOS in lysates of WT or AR KO BMMs treated with 500 ng/ml LPS for 16 h in the presence or

absence of 3-MA (3 mM) or NH₄Cl (10 mM). Data are expressed as the mean $\pm$ SD from three independent experiments. *P < 0.05, **P < 0.01. Data are representative of three independent experiments.

Figure 4

AR deficiency mediates the autophagic degradation of IKKβ and IKKγ in LPS-stimulated macrophages. a, c WT or AR KO BMMs were incubated for 16 h in the presence of LPS (500 ng/ml), LPS+3-MA (3 mM), or LPS+NH₄Cl (10 mM), followed by immunostaining for LC3. Lower graphs represent quantification of the

number of autophagosomes per cell. Scale bars = 20 μ m. b, d Immunoblot analysis for LC3, IKK β , IKK γ , and iNOS in lysates of WT or AR KO BMMs treated with 500 ng/ml LPS for 16 h in the presence or absence of 3-MA (3 mM) or NH4Cl (10 mM). Data are expressed as the mean $\pm$ SD from three independent experiments. *P < 0.05, **P < 0.01. Data are representative of three independent experiments.

Figure 5

AR deficiency facilitates IKK β and IKK γ to colocalize with autophagosomes and lysosomes in LPS-stimulated macrophages. Confocal microscopy of BMMs from WT or AR KO mice treated with or without 500 ng/ml LPS 1 h and costained for a beclin1 and IKK β , b beclin1 and IKK γ , c LC3 and IKK β , d LC3 and IKK γ , e LAMP1 and IKK β , f LAMP1 and IKK γ . Scale bars = 20 μ m. Right graphs represent quantification of colocalization of signals using Manders' overlap coefficient. Data are expressed as the mean $\pm$ SD from three independent experiments, *P < 0.05, **P < 0.01. Data are representative of three independent experiments.

Figure 5

AR deficiency facilitates IKK β and IKK γ to colocalize with autophagosomes and lysosomes in LPS-stimulated macrophages. Confocal microscopy of BMMs from WT or AR KO mice treated with or without 500 ng/ml LPS 1 h and costained for a beclin1 and IKK β , b beclin1 and IKK γ , c LC3 and IKK β , d LC3 and IKK γ , e LAMP1 and IKK β , f LAMP1 and IKK γ . Scale bars = 20 μ m. Right graphs represent quantification of colocalization of signals using Manders' overlap coefficient. Data are expressed as the mean $\pm$ SD from three independent experiments, *P < 0.05, **P < 0.01. Data are representative of three independent experiments.

Figure 6

Connecting the IKK complex and autophagy pathway. Confocal microscopy images of BMMs from WT or AR KO mice treated with or without 500 ng/ml LPS for 1 h, and co-stained for (a) polyUb and IKK β , (b) polyUb and IKK γ , (c) p62 and IKK β , and (d) p62 and IKK γ . Right graphs represent quantification of colocalization of signals using Manders' overlap coefficient. Scale bars = 20 μ m. Data are expressed as the mean $\pm$ SD from three independent experiments, *P < 0.05, **P < 0.01. Data are representative of three independent experiments.

Figure 6

Connecting the IKK complex and autophagy pathway. Confocal microscopy images of BMMs from WT or AR KO mice treated with or without 500 ng/ml LPS for 1 h, and co-stained for (a) polyUb and IKKβ, (b) polyUb and IKKγ, (c) p62 and IKKβ, and (d) p62 and IKKγ. Right graphs represent quantification of colocalization of signals using Manders' overlap coefficient. Scale bars = 20 μm. Data are expressed as the mean ± SD from three independent experiments, *P < 0.05, **P < 0.01. Data are representative of three independent experiments.