

Anti-inflammatory effect of *Aiouea padiformis* extract via inhibition of the ATPase activity of NLRP3

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Abstract

Aiouea padiformis belongs to the family Lauraceae and is primarily found in tropical regions. Although other plants in this family have been shown to have anti-inflammatory effects, the anti-inflammatory properties of *A. padiformis* extract (AP) have not yet been investigated. In this study, we examined the effects of AP on the NLRP3 inflammasome and aimed to elucidate the mechanism behind these effects. AP treatment inhibited interleukin-1 beta (IL-1 β) secretion without affecting cell viability in J774A.1 and THP-1 cells. AP treatment had no effect on NF- κ B signaling, potassium efflux, or intracellular reactive oxygen species (ROS) production, all of which are associated with NLRP3 activation. However, interestingly, AP treatment significantly reduced the ATPase activity of NLRP3. To confirm whether AP functions in vivo, we used zebrafish larvae with lipopolysaccharide (LPS)-induced inflammation. Confirming the results observed in cellular experiments, a decrease in the number of neutrophils was observed in zebrafish embryos following AP treatment. Our results show that AP hindered NLRP3 inflammasome assembly through ATPase activity suppression, resulting in reduced inflammation in vivo. This study demonstrates the potential of AP as a treatment for inflammatory diseases associated with NLRP3 inflammasomes.

Introduction

Aiouea padiformis (Standl. & Steyererm.) R. Rohde, formerly known as *Cinnamomum padiforme* (Standl. & Steyererm.) Kosterm or *Phoebe padiformis* Standl. & Steyererm., belongs to the family Lauraceae¹. It is primarily found in tropical regions of North and South America. Although the metabolomics profile and anti-inflammatory activity of other members of the family Lauraceae have been investigated²⁻⁴, the mechanisms underlying these anti-inflammatory effects remain unknown.

Inflammation is a critical component of the innate immune system, triggered by the presence of external stressors. It prevents injury and infection and promotes the recovery of damaged tissues; however, excessive inflammation can cause various autoinflammatory diseases and must therefore be carefully regulated. The nucleotide-binding oligomerization domain, leucine-rich repeat-containing (NLR) protein 3 (NLRP3), is one of the pattern recognition receptors and is a critical part of the immune system. It forms an inflammasome complex with an adaptor protein, ASC and an effector protein, pro-caspase-1⁵. To activate NLRP3 inflammasome, two indispensable processes, priming and activation, must occur sequentially⁶. In the priming step, the transcription factor NF- κ B upregulates the expression of NLRP3 and the inflammatory cytokine pro-IL-1 β ⁷. Once primed, the NLRP3 inflammasome can be activated by various signals, including the production of ROS⁸ and potassium ion (K⁺) efflux^{9,10}. When activated, the NLRP3 inflammasome forms oligomers that, in turn, activate pro-caspase-1. Active caspase-1 then cleaves pro-IL-1 β and gasdermin D (GSDMD) into their active forms. Processed GSDMD forms pores in the cell membrane, causing the release of IL-1 β and the initiation of pyroptosis^{11,12}.

Mutations in the NLRP3 gene commonly result in the overactivation of the NLRP3 inflammasome. This is closely associated with various diseases, including cryopyrin-associated autoinflammatory syndromes

(CAPS)^{13–15}, type 2 diabetes¹⁶, Alzheimer's disease¹⁷, and Parkinson's disease^{18,19}. Inhibiting the release of the cytokine IL-1 β , a major product of the NLRP3 inflammasome, can be beneficial in the treatment of these diseases. However, because targeting IL-1 β may suppress all IL-1 β -mediated immune responses, it potentially cause immunodeficiency, leading to an increased susceptibility to infections²⁰. Therefore, ongoing research aims to discover substances that can specifically inhibit the NLRP3 inflammasome, and this constitutes the primary focus of our study.

Natural products are compounds derived from living organisms and exhibit a wide variety of chemical structures and biological activities. These compounds play a crucial role in the research and development of drugs for the treatment of various diseases. Medications derived from natural products tend to have fewer side effects than those based on synthetic chemicals. This is a notable advantage, especially in the treatment and prevention of chronic diseases, where long-term medication is often required²¹. Therefore, our study aims to investigate the potential therapeutic mechanism of specific inhibition of the NLRP3 inflammasome through AP treatment. We believe that this research is notable, as it could offer novel therapeutic strategies to alleviate various NLRP3-associated diseases with, while minimizing the risk of adverse effects.

Results

AP specifically reduces NLRP3 inflammasome activation

To identify natural compounds with anti-inflammatory properties, we collected plant extracts from 45 species native to Nicaragua. We assessed whether these extracts reduced IL-1 β secretion. The levels of IL-1 β were quantified relative to untreated samples using Western blot with ImageJ (ver. 1.54d) (Supplemental Table 1). Among the 45 plant extracts tested, we selected candidates that did not affect cell viability, showed concentration-dependent reduction in IL-1 β secretion, and did not inhibit the NF- κ B signal. Among these selected candidates, AP appeared to effectively reduce IL-1 β and was therefore used for subsequent experiments.

To confirm the anti-inflammatory effects of AP, we first investigated whether the AP extract was toxic to cells. We measured the viability of mouse J774.A1 and human THP-1 cells after AP treatment. The viability of both types of cells did not significantly decrease, even when treated with the highest concentration of 100 μ g/ml of AP (Fig. 1a–b). We then investigated the effect of AP treatment on IL-1 β secretion. Primed J774A.1 cells were exposed to nigericin and adenosine triphosphate (ATP), well-known NLRP3 activators, showed a significant increase in IL-1 β secretion. However, when the cells were treated with both the activators and AP, IL-1 β secretion decreased in a concentration-dependent manner. The effects of AP were compared with those of MCC950, an NLRP3-specific inhibitor²³. As shown in Fig. 1c–d, a high concentration of AP inhibited NLRP3 activation to a similar extent as MCC950. Similar results were obtained when phorbol 12-myristate 13-acetate (PMA)-primed THP-1 cells were treated with nigericin alone or in combination with AP or MCC950 (Fig. 1e).

To investigate whether AP specifically suppressed NLRP3 inflammasome activity, we examined two other types of inflammasomes: Absent in Melanoma 2 (AIM2), activated by dsDNA treatment, and NOD-like receptor family CARD domain-containing protein 4 (NLRC4), activated by flagellin. We measured the secretion of IL-1 β after activating AIM2 and NLRC4 inflammasome. In both cases, AP treatment did not affect IL-1 β production, indicating that AP specifically blocked the activation of the NLRP3 inflammasome (Fig. 1f–g).

AP does not impact the NF- κ B signaling pathway

To investigate whether AP regulates the priming step of the NLRP3 inflammasome, we assessed NF- κ B activity by transfecting cells with an NF- κ B luciferase reporter gene. Treatment with TNF- α significantly increased NF- κ B signaling, and co-treatment with TNF- α and AP had no effect on the elevated NF- κ B signaling (Fig. 2a). To further confirm these findings, we examined the phosphorylation levels of p65, a subunit of NF- κ B, by performing a Western blot on NLRP3 inflammasome-activated J774A.1 cells. AP treatment did not affect the levels of phosphorylated p65 (Fig. 2b). These results indicate that AP does not influence NF- κ B signaling.

AP suppression of the NLRP3 inflammasome is not dependent on K⁺ efflux or increased intracellular ROS levels

After confirming that AP does not suppress the priming step of the NLRP3 inflammasome, we investigated the mechanism by which AP suppresses NLRP3 inflammasome activation. The NLRP3 inflammasome is activated by two main pathways: K⁺ efflux and increased intracellular ROS levels.

To assess the impact of AP treatment on K⁺ efflux, we treated cells with imiquimod, which activates the NLRP3 inflammasome independently of K⁺ efflux²⁴. As shown in Fig. 3a, AP treatment continued to reduce the IL-1 β level in a concentration-dependent manner. This indicates that the inhibition of the NLRP3 inflammasome by AP is not dependent on its effect on K⁺ efflux.

To determine whether AP regulates intracellular ROS levels to inhibit the NLRP3 inflammasome, cells were treated with ATP to increase ROS levels. After treatment with AP, ROS levels remained constant. However, when cells were treated with N-acetylcysteine (NAC), a well-known ROS scavenger, ROS levels significantly decreased. This confirmed that AP did not influence intracellular ROS levels (Fig. 3b).

AP inhibits ATPase activity and ASC oligomerization of the NLRP3 inflammasome

Never in Mitosis Gene A-related kinase 7 (NEK7) is essential for promoting oligomerization of the NLRP3 inflammasome, which is necessary for its activation^{25,26}. We examined the effect of AP on the interaction between NEK7 and the NLRP3 inflammasome using co-immunoprecipitation (Co-IP) of the two proteins. No significant differences in the amount of NEK7-NLRP3 complexes were found between

untreated samples and those treated with AP. Therefore, we confirmed that AP did not interfere with the interaction of NEK7 and NLRP3 (Fig. 4a).

ATPase activity is essential for the assembly of the NLRP3 inflammasome²⁷. Many NLRP3 inhibitors are known to impede the ATPase activity of NLRP3^{28,29}; therefore, we set out to determine whether AP affects ATPase activity. As shown in Fig. 4b, AP treatment significantly reduced the ATPase activity of NLRP3 compared to that of the control, suggesting that treatment with AP suppressed NLRP3 inflammasome activation by hindering inflammasome assembly. We subsequently investigated ASC oligomerization, a marker of inflammasome assembly³⁰. As expected, AP treatment attenuated ASC oligomerization in a concentration-dependent manner (Fig. 4c). To confirm these results, confocal microscopy was used to assess the extent of speck formation, which represents inflammasome assembly. Consistent with previous data, speck formation was significantly reduced by AP treatment. These results revealed that the AP-mediated inhibition of NLRP3 can be attributed to the prevention of inflammasome assembly (Fig. 4d–e).

AP alleviates inflammatory responses induced in the zebrafish embryos

To evaluate the anti-inflammatory effects of AP at the organism level, we used a zebrafish (*Danio rerio*) model. Zebrafish embryos are an effective tool to study inflammation during early development^{31,32}. Inflammation can be induced through various methods, including LPS immersion, direct injection of LPS into the yolk sac, and tail-cut injuries³³.

We conducted a primary experiment by inducing inflammation in 1 d post-fertilization (dpf) zebrafish embryos by treating the larvae with 10 µg/ml LPS. Following an induction period of 2 d, the embryos were fixed. The resulting inflammatory response was assessed by monitoring the number of myeloid cells using Sudan Black B staining³⁴. Inflammation was successfully induced in the LPS-treated zebrafish, and co-treatment with 10 µg/ml of AP markedly reduced this inflammation (Fig. 5a). Further quantification revealed a significant reduction in the levels of the inflammatory markers in embryos treated with AP compared to those that were not (Fig. 5b). Additionally, under the same conditions, whole-mount in situ hybridization (WISH) analysis targeting neutrophil-specific *mpx* expression revealed that AP treatment reduced the LPS-induced increase in *mpx*-positive cells (Fig. 5c). In addition to the LPS-immersion experiments, we induced inflammation by directly injecting LPS into the zebrafish yolk. This approach led to noticeable inflammation, particularly around the injection site and the anterior part of the embryos. The enhanced inflammatory response was significantly alleviated by incubation with AP (Fig. 5d–e).

Taken together, the findings of our experiments on the zebrafish model verify the potent anti-inflammatory effects of AP at the organismal level, further emphasizing the broader potential of this natural product as an anti-inflammatory agent.

Discussion

Anti-inflammatory activity has been reported in several plant species belonging to the genus *Aiouea*. An ethanol extract of xylopodium from *A. trinervis* was found to contain three types of butanolides that exhibit antitrypanosomal activity ². Some plants from the family Lauraceae, including *A. saligna* Meisn., have been shown to inhibit the release of prostaglandin E₂, one of the main mediators of inflammation ³. Furthermore, the essential oils from *A. maguireana* were found to contain oxygenated sesquiterpenes, which are known to have anti-inflammatory properties ⁴. Out of 45 extracts from Nicaraguan plants, AP, a methanol extract of *A. padiformis*, showed the highest anti-inflammatory activity with no cytotoxicity. In this study, we aimed to demonstrate the inhibitory effect of AP on NLRP3 inflammasome activation and elucidate its underlying mechanism.

First, we demonstrated that AP is not toxic to mouse or human cell lines but can inhibit the secretion of IL-1 β upon activation of the NLRP3 inflammasome. We also found that AP did not inhibit IL-1 β produced by AIM2 or NLRC4 inflammasomes, indicating that its inhibition is specific to the NLRP3 inflammasome. Moreover, treatment with AP did not affect NF- κ B signaling, a critical process in the priming step of inflammasome activation, suggesting that AP treatment is unrelated to the priming step. Furthermore, AP treatment did not affect two crucial factors that activate the NLRP3 inflammasome: K⁺ efflux and intracellular ROS levels.

To investigate the inhibitory mechanism of AP on NLRP3 inflammasome activation, we measured ATPase activity, which is essential for NLRP3 inflammasome assembly. AP treatment significantly reduced the ATPase activity of NLRP3, effectively suppressing ATP-dependent oligomerization. Furthermore, AP treatment reduced the occurrence of ASC oligomerization, another inflammasome assembly marker.

Finally, we assessed the anti-inflammatory effects of AP at an organismal level using a zebrafish inflammation induction model. Zebrafish, a vertebrate model frequently used in biomedical research, share physiological similarities with humans ^{35,36}. During the embryonic stage, the distribution of macrophages and neutrophils, which play critical roles in the innate immune system, is similar to that in humans. Markers related to the innate immune system that are closely associated with the induction of inflammation and its associated diseases. Consequently, in zebrafish, the expression of well-conserved innate immune markers, including neutrophils, serves as a vital tool for monitoring inflammatory processes ^{31,37}. In this study, we established zebrafish inflammation models using LPS treatment or physical injury to induce inflammation. We found that the inflammatory response induced by these methods was alleviated by the natural compound AP. These findings, along with the results from our cellular experiments, underscore the anti-inflammatory potential of AP.

Inflammation is a critical defense mechanism employed by the body in response to external damage. However, because chronic or abnormal inflammation can lead to various diseases, the development of anti-inflammatory agents is crucial. Many drugs, such as anakinra and canakinumab, have been

developed to reduce IL-1 β levels and have received approval from the Federal Drug Administration for clinical use^{38,39}. However, directly inhibiting IL-1 β , which is essential for preventing infection, can lead to notable side effects. Consequently, there is growing interest in researching the regulation of specific inflammasomes as an approach to modulating the immune response. The NLRP3 inflammasome, in particular, is closely associated with many diseases, and several studies have focused on the development of NLRP3 inhibitors. Natural products, such as AP, are valuable tools in pharmaceutical development due to their diverse chemical structures and biological activity. Pharmaceuticals based on natural products tend to have fewer side effects than synthetic chemical compounds, increasing their potential for the treatment of chronic inflammatory conditions²¹.

Conclusion

Our findings suggest that AP, which specifically inhibits activation of the NLRP3 inflammasome, could be a valuable candidate for the treatment of inflammatory-related diseases. Because AP is extracted from plant leaves, stems, and fruits, further research should analyze the specific components of the extract responsible for its anti-inflammatory activity.

Materials and methods

Zebra fish

All experimental protocols and procedures involving the embryos were approved by the Institutional Animal Care and Use Committees at Kyung Hee University Hospital at Gangdong (IACUC: KHNMC AP 2023) and were performed in accordance with approved guidelines.

Plant sample collection

The collection and preservation of plants material were conducted under the agreement for sustainable exploitation of the Nicaraguan flora between International Biological Material Research Center (IBMRC) in Korea Research Institute of Bioscience and Biotechnology (KRIBB) of Korea and Universidad Nacional Autonoma de Nicaragua-Leon of Nicaragua. All research samples follow the regulation of Nicaragua institutional, national, and international guidelines and legislation, with all necessary permissions and licenses obtained to ensure compliance with ethical and legal requirements.

Reagent and antibodies

Aiouea padiformis (Standl. & Steyerl.) R. Rohde was provided by Korea Research Institute of Bioscience and Biotechnology; KRIBB. Dulbecco's modified Eagle medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640, Penicillin-streptomycin, fetal bovine serum (FBS), pure down protein A/G-agarose (P9203-200), and phosphatase inhibitor cocktail (P3200-005) were purchased from GenDEPOT (Baker, TX, USA). The protease inhibitor cocktail (PPI1015) was purchased from Quartett GmbH (Berlin, Germany). Antibodies against α -tubulin (sc-5286), NIMA-related kinase 7 (NEK7; B-5) (sc-393539), c-Myc (9E10) (sc-

40) and ASC (sc-514414) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human IL-1 β (AF-401-NA) and mouse IL-1 β (AF-201-NA) antibodies were purchased from R&D Systems (Minneapolis, MN, USA). Anti-Caspase-1 (A0964) was purchased from Abclonal Technology (Woburn, MA, USA). Phospho-NF- κ B p65 (93H1), NF- κ B p65 (8242) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Lipopolysaccharide from *P. gingivalis* (LPS, Ultrapure) (tlrl-ppglps), nigericin (tlrl-nig), ATP (tlrl-atpl), and flagellin (FLA-ST; tlrl-epstfla) were purchased from InvivoGen (San Diego, CA, USA). Highly cross-adsorbed Mouse IgG (H + L) secondary antibodies (A32723), Lipofectamine™ 2000 (11668019) and 3000 (L3000015) were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). Phorbol 12-myristate 13-acetate (PMA; HY-18739) and Imiquimod (IQM, (HY-B0180)) were purchased from MedChem Express (South Brunswick Township, NJ, USA). EZ-CYTOX (EZ-500) was purchased from DoGenBio (Shanghai, China). N-acetyl-L-cysteine (NAC; A9165-25G) and Disuccinimidyl suberate (DSS; 68528-80-3) were purchased from Sigma-Aldrich (St Louis., MO, USA). The cellular reactive oxygen species (ROS) assay kit (DCFDA/H2DCFDA) (ab113851) and nuclei stained with mounting medium (DAPI) (ab104139) were purchased from Abcam (Cambridge, UK). Bright-Glo™ Luciferase assay system (E2610), pGL4.32 Luciferase Reporter vector (E8491), and ADP-Glo™ Max Assay (V7001) were purchased from Promega (Madison, WI, USA).

Cell culture and stimulation

THP-1 and J774A.1 cells were collected and cultured as previously described (ATCC). We treated PMA (500 nM) for 3 h to differentiate THP-1 cells, and incubated cells for 2 days. J774A.1 cells were incubated for 1 day. Subsequently, we treated LPS (100 ng/mL) for 5 h in the cells for priming. After the cells were primed with LPS for 3 h, AP (10 μ g/mL, 50 μ g/mL, 100 μ g/mL) was further treated for 2 h. To activate NLRP3 inflammasome, we treated Nigericin (10 mM) or ATP (5 mM) for 30 mins or 1 h, or Imiquimod (200 μ M) for 1 h in the cells. To activate AIM2 or NLRC4 inflammasome, we transfected dsDNA (2 μ g/mL) or flagellin (1.25 μ g/mL) for 3 h using Lipofectamine™ 2000.

In vitro cell viability assay

In a 96-well plate, differentiated THP-1 cells were seeded 2 days before the experiment. J774A.1 cells were seeded 1 day before the experiment. The cells were treated with AP (10 μ g/mL, 50 μ g/mL, 100 μ g/mL) for 2 h. After treatment, we treated EZ-CYTOX for 1 h in THP-1 and 30 mins in J774A.1. The absorbance was measured at 450 nm wavelengths using an iMark™ Microplate Absorbance Reader (1681130; Bio-Rad Laboratories, Hercules, CA, USA).

ASC oligomerization and ASC speck staining

To investigate ASC oligomerization, pellets of THP-1 cells were resuspended with phosphate-buffered saline (PBS) for washing and incubated with DSS (2.5 mM) for 30 mins at room temperature (25°C). ASC oligomerization was analyzed by immunoblotting.

ASC speck staining was conducted in NLRP3 inflammasome-activated J774A.1 cells. The cells were fixed with 4% paraformaldehyde in PBS for 10 mins at room temperature and washed thrice with ice-cold

phosphate-buffered saline with 0.1% tween 20 (PBS-T). Permeabilization was conducted using PBS containing 0.2% Triton X-100 for 10 mins at room temperature and washed three times with ice-cold PBS-T for 5 mins. Next, the cells were blocked with 1% bovine serum albumin (BSA) and 22.52 mg/mL glycine in PBS-T and incubated with ASC antibody overnight at 4°C. Then, the cells were washed three times with PBS-T for 5 mins and incubated with AF488-conjugated anti-mouse IgG antibody in 1% BSA for 1 h at room temperature in the dark. The cells were washed three times with ice-cold PBS-T and cells were mounted in a medium containing DAPI for nuclear staining. A confocal laser-scanning microscope (Carl Zeiss, LSM710) was used for imaging.

Measurement of cellular ROS assay

ROS staining was conducted in NLRP3 inflammasome-activated J774A.1 cells. In a 4-well culture slide, J774A.1 cells were seeded 1 day before the experiment. Cells were primed by treating them with LPS (100 ng/mL) for 5 h, followed by AP (100 µg/mL) treatment for 2 h. The medium was removed, and the cells were washed with 1× buffer (250 µL/well). Subsequently, the cells were treated with DCFDA solution (20 µM) for 1 h at 37°C in the dark. The solution was removed, and cells were washed with 1× buffer (250 µL/well). The cells were mounted in a medium containing DAPI for nuclear staining. Images were obtained using a confocal laser-scanning microscope (Carl Zeiss, LSM710).

Measurement ATPase activity of NLRP3

Purified mouse recombinant NLRP3 (0.139 mg/mL) was incubated with AP or DMSO in ATPase reaction buffer (100 mM Tris, pH7.8, 2.8 mM EDTA, 100 mM MgCl₂, 15 mM KCl, 665 mM NaCl) for 3 h at room temperature. Ultra-pure ATP (1 mM) was added for another 40 min at 37°C. The ATP hydrolysis by NLRP3 was determined by an ADP-Glo™ Max assay (Promega, Madison, USA) according to the manufacturer's protocol.

NF-κB luciferase activity reporter gene assay

In a 96-well white plate, 293T cells (0.2×10^5 cells/well) were seeded 1 day before the transfection. The cells were transfected with pGL4.32 Luciferase Reporter Vector (0.1 µg/well) using Lipofectamine™ 3000. After 24 h, nuclear factor-kappa B (NF-κB) activity was activated by tumor necrosis factor-alpha (TNF-α) (20 ng/mL) for 5 h with or without AP (10 µg/mL, 50 µg/mL, 100 µg/mL). Luciferase activity assay was conducted using a Bright-Glo™ Luciferase Assay System (Promega, Madison, USA) according to the manufacturer's protocol, using a BMG LabTech FLUOstar OPTIMA microplate reader.

Immunoprecipitation

In a 100pi dish, HEK 293FT cells were seeded 1 day before the transfection. The cells were transfected with NLRP3-Myc vector (10 µg) using Lipofectamine™ 2000. After 24 h, we treated AP (10 µg/mL, 50 µg/mL, 100 µg/mL) for 2 h, and lysed with IP lysis buffer (Tris-HCl pH7.4 30 mM, EDTA 2 mM, NaCl 120 mM, KCl 2 mM, NP-40 0.2%, glycerol 10%, and protease inhibitor cocktail). The lysates were incubated with Myc antibody overnight at 4°C, with additional treatment of protein A/G beads for 1 h. The beads were rinsed and eluted. NLRP3-NEK7 binding was analyzed with these samples by immunoblotting.

Statistical analysis

Unless stated otherwise, differences between treatment groups were evaluated using one-way analysis of variance (ANOVA) and Tukey's post hoc test. In the in vitro assay, differences between stimulated and control cells were assessed using the Wilcoxon single-rank test. All statistical analyses were carried out using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com), and a p-value of < 0.05 was considered significant.

Zebrafish husbandry and AP preparations

Zebrafish embryos used in this study are the Wild-Type TAB5 strain. The embryos were consistently incubated at 28.5°C. The natural product AP, provided in powder form, is stable at room temperature. For experimental use, it was diluted with 500 µL DMSO and 1.5 mL of PBS to achieve a concentration of 10 mg/mL. This solution was then stored at -20°C.

Zebrafish harvest and inflammation induction

Fertilized embryos were harvested and incubated in E3 solution at 28.5°C. At 1 day post-fertilization (dpf), embryos underwent a dechoriation procedure. They were then raised in the incubator until further use. The LPS (*Escherichia coli* 055:B5; Sigma) was used to induce inflammation with either method of immersion or injection. Twenty five embryos were placed in a 60 mm petri dish containing 6 mL of solution. After dechoriation, the larvae were treated with E3 solution with 1-phenyl-2-thiourea (PTU) containing 0.125% DMSO and 10 µg/mL LPS and 10 µg/mL AP. The solution in the petri dish was replaced daily. After 48 h, all samples were fixed for subsequent experiments. For the LPS injection method, 3 dpf larvae were anesthetized using tricaine for approximately 2 mins. Each embryos were injected with 2 nL of either a PBS mixture or 0.5 mg/mL LPS mixture into the yolk. The PBS mixture contained 0.5 µL phenol red, 4.5 µL 1× PBS and the LPS mixture contained 0.5 µL phenol red, 4 µL 1× PBS, and 0.25 µL LPS. Post-injection, all injected larvae were treated with either PTU (DMSO) control or 10 µg/mL AP mixture. 5.5 h post-natural product treatment, samples were fixed samples for the next phase of experiment. For tail cut injury, 3 dpf larvae were anesthetized using tricaine.

Sudan black B stain and Whole-mount in situ hybridization (WISH)

All embryos were fixed in 4% Paraformaldehyde (PFA) throughout the night. Sudan black stain method for wounding zebrafish larvae protocols was previously described²². The embryo used in WISH was washed in PBS after overnight storage in methanol at -20°C. After rehydration and permeabilization with acetone at -20°C. Permeabilized samples were combined with digoxigenin (DIG)-labeled RNA probes in hybridization buffer (50% formamide, 5 × SSC, 500 µg/mL Torula yeast tRNA, 50 µg/mL heparin, 0.1% Tween-20 and 9 mM citric acid (pH 6.5)) were hybridized in a 65°C water bath. After hybridization, samples were washed using 2× and 0.2× SSC solutions. The washed samples were incubated at 4°C with alkaline phosphate-conjugated DIG antibodies (1:5000, Roche, Basel, Switzerland). For signal

development, samples were mixed with NBT/BCIP substrate (Promega) and alkaline phosphate reaction buffer (100 mM Tris, pH9.5, 50 mM MgCl₂, 100 mM NaCl and 0.1% Tween-20) at room temperature. Incubate until the signal develops to a certain stage and then stops developing.

Declarations

Acknowledgements

The collection and preservation of plants material were conducted under the agreement for sustainable exploitation of the Nicaraguan flora between International Biological Material Research Center (IBMRC) in Korea Research Institute of Bioscience and Biotechnology (KRIBB) of Korea and Universidad Nacional Autonoma de Nicaragua-Leon of Nicaragua by following the regulation of Nicaragua. Molecular biological experiments using the plant extracts were conducted based on the agreement between Ajou University School of Medicine in Korea and IBMRC.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by S. L. and Q. Y. The first draft of the manuscript was written by S. L. and Q. Y. and all authors commented on the previous versions of the manuscript. Y. H. P. and Y. L. supervised the project. All authors read and approved the final manuscript.

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Data availability

The datasets generated and/or analyzed during the current study are available in the manuscript and figures.

Competing interests

The authors declare no conflict of interest.

Ethics approval

All zebra fish experimental protocols and procedures involving the embryos were approved by the Institutional Animal Care and Use Committees at Kyung Hee University Hospital at Gangdong (IACUC: KHNMC AP 2023).

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Figures

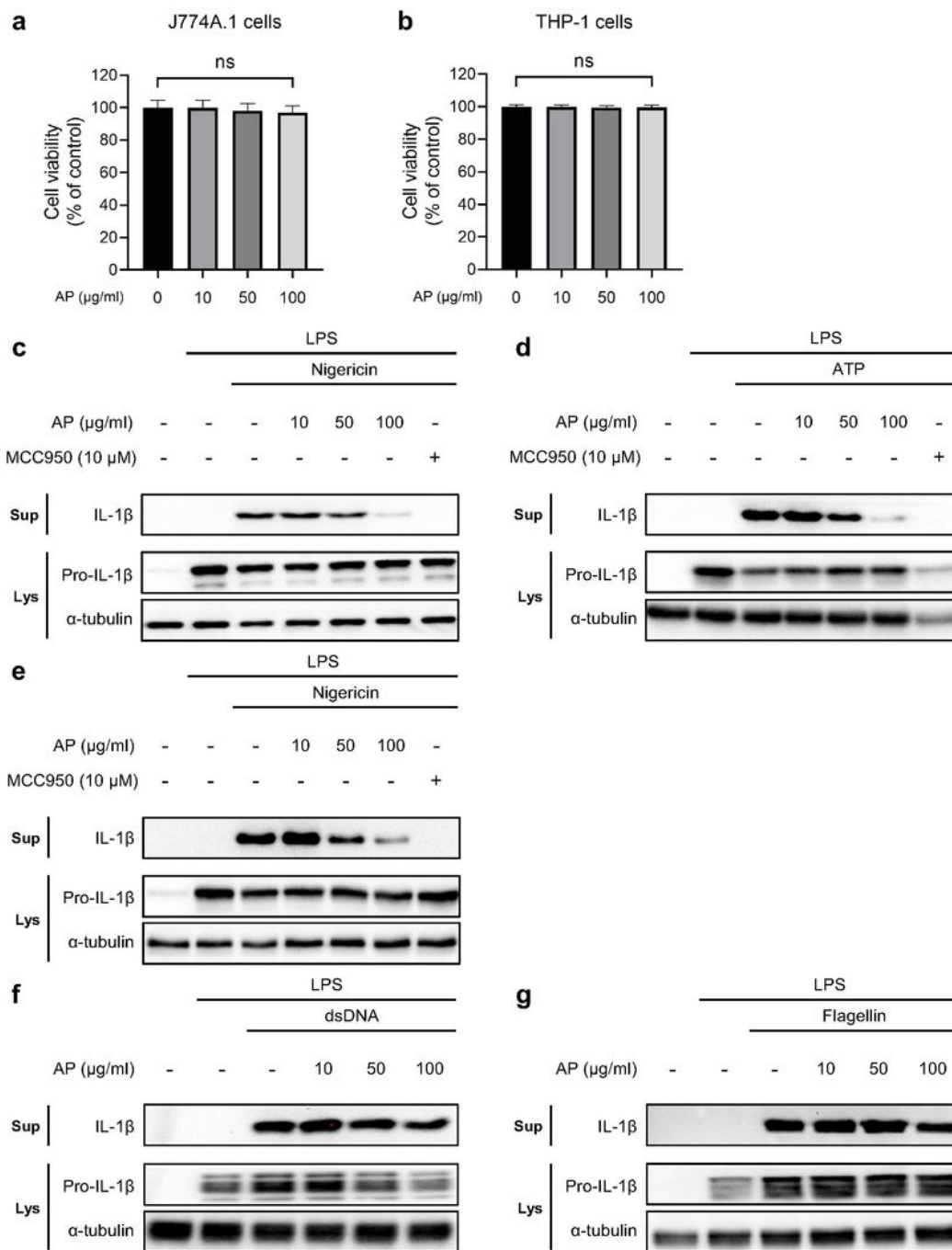


Figure 1

AP specifically reduces NLRP3 inflammasome activation

(a–b) Cell viability assays of J774A.1 cells (a) and differentiated THP-1 cells (b) treated with AP (10 μg/mL, 50 μg/mL, 100 μg/mL) for 2 h. Cell viability was analyzed by EZ-Cytox, measured at a wavelength of 450 nm. Ns: not significant. (c–e) Western blots of IL-1β in the supernatants (Sup), and pro-IL-1β and

α -tubulin in the soluble lysates (Lys) of treated cells. LPS-primed J774A.1 cells were treated with AP for 2 h and activated for 30 min with nigericin (10 μ M) (c) or ATP (5 mM) (d) with or without MCC950, differentiated, and LPS-primed THP-1 cells were treated with AP for 2 h and activated for 30 min with nigericin (10 μ M) with or without MCC950 (e). (f–g) LPS-primed J774A.1 cells were treated with AP for 2 h and activated for 3 h with transfection of dsDNA (2 μ g/mL) (f) or flagellin (1.25 μ g/mL) (g).

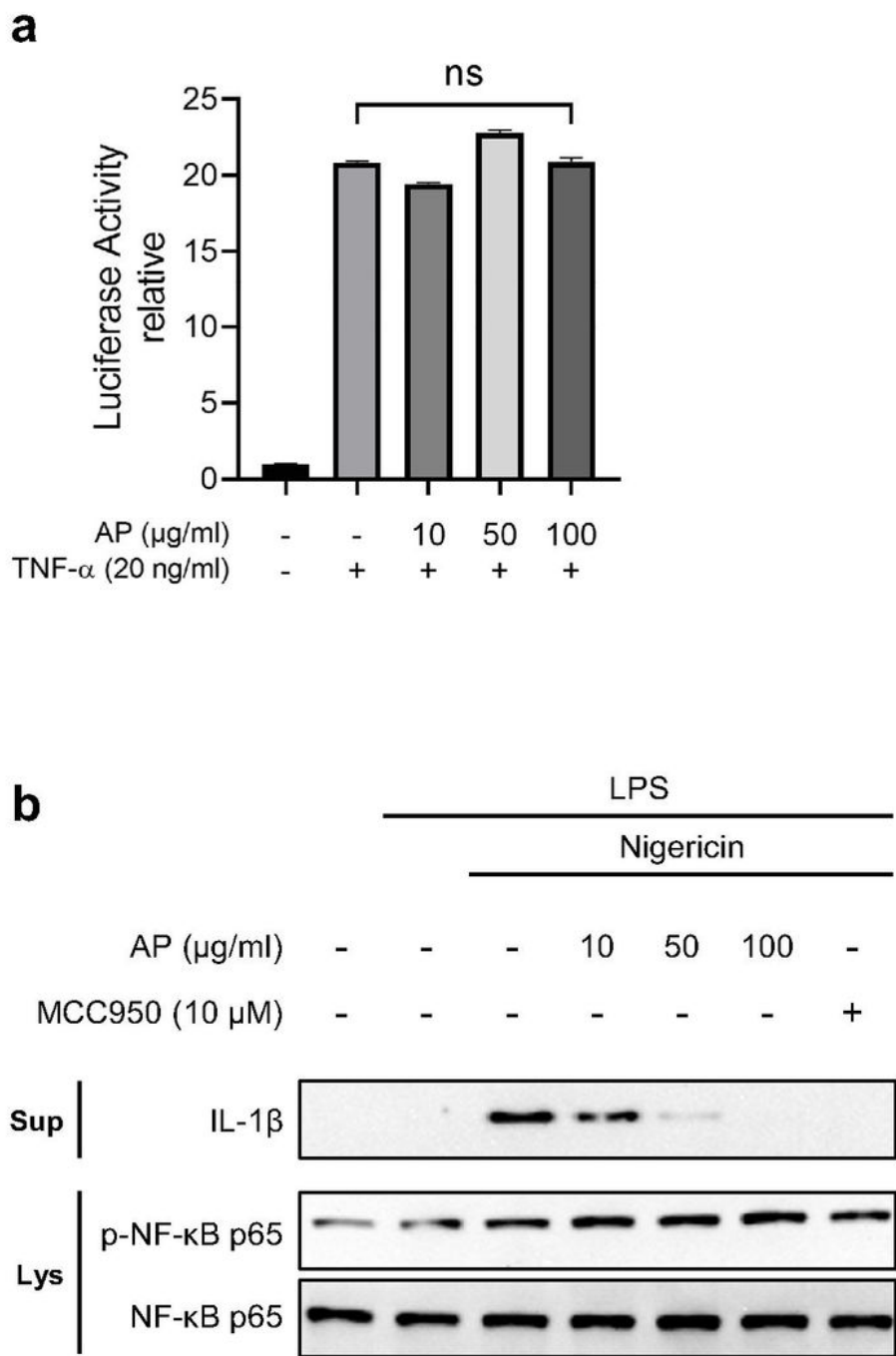


Figure 2

AP does not affect the NF-κB signaling pathway

(a) 293T cells were transfected with the pGL4.32 luciferase reporter vector and treated with tumor necrosis factor-α (TNF-α; 20 ng/mL) for 5 h with or without AP. Luciferase activity was analyzed using the Promega Bright-Glo™ Luciferase Assay System. (b) Lipopolysaccharide (LPS)-primed J774A.1 cells were treated with AP for 2 h and activated for 30 min with nigericin (10 μM). IL-1β in the supernatants (Sup), and phospho-NF-κB and NF-κB in the soluble lysates (Lys) were analyzed using a Western blot.

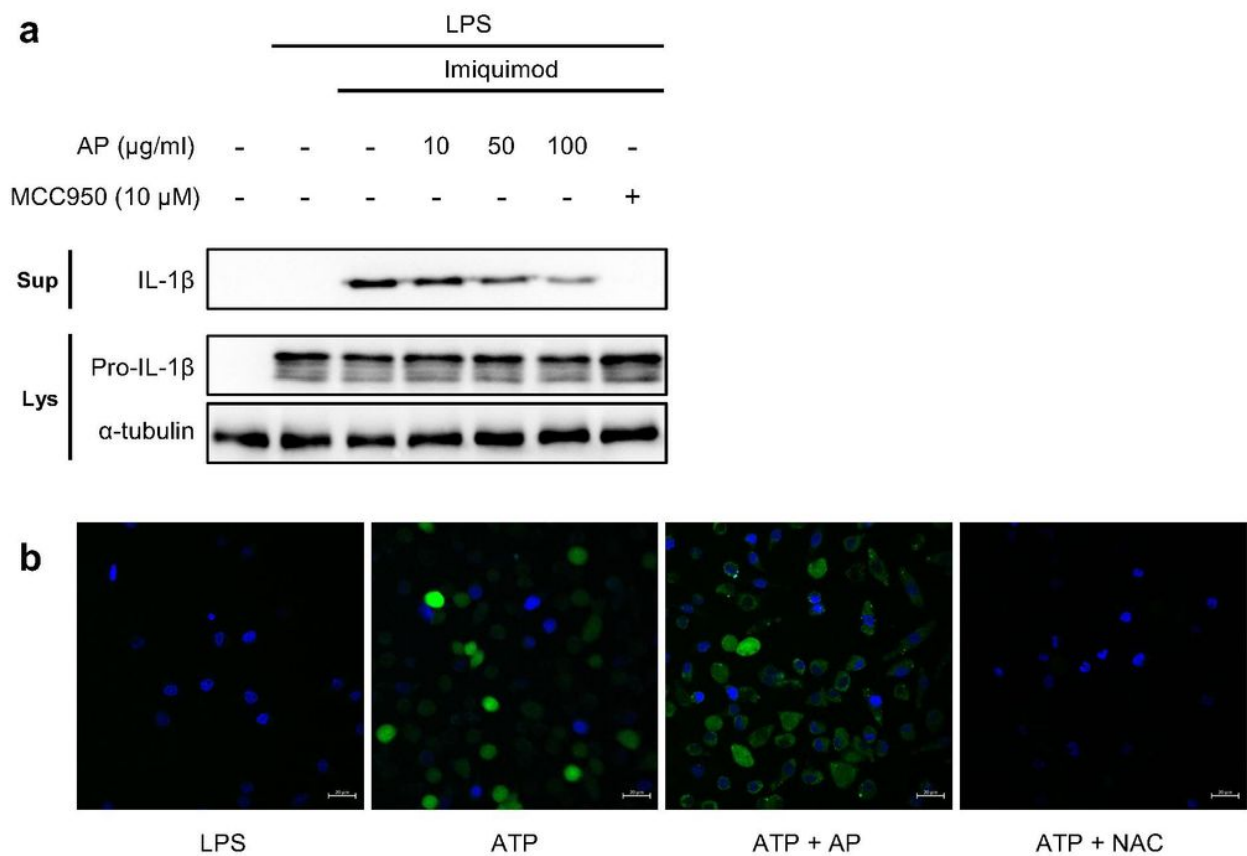


Figure 3

AP inhibits NLRP3 inflammasome, regardless of K⁺ efflux and ROS

(a) Lipopolysaccharide (LPS)-primed J774A.1 cells were treated with AP for 2 h and activated with Imiquimod (200 μM) for 1 h. (b) LPS-primed J774A.1 cells treated with AP for 2 h and activated with ATP

(5 mM) for 10 min. Representative immunofluorescence images of ROS (DCF-DA; green color) were taken by a confocal laser-scanning microscope (Carl Zeiss, LSM710, scale bar, 20 μ m).

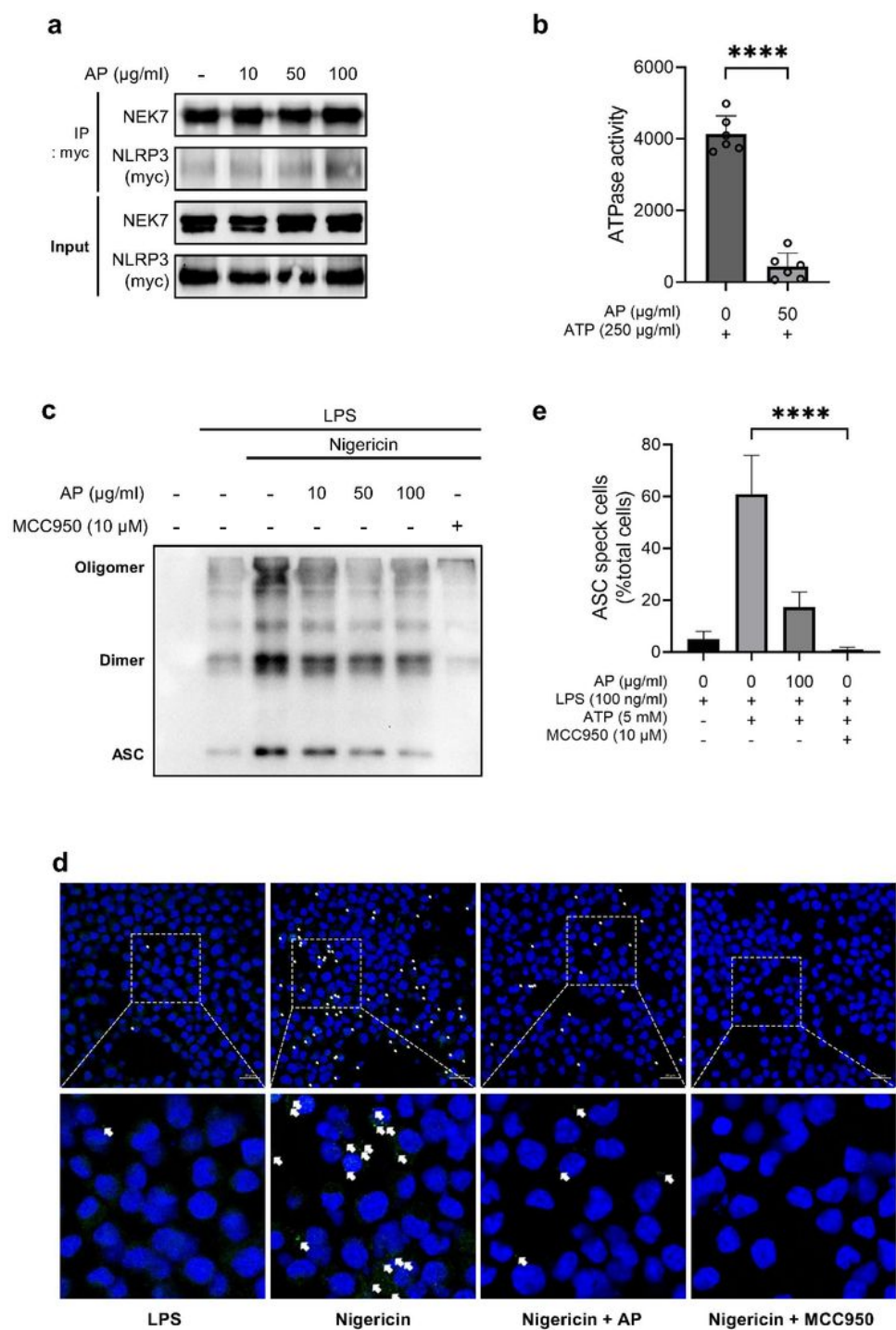


Figure 4

AP blocks NLRP3 inflammasome assembly by hindering ATPase activity of NLRP3

(a) HEK 293FT cells were transfected with NLRP3-Myc and treated with or without AP for 2 h. The interaction between NLRP3 and NEK7 was analyzed by immunoprecipitation and immunoblot. (b) The ATPase activity of NLRP3 with or without AP was measured by luminescence using the ADP-Glo™ Max assay. (c) Lipopolysaccharide (LPS)-primed THP-1 cells were treated with AP for 2 h and activated with nigericin (10 μ M) for 30 min. The cell pellet was cross-linked by DSS (2.5 mM) for 30 min. ASC oligomerization was analyzed by immunoblot. (d–e) LPS-primed J774A.1 cells treated with AP for 2 h and activated with nigericin (10 μ M) for 30 min. Representative immunofluorescence images of ASC speck formation (indicated by arrows) were taken by a confocal laser-scanning microscope (Carl Zeiss, LSM710, scale bar, 20 μ m) (d). Graph representing the number of cells containing ASC specks in all treatments (e).

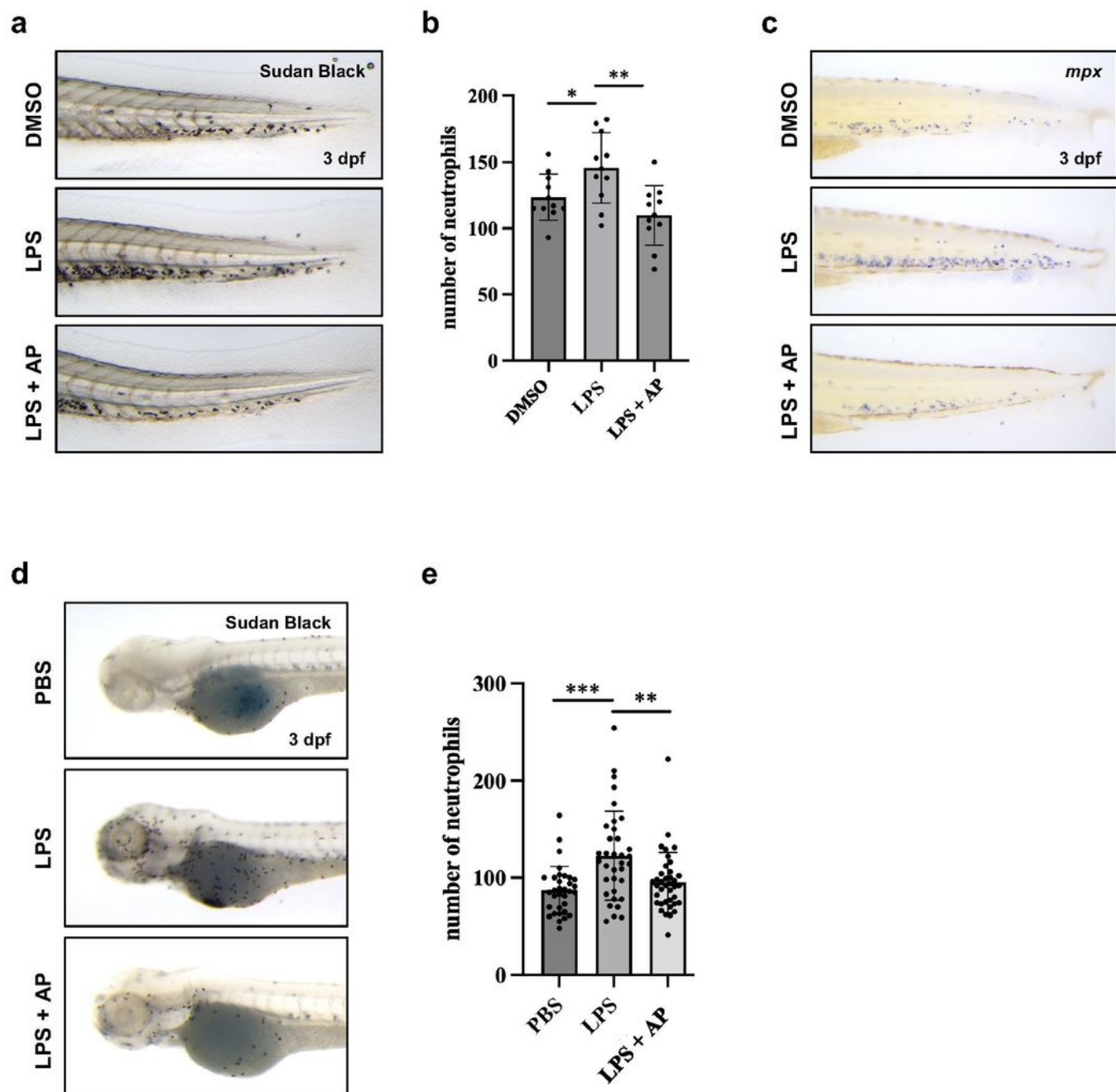


Figure 5

AP alleviates inflammatory response induced by LPS treatment in zebrafish

(a) Representative images of posterior areas of Sudan Black B-stained zebrafish embryos after LPS induction and AP treatment at 3 days post-fertilization (dpf). The upper panel shows the control group with DMSO (0.025%) treatment, the middle panel shows LPS inflammation group, and the bottom panel

shows the LPS inflammation group after treatment with 10 µg/mL AP. (b) Quantification of neutrophils in the posterior region of zebrafish after LPS induction and AP treatment. (c) Representative images of the caudal hematopoietic tissues after LPS induction and AP treatment at 3 dpf. Whole-mount in situ hybridization was conducted using an *mpx*probe. (d) Representative images of anterior Sudan Black B-stained zebrafish embryos following direct LPS injection and AP treatment at 3 dpf. (e) Quantification of the number of neutrophils in the anterior zebrafish embryos after LPS induction and AP treatment. All graphs represent the mean ± S.E.M. of individual values. P-values were calculated using an unpaired two-tailed Student's t-test. *P < 0.05; **P < 0.01; ***P < 0.001.

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