

# $\alpha$ -linolenic Acid Regulates Macrophages NF- $\kappa$ B/NLRP3 Signal Pathway via Binding GPR120 to Inhibit Inflammation in Type 2 Diabetes

**Yuanyuan Liu**

Ningxia Medical University

**Mixue Guo**

Ningxia Medical University

**Yiwei Li**

Ningxia Medical University

**Ting Wang**

Ningxia Medical University

**Yi Ren**

Ningxia Medical University

**Rui Wang**

Ningxia Medical University

**Xin Jiang**

Ningxia Medical University

**Xiaoxia Zhang**

Ningxia Medical University

**Hao Wang** (✉ [wanghaograde@126.com](mailto:wanghaograde@126.com))

Ningxia Medical University

---

## Research Article

**Keywords:**  $\alpha$ -linolenic acid (ALA), type 2 diabetes mellitus (T2DM), RAW264.7, GPR120, NLRP3 inflammasome

**Posted Date:** May 31st, 2022

**DOI:** <https://doi.org/10.21203/rs.3.rs-1702165/v1>

**License:**   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

## Background

Type 2 diabetes mellitus (T2DM) is one of the metabolic diseases that increasingly common worldwide. Recent studies confirm that chronic low-grade inflammation is closely associated with metabolic syndromes, and anti-inflammatory therapy is a potential approach for treating T2DM. Accumulating evidence suggests that G-protein coupled receptor 120 (GPR120) activation is a feasible solution to ameliorating chronic inflammation and improving glucolipid metabolism.  $\alpha$ -linolenic acid (ALA) has proved to benefit for chronic metabolic diseases. However, the triggering mechanisms of ALA alleviated the inflammation in T2DM by enhancing the expression of GPR120 are still ill-understood. In this study, we investigated the anti-inflammatory potential of ALA in T2DM and uncovered the underlying mechanisms.

## Methods

*In vivo*, SD rats were randomly allocated into 4 groups: pair-fed (PF) with corn oil (CO) group (PF/CO), DM with CO group (DM/CO), PF with ALA group (PF/ALA), DM with ALA group (DM/ALA). A diabetic rat model was generated by a single intraperitoneal injection of streptozotocin-nicotinamide (STZ-NA). Each group was fed diet with or without ALA. *In vitro*, lipopolysaccharide (LPS) stimulated RAW264.7 mouse macrophages cellular inflammation model was established. The effect of ALA on liver macrophages (M $\phi$ s) in T2DM rat was measured by immunofluorescence (IF). The concentrations of TNF- $\alpha$ , IL-6, IL-1 $\beta$ , IL-18 and IL-10 in RAW264.7 cell culture supernatants were analyzed with ELISA kits. The protein expressions of GPR120,  $\beta$ -arrestin2, NLRP3, caspase-1, TLR-4 and NF- $\kappa$ B signaling pathway were investigated with western blot analysis. Furthermore, LPS-induced RAW264.7 cells were treated with AH7614, the GPR120 antagonist, to confirm the effect of ALA dependent on GPR120.

## Results

It was shown that the expression of liver M $\phi$ s was significantly attenuated in DM/ALA group compared with that in the DM/CO group. In addition, ALA decreased the levels of proinflammatory cytokines (TNF- $\alpha$ , IL-6, IL-1 $\beta$  and IL-18) and increased the levels of anti-inflammatory cytokines (IL-10) in LPS-induced RAW264.7 mouse macrophages. Moreover, ALA markedly inhibited the up-regulation of Toll-like receptor 4 (TLR4), the activation of nuclear factor kappa B (NF- $\kappa$ B) and the expression of the inflammasome components such as NOD-like receptor protein 3 (NLRP3) and caspase-1 in LPS-stimulated RAW264.7 cells. Of note, our observations indicated that expressions of GPR120 and  $\beta$ -arrestin2 were enhanced by the treatment with ALA, suggesting that GPR120 and subsequent recruited  $\beta$ -arrestin2 participated in anti-inflammatory effect. Intriguingly, blocked with GPR120 antagonist, AH7614, the inhibitory effects of ALA on inflammation and TLR4/NF- $\kappa$ B pathway activity in LPS-stimulated RAW264.7 mouse macrophages were weakened.

# Conclusion

Collectively, ALA suppressed NF- $\kappa$ B signal pathway and NLRP3 inflammasome activation of macrophage by binding to the GPR120 receptor to ameliorate T2DM.

## 1. Introduction

According to the latest global diabetes map released of the International Diabetes Federation (IDF), a total of 463 million people are estimated to be living with diabetes in 2019, it is predicted that 579 million people will have diabetes in 2030 and the number will increase by 51% (700 million) in 2045, 90% of which are type 2 diabetes mellitus (T2DM). Diabetes has become one of the rapidly-growing diseases[1]. T2DM is a chronic metabolic disease, mainly characterized by hyperglycemia and insulin resistance, which can cause serious damage to the body target organs, lead to numerous complications, and seriously affect the health and quality of life of patients[2–4]. Currently, chronic low-grade inflammation is considered to be one of the leading causes of T2DM[5]. The clinical and experimental animal studies have shown that in the occurrence and development of T2DM, inflammation induces insulin resistance to produce hyperglycemia which also promotes the progression of inflammation, leading to the occurrence of various complications including nephropathy[6], vasculopathy[7], retinopathy[8] and peripheral neuropathy[9]. Hence, the improved understanding of the mechanisms linking inflammation to diabetes mellitus has stimulated interest in targeting inflammatory pathways to improve diabetes prevention and care.

In the inflammatory mechanism of T2DM, hepatic macrophages (mainly Kupffer cells) promote the expression of inflammatory cytokines such as interleukin (IL)-1 $\beta$ , IL-6, IL-18 and tumor necrosis factor (TNF)- $\alpha$ , cause abnormal phosphorylation of insulin receptor substrates, interfere with normal insulin signal transduction and lead to insulin resistance through activating the NF- $\kappa$ B or JUNK signaling pathway, which causes diabetes[10, 11]. It is now generally accepted that tissue-resident macrophages play major roles in the regulation of tissue inflammation[12]. According to different functions and phenotypes, macrophages can be divided into the classically activated, pro-inflammatory phenotype (M1-type macrophages) and the alternatively activated phenotype (M2-type macrophages). Representative pro-inflammatory indicators of M1 involve inducible nitric oxide synthase (iNOS) and TNF- $\alpha$ , which have been shown to increase tissue inflammation in chronic metabolic diseases[13]. Arginase-1 (Arg-1) and anti-inflammatory IL-10 in M2 are implicated in immunosuppression, tissue remodeling and homeostasis[14]. M2-type macrophages, which are different from M1-type macrophages, play a key role in the regression of inflammation by suppressing the secretion of pro-inflammatory cytokines and producing anti-inflammatory regulators[15–17]. Imbalance of M1/M2 polarization or repolarization of resident macrophages is usually associated with various diseases, such as inflammatory, auto-immune disorders and chronic infections[18–20].

NLRP3 inflammasome is a protein complex composed of nucleotide binding oligomerization domain like receptor protein 3 (NLRP3), apoptosis associated speck-like protein (ASC) and pro-caspase-1, which is an

important part of inflammatory immune response and a major “receptor” of inflammatory response[21, 22]. Under normal circumstances, NLRP3 binds to molecular chaperones such as heat shock protein (HSP) 90 and exists in the cells in an inactive form. Upon stimulation by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), NLRP3 is activated as a key component in the complex, recruiting the connector molecule ASC, which co-assembles with the effector molecule caspase-1 into inflammasome to triggering a series of inflammatory cascade reactions and participating in the occurrence and development of inflammation in various chronic diseases[23]. Accumulating evidence has shown that NLRP3 plays a significant role in the development of T2DM[24–26]. Studies have demonstrated that T2DM and other diseases can cause increased expression of NLRP3 inflammasome in peripheral blood mononuclear cells of patients, which can be used as a marker to predict the onset of T2DM severity events[24]. The NLRP3 inflammasome is involved in macrophage-mediated inflammation and regulates insulin secretion[25]. In addition, Zhou R et al. found that NLRP3 gene knockout could significantly increase glucose tolerance and insulin sensitivity[26].

$\alpha$ -linolenic acid (ALA) is an essential fatty acid that cannot be synthesized by the human body and must be obtained as nutrients in the diet[27]. As the precursor of two important longer chain omega-3 fatty acids ( $\omega$ -3 FAs) eicosapentaenoic acid (EPA, 20:3n-5) and docosahexaenoic acid (DHA, 22:3n-6), ALA has been reported to have a variety of nutritional, health care and pharmacological effects, mainly including cardiovascular-protective, neuro-protective, anti-cancer, anti-osteoporotic, anti-inflammatory, and antioxidative effects[27, 28]. Among researchers including our lab have demonstrated that dietary ALA-rich flaxseed oil (FO) has pleiotropic benefits in chronic metabolic diseases, such as alcoholic liver disease (ALD)[29], polycystic ovarian syndrome (PCOS)[30], atherosclerosis (AS)[31] and colitis[32]. Furthermore, animal model studies of T2DM also indicated that an ALA-rich diet improved hyperglycemic and hypertriglyceridemia in diabetic mice[33, 34]. Although an ALA-rich diet has significant beneficial effects on variety chronic metabolic diseases, these studies are difficult to directly demonstrate the effect of improvement in these diseases. In addition, the effects of ALA on T2DM and the underlying mechanisms are poorly understood.

G protein-coupled receptor 120 (GPR120), as a member of a family of lipid binding free fatty receptors including GPR40, GPR41 and GPR43, which has been demonstrated to be specifically activated by long-chain fatty acids (LCFAs)[35]. Da Young Oh et al. discovered that GPR120 is the only lipid sensing G protein-coupled receptor (GPCR) which is highly expressed in adipose tissue, proinflammatory CD11c<sup>+</sup> macrophages (BMDCs), mature adipocytes, and monocytic RAW264.7 cells[36]. Furthermore, a research has shown that DHA stimulation leads to the recruitment of  $\beta$ -arrestin2 to the plasma membrane where it colocalizes with GPR120. Following DHA-stimulated internalization of the GPR120- $\beta$ -arrestin2 complex, and then  $\beta$ -arrestin2 can interplay with TAB1. It has been reported that TAB1 is the activating protein for TAK1 and above phenomenon suppresses TAK1 activation and downstream signaling to the IKK $\beta$ /NF- $\kappa$ B and JNK/AP1 system[36, 37]. Besides, an animal study showed that a GPR120-selective agonist improves insulin resistance and chronic inflammation in obese mice[38].

Since chronic inflammation is a mechanistic feature of insulin resistance and the underlying mechanisms of anti-inflammatory of ALA on T2DM remain elusive, we postulated that ALA may alleviate the inflammation in T2DM by enhancing GPR120 expression. In the present study, we investigate the effectiveness of ALA on the liver macrophages of T2DM model rats and the anti-inflammatory mechanism of ALA was uncovered in LPS-stimulated RAW264.7 murine macrophages.

## **2. Materials And Methods**

### **2.1 Animals and diet**

All experimental procedures were approved by the Ethics Committee of Ningxia Medical University (No.2020-034). Male Sprague-Dawley (SD) rats (200–250 g) were purchased from Central Animal House of Ningxia Medical University. They were placed in a polycarbonate cage with a temperature control chamber (ambient temperature  $22 \pm 2^{\circ}\text{C}$ , air humidity 40%-70%), 12 h dark/light cycle. During the whole experiments period, animals were fed with a balanced commercial diet and water ad libitum. All diets for rats feeding were purchased from TROPHIC Animal Feed High-tech Co., Ltd., Nantong, China.

### **2.2 Diabetic rats**

The rats were fasted 12 h before the streptozotocin (STZ)-nicotinamide (NA) injection. T2DM was induced by the intraperitoneal (i.p) administration of a single dose (65 mg/kg BM) of STZ freshly dissolved in 0.1 M citrate buffer(pH = 4.5), 15 min after i.p administration of 110 mg/kg BM of NA dissolved in normal saline. Animals were allowed to drink 20% glucose solution overnight to overcome drug-induced hypoglycemia. Rats in the pair-fed (PF) group were i.p injected with an equal volume of citrate buffer and normal saline. Hyperglycemia was confirmed according to the levels of fasting blood glucose (FBG), which was determined in day 3 and 7 after injection. Rats with FBG levels above 13.9 mmol/L were considered to T2DM in the experiment[39], followed by feeding various types of diets.

### **2.3 Experimental design**

Rats were randomly divided into 4 groups (8 rats/group): (a) PF with corn oil (CO) group (PF/CO), PF rats were fed 10% w/w CO diet as CO control; (b) DM with CO group (DM/CO), DM rats were fed 10% w/w CO diets; (c) PF with  $\alpha$ -Linolenic acid (ALA) group (PF/ALA), PF rats were fed 10% w/w ALA diet as ALA control; (d) DM with ALA group (DM/ALA), DM rats were fed 10% w/w ALA diet. The standard rodent chow pellets were powdered and mixed with ALA and re-pelleted so as to contain either 10% w/w. Animals from PF/ALA group and DM/ALA group received 10% w/w ALA diets. As control, PF/CO group and DM/CO group received 10% w/w CO diet. Feeds were prepared every week and packed sealed bags in quantities sufficient for 1 day's feed. Feeds in the plastic bags were flushed with nitrogen, sealed, and stored at  $-20^{\circ}\text{C}$ . Feeds which were not consumed by animals were discarded daily. After 5 weeks of feeding, rats were then euthanized and associated indications were investigated.

### **2.4 Cell culture and stimulation**

Murine macrophage RAW264.7 cells (ATCC: TIB-71) were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, BI, Israel), 100 mg/mL streptomycin, 100 U/mL penicillin and incubated in 5% CO<sub>2</sub> at 37°C. ALA (Sigma-Aldrich, USA) and GPR120 antagonist AH7614 (APE\*<sup>BIO</sup>, USA) were dissolved with DMSO to a concentration of 20 mM, and then diluted in DMEM to a working concentration of 20 µM. RAW264.7 murine macrophage cells were seeded in 6-well plates. After 12 h of culture, cells were treated for 12 h with AH7614 followed by 2 h incubation with ALA or its vehicle solution. After incubation, cells were stimulated with LPS (Sigma-Aldrich, USA) for 24 h, and then the cell culture supernatants or the cells were collected for ELISA or western blotting. The working concentration of LPS was 100 ng/mL.

## 2.5 Immunofluorescence (IF) of liver tissue

To determine the role of hepatic Mφs in T2DM after ALA treatment, IF was used to investigate the liver tissue. In brief, after dehydration and rehydration, paraffin sections of liver were incubated with EDTA (pH 8.0) solution at microwave oven for 20 min to renovate antigen. Subsequently the slides were blocked with 2% bovine serum albumin (BSA) for 1 h at room temperature. After washing with 0.5% BSA for three times, samples were incubated with rabbit anti-rat CD68 antibody (1:100 dilution, Servicebio, China) at 4°C overnight. After rinsing with 0.5% BSA, the slides were incubated with CY-3 labeled goat anti-rabbit antibody (1:200 dilution, Servicebio, China) for 1 h at room temperature under dark condition. After three times of washing, slides were stained with the sealing tablet containing DAPI to observe the nucleus. Images were captured with Olympus BX51 microscope (Aomori Olympus, Japan). Positive areas in 20 optical fields (200× magnification) within the liver region were then observed by blinders of the experiments.

## 2.6 Enzyme-linked immunosorbent assay (ELISA)

Supernatants of *in vitro* cell culture in each group were respectively collected and centrifuged at 1000 × g for 20 min, and the supernatants were collected for the determination of inflammatory cytokines concentrations. The tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1β, IL-18 and IL-10 were measured respectively by ELISA kits according to the manufacturer's instructions (Shanghai, Jianglai biotech, Shanghai, China). Optical density was measured at 450 nm within 15 min using an automated microplate reader (Thermo Scientific, United States). Each sample was tested in triplicate.

## 2.7 Western blot

Total proteins of RAW264.7 cells were extracted using a commercial kit (Keygen, No. KGP903, Nanjing, China). Protein concentrations were detected with a BCA protein assay kit (Keygen, No. KGBSP002, China). Equal amounts of protein extracted (30–50 µg) samples were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to a polyvinylidene difluoride (PVDF) (Millipore, Bedford, MA, United States) membrane electronically. The membranes were blocked with 5% non-fat milk and then incubated with primary antibodies at 4°C overnight as follows: rabbit anti-mouse NLRP3 (1:500 dilution, Abcam, United State), mouse monoclonal caspase-1 (1:500

dilution, Santa Cruz, United States), rabbit anti-mouse NF- $\kappa$ B (1:500 dilution, Abcam, United State), mouse monoclonal Toll-like receptors-4(TLR4) (1:500 dilution, Santa Cruz, United States), rabbit polyclonal Arg-1 (1:500 dilution, Abcam, United State), rabbit monoclonal iNOS (1:1,000 dilution, Abcam, United State), and mouse monoclonal GAPDH (1:1,000 dilution, China). After washing with 1×TBST buffer for three times, membranes were incubated with HRP-conjugated anti-mouse (1:1,000 dilution, Abbkine, China) or anti-rabbit (1:1,000 dilution, Abbkine, China), respectively. After washing, membranes were visualized with ECL chemiluminescent kit (Thermo Scientific, United State) and measured using the Azure c400 (Thermo Scientific, United State). Images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, United State).

## 2.8 Statistical analysis

All data were statistically analyzed using Prism 8.0 (GraphPad Software Inc., USA). The results were presented as the mean  $\pm$  standard deviation (SD). Differences among multiple comparisons were performed using two-way analysis of variance (ANOVA). Differences between two groups were analysis by Student's t test (2-tailed) based on normal distribution. Correlation analysis was performed using the Spearman method.  $P < 0.05$  was considered statistically significant.

## 3. Results

### 3.1 ALA intervention decreased the percentage of M $\phi$ s from liver in T2DM rats

To study M $\phi$ s of hepatic in T2DM rats, we determined the quantity and localization of M $\phi$ s in liver by IF assay. Images from IF showed that the fluorescence intensity of M $\phi$ s in PF/CO group was  $0.98 \pm 0.18\%$ , and that in the DM/CO group was significantly increased to  $1.78 \pm 0.05\%$  (Fig. 1A and B,  $P < 0.01$ ), indicating that M $\phi$ s infiltration appeared in the liver of T2DM rats. Compared with the DM/CO group, the fluorescence intensity of M $\phi$ s decreased to  $0.88 \pm 0.02\%$  after ALA intervention (Fig. 1A and B,  $P < 0.001$ ), which was similar to the PF/CO group. Thus, we speculated that the chronic low-grade inflammation of T2DM could be reduced by improving the infiltration of M $\phi$ s in hepatic after ALA intervention. In conclusion, ALA intervention can reduce the level of M $\phi$ s in liver, which may provide a new idea for treatment of T2DM.

### 3.2 ALA suppressed NLRP3 inflammasome and Nuclear Factor- $\kappa$ B in RAW264.7

The above finding indicated that in T2DM, the polarization of pro-inflammation M $\phi$ s could be attenuated by a dietary ALA intervention treatment. In addition, relevant animal studies have shown that dietary ALA-rich FO significantly reduced the levels of plasma inflammatory cytokines in T2DM model rats, including IL-1 $\beta$ , IL-6, IL-17A and TNF- $\alpha$ [40]. Due to NLRP3 inflammasome and NF- $\kappa$ B pathway play a crucial role in inflammation, we investigated whether anti-inflammatory effect of ALA on T2DM was associated with

the suppression of NLRP3 inflammasome/NF- $\kappa$ B activation *in vitro*. The lipopolysaccharide (LPS) stimulated RAW264.7 mouse macrophages cellular inflammation model was established to understand the molecular mechanism by which ALA inhibits the release of cytokines from RAW264.7 cells. Western blot was utilized to determine the expressions of caspase-1, NLRP3, NF- $\kappa$ B and TLR4. As a result, RAW264.7 cells stimulated by LPS significantly increased the expression of caspase-1 ( $P < 0.001$ , Fig. 2A, B), NLRP3 ( $P < 0.0001$ , Fig. 2A, C) and TLR4 ( $P < 0.01$ , Fig. 2A, E) compared with those in the control group. In contrast, the treatment with ALA reduced the representations of NLRP3, caspase-1 and TLR4 in RAW264.7 cells greatly of that in the LPS group. In addition, LPS induced a notable increase of NF- $\kappa$ B, but a significant reduction of which was observed in ALA treatment group ( $P < 0.05$ , Fig. 2A, D), suggesting that ALA may inhibit the activation of NLRP3 inflammasome depending on NF- $\kappa$ B signaling pathway.

### **3.3 ALA repression of LPS-induced NLRP3 inflammasome and NF- $\kappa$ B activation depends on GPR120**

To verify whether ALA inhibits LPS-induced inflammatory response in RAW264.7 cells by activating GPR120 receptors, the GPR120 antagonist, AH7614, as well as ALA were co-cultured with RAW264.7 cells, and ultimately the expression levels of NLRP3 inflammasome and NF- $\kappa$ B pathway were assessed. As displayed in Fig. 3, the expression levels of caspase-1 ( $P < 0.05$ , Fig. 3A, B), NLRP3 ( $P < 0.05$ , Fig. 3A, C), NF- $\kappa$ B ( $P < 0.05$ , Fig. 3A, D) and TLR4 ( $P < 0.0001$ , Fig. 3A, E) in the LPS stimulated group were higher compared with those in the control group. Interestingly, AH7614 treatment clearly mitigated the suppressive effects of ALA on the expression levels of caspase-1, NLRP3, NF- $\kappa$ B and TLR4 in the LPS-induced RAW264.7 cells ( $P > 0.05$ , Fig. 3).

### **3.4 ALA suppressed the release of inflammatory factors from RAW264.7 mouse macrophages**

To measure the contents of inflammatory factors in cell culture supernatants, ELISA was used. The data showed that the concentrations of IL-1 $\beta$ , IL-18, TNF- $\alpha$  and IL-6 in the supernatants of RAW264.7 cells stimulated with LPS were increased significantly than those in the control group ( $P < 0.0001$ ). However, the treatment with ALA decreased the release of IL-1 $\beta$ , IL-18, TNF- $\alpha$  and IL-6 compared with those in the LPS group ( $P < 0.0001$ , Fig. 4). Notably, AH7614 clearly weakened the inhibiting effect of ALA on inflammatory factors expression. As displayed in Fig. 4, with the treatment of AH7614 in RAW264.7, the suppressive effects of ALA on the expression of IL-1 $\beta$ , IL-18, TNF- $\alpha$  and IL-6 in the LPS-induced group were eliminated ( $P > 0.05$ ). The results indicated that ALA suppressed the release of inflammatory factors from RAW264.7 mouse macrophages, but AH7614 reversed these inhibiting effect.

### **3.5 ALA alleviated inflammatory response in the LPS-induced RAW264.7 cells by enhancing GPR120**



In order to further verify the mechanism of ALA inhibiting the generation of NLRP3 inflammasome and the transduction of NF- $\kappa$ B pathway, we next investigated the influence of ALA on GPR120 and  $\beta$ -arrestin-2 expression in murine macrophage RAW264.7 cells by treating the cells with ALA for 2 h followed by harvesting and lysis. Subsequent western blot analysis showed that the expression of  $\beta$ -arrestin-2 was notably decreased in response to LPS ( $P < 0.01$ , Fig. 5A and D). On the contrary, both the expressions of GPR120 ( $P < 0.05$ ) and  $\beta$ -arrestin-2 ( $P < 0.001$ ) were observably increased after ALA treatment (Fig. 5A, C and D). However, all of these effects of ALA were completely abrogated by the inhibitor of GPR120 (Fig. 5B, C and D).

### **3.6 ALA significantly reduced inflammation in LPS-induced RAW264.7 cells, including suppression of M1 macrophages and activation of M2 macrophages**

To further investigate the effects of ALA on M1/M2 M $\phi$  polarization, murine macrophage RAW264.7 cells were stimulated with LPS for 24 h and then co-cultured with ALA. ELISA was used to examine the expression of TNF- $\alpha$  and IL-10 in the supernatants of medium after culture experiments. The levels of TNF- $\alpha$  in groups of LPS + ALA were reduced compared to LPS group ( $P < 0.0001$ , Fig. 6A). On the contrary, the level of IL-10 was increased in group of ALA + LPS compared with LPS group ( $P < 0.0001$ , Fig. 6B). All of these alleviated effects were reversed due to the intervention of AH7614.

To examine the hypothesis that M1/M2 M $\phi$  polarization was induced by ALA, we further evaluated the expression of M $\phi$  phenotypical markers upon ALA treatment *in vitro* by western blot. The results indicated that LPS + ALA reduced protein level of iNOS compared to LPS group ( $P < 0.05$ , Fig. 6C and D). Furthermore, the expression of M2 M $\phi$  phenotypical marker Arg-1 in group of ALA + LPS was increased compared to LPS group ( $P < 0.001$ , Fig. 6C and F). Interestingly, the intervention of ALA showed no effect on iNOS and Arg-1 under the AH7614 treatment ( $P > 0.05$ , Fig. 6D, E and F).

## **4. Discussion**

T2DM is a leading cause of morbidity and mortality worldwide. In recent decades, accumulating studies have demonstrated that inflammation mediates insulin resistance and hyperglycemia participates in the pathogenesis of T2DM[41, 42]. It is known that chronic inflammation, mediated in large part by pro-inflammatory macrophage population, contributes directly to the induction and perpetuation of metabolic diseases including obesity, insulin resistance (IR) and T2DM[43]. As a part of the immune system, the liver plays a key role by secreting acute-phase protein, complement components, chemokines and cytokines. Moreover, relevant studies have shown that liver macrophages infiltrate metabolic organs under obese conditions and contribute to low-grade inflammation that impairs insulin action, leading to the development of IR[44, 45]. In clinical and experimental studies, the consumption of  $\omega$ -3 FAs has exhibited a systemic anti-inflammatory benefit in a variety of inflammatory human diseases, including diabetes, AS, asthma, and arthritis[46, 47]. Compared with EPA and DHA, the study of ALA is relatively poor. However, as the only dietary source for  $\omega$ -3 FAs produced from the ground, the functional effects and health benefits of ALA deserves further study. Thus, to confirm the influence of dietary ALA on T2DM,

the immunofluorescence results of hepatic macrophages showed that the proportions of CD68<sup>+</sup> cells was significantly decreased with the dietary ALA administration *in vivo*, suggesting that the anti-inflammation effect of ALA intervention might be mainly due to the inhibition of inflammatory macrophages. We speculated that the anti-inflammation role of dietary ALA on T2DM might be through regulating macrophage polarization to improve IR, but the exact evidence needs to further verify *in vitro*.

Next, the experiment were designed to reveal the underlying mechanisms of anti-inflammatory effect of ALA due to perform on macrophages. RAW264.7 cells, a murine macrophage cell line, were stimulated with LPS to establish an *in vitro* inflammatory model, which is characterized with an excessive inflammatory response and abnormally high levels of pro-inflammatory cytokines[48, 49]. It has been demonstrated that macrophages play a key role in obesity-induced chronic low-grade inflammation, which is tightly related to macrophage infiltration into peripheral tissues and IR[50]. Emerging evidence has shown that NLRP3 inflammasome is a key target for  $\omega$ -3 FAs to suppress inflammation and exert their anti-inflammatory activity in inflammatory disorders[51]. Moreover,  $\omega$ -3 FAs supplementation could suppress HFD-induced NLRP3 inflammasome activation and prevent NLRP3 inflammasome-dependent IR. The NLRP3 inflammasome is a critical component of the innate immune system that mediates caspase-1 activation and the secretion of proinflammatory cytokines IL-1 $\beta$ /IL-18 in response to microbial infection and cellular damage[52]. Under the pathology, macrophages exposed to priming stimuli, such as ligands for Toll-like receptors (TLRs), NOD-like receptors (NLRs), or cytokine receptors which activate the transcription factor NF- $\kappa$ B[53]. And then, NF- $\kappa$ B acts as an initiation signal to activate the NLRP3 inflammasome, recruit and activate caspase-1, induce apoptosis and the maturation and secretion of proinflammatory cytokines, thereby promoting inflammation. In the present study, LPS-stimulated expression of TLR4, NF- $\kappa$ B, NLRP3 and caspase-1 as well as production of IL-1 $\beta$  and IL-18 tended to be reduced by ALA, which was consistent with previous studies[51].

A research has indicated that DHA induces GPR120 activation leading to the recruitment of  $\beta$ -arrestin-2, and the subsequent internalization of the GPR120/ $\beta$ -arrestin-2 complex leads to its binding to TAK1 binding protein 1 (TAB1), which interferes with TAB1 binding to TAK1 and inhibits TAK1 phosphorylation and activation[36]. Furthermore, since TAB1/TAK1 binding is a convergent signaling point of both LPS and TNF- $\alpha$  stimulation, TAK1 activation stimulates both IKK $\beta$ /NF- $\kappa$ B and JNK/AP1 pathway and participates in low grade tissue inflammation[36]. Moreover, in another study, it was found that the stimulation of macrophages with  $\omega$ -3 FAs, including EPA and DHA, abolished NLRP3 inflammasome activation and suppressed subsequent caspase-1 activation and IL-1 $\beta$  secretion via GPR120 and its downstream scaffold protein  $\beta$ -arrestin-2[51]. Our data revealed that the expression of GPR120 and  $\beta$ -arrestin-2 in LPS-induced RAW264.7 cells were markedly increased with ALA supplementation, demonstrating that ALA may prevent NLRP3 inflammasome-dependent inflammation and metabolic disorder via GPR120. Subsequently, the impacts of ALA on LPS-stimulated inflammation consequences were further determined. In parallel, ALA prominently inhibited the increases of TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and IL-18 induced by LPS-stimulated macrophage activation, which are important proinflammatory cytokines.

Taken together, we reasoned that GPR120 functions as ALA receptor and mediates robust and broad anti-inflammatory effects, particularly in macrophages. In order to determine the crucial role of GPR120 in the effectiveness of ALA on the development of inflammation, we used GPR120 antagonist, AH7614, to demonstrate that ALA cause GPR120-mediated anti-inflammatory effects *in vitro*. In this experiment, ALA intervention observably decreased the activation of the NLRP3 inflammasome and the expression of GPR120 in LPS-induced RAW264.7 cells but was without effect in the GPR120 KD (under the use of AH7614). Consistent with these results, ALA treatment led to a decrease in M1-type macrophage with reduced pro-inflammatory markers iNOS and the secretion of TNF- $\alpha$ , as well as an increase in M2-type macrophage with promoted anti-inflammatory markers Arg-1 and the secretion of IL-10 in LPS-stimulated murine macrophages. As expected, restraining the expression of GPR120 abolished the anti-inflammatory effect of ALA. These results together highlight the fact that ALA exerts its anti-inflammatory property largely by stimulating GPR120 activation and modulating macrophage polarization.

In summary, we have found that ALA can suppress NF- $\kappa$ B signal pathway and NLRP3 inflammasome activation of LPS-induced RAW264.7 macrophages inflammatory process by binding to the GPR120 receptor. Furthermore, we proved that this suppressing effect of ALA was strictly dependent on GPR120 activation. Combined with previous studies[54], it's reasonable to speculate that ALA may improve the occurrence and development of T2DM by ameliorating insulin sensitivity through anti-inflammatory effect. In addition, GPR120 acts as an important control point of anti-inflammatory and insulin sensitizing responses. Thus, this study highlighted that the ALA mainly ameliorated chronic inflammation via stimulating GPR120 activation, which potentially served as an auxiliary functional food for T2DM.

## Abbreviations

T2DM

Type 2 diabetes mellitus

GPR120

G-protein coupled receptor 120

ALA

$\alpha$ -linolenic acid

PF

Pair-fed

CO

Corn oil

STZ-NA

Streptozotocin-nicotinamide

LPS

Lipopolysaccharide

M $\phi$ s

Macrophages

IF

Immunofluorescence  
TLR4  
Toll-like receptor 4  
NF-κB  
Nuclear factor kappa B  
iNOS  
Inducible nitric oxide synthase  
Arg-1  
Arginase-1  
NLRP3  
NOD-like receptor protein 3  
IL  
Interleukin  
TNF-α  
Tumor necrosis factor  
ASC  
Apoptosis associated speck-like protein  
HSP  
Heat shock protein  
PAMPs  
Pathogen-associated molecular patterns  
DAMPs  
Damage-associated molecular patterns  
ω-3 FAs  
Omega-3 fatty acids  
EPA  
Eicosapentaenoic acid  
DHA  
Docosahexaenoic acid  
ALD  
Alcoholic liver disease  
PCOS  
Polycystic ovarian syndrome  
AS  
atherosclerosis  
LCFAs  
Long-chain fatty acids  
GPCR  
G protein-coupled receptor.

# Declarations

## Acknowledgements

No applicable.

## Authors' contributions

Conceptualization, H. W., XX. Z. and YY. L.; methodology, YY. L., MX. G., YW. L., T. W., Y. R., R. W., X. J.; software, YY. L.; validation, H. W., XX. Z. and YY. L.; formal analysis, YY. L.; investigation, YY. L., MX. G., YY. L., T. W., Y. R., R. W. and X. J.; resources, H. W.; data curation, YY. L., MX. G., YW. L. and T. W.; writing—original draft preparation, YY. L.; XX. Z. and H. W.; writing—review and editing, H. W. and YY. L.; visualization, YY. L.; supervision, XX. Z. and H. W.; project administration, H. W.; funding acquisition, H. W. and XX. Z. All authors have approved the final draft of the manuscript.

## Funding

This work was supported by the National Natural Science Foundation of China (Grant No. 82160691), Ningxia Natural Science Foundation, China (Grant No. 2020AAC03132, 2021AAC05010), the Research Project of Ningxia Medical University (Grant No. XZ2021003), Orthopedic Department Group Project of Ningxia Medical University (Grant No. XY201627 and XY201529).

## Ethics approval and consent to participate

All experimental procedures were approved by the Ethics Committee of Ningxia Medical University (No.2020-034), and carried out in accordance with the 2011 revised form of The Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

## Author details

<sup>1</sup> Department of Pathogenic Biology and Medical Immunology, School of Basic Medical Sciences, Ningxia Medical University, Yinchuan, China. <sup>2</sup> Key Laboratory of Fertility Preservation and Maintenance of Ministry of Education, Ningxia Medical University, Yinchuan, China. <sup>3</sup> Clinical Medical College, Ningxia Medical University, Yinchuan, China. <sup>4</sup> College of Traditional Chinese Medicine, Ningxia Medical University, Yinchuan, China.

# References

1. Saeedi P, Petersohn I, Salpea P, Malanda B, Karuranga S, Unwin N, Colagiuri S, Guariguata L, Motala AA, Ogurtsova K *et al*: Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9(th) edition. *Diabetes Res Clin Pract* 2019, 157:107843.
2. Van der Schaft N, Schoufour JD, Nano J, Kiefte-de JJ, Muka T, Sijbrands E, Ikram MA, Franco OH, Voortman T: Dietary antioxidant capacity and risk of type 2 diabetes mellitus, prediabetes and insulin resistance: the Rotterdam Study. *EUR J EPIDEMIOLOG* 2019, 34(9):853-861.
3. Li X, Zhen M, Zhou C, Deng R, Yu T, Wu Y, Shu C, Wang C, Bai C: Gadofullerene Nanoparticles Reverse Dysfunctions of Pancreas and Improve Hepatic Insulin Resistance for Type 2 Diabetes Mellitus Treatment. *ACS NANO* 2019, 13(8):8597-8608.
4. Shin JJ, Lee EK, Park TJ, Kim W: Damage-associated molecular patterns and their pathological relevance in diabetes mellitus. *AGEING RES REV* 2015, 24(Pt A):66-76.
5. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA *et al*: Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J CLIN INVEST* 2003, 112(12):1821-1830.
6. Liu SY, Chen J, Li YF: Clinical significance of serum interleukin-8 and soluble tumor necrosis factor-like weak inducer of apoptosis levels in patients with diabetic nephropathy. *J Diabetes Investig* 2018, 9(5):1182-1188.
7. Qiu T, Li M, Tanner MA, Yang Y, Sowers JR, Korthuis RJ, Hill MA: Depletion of dendritic cells in perivascular adipose tissue improves arterial relaxation responses in type 2 diabetic mice. *METABOLISM* 2018, 85:76-89.
8. Chen H, Zhang X, Liao N, Mi L, Peng Y, Liu B, Zhang S, Wen F: Enhanced Expression of NLRP3 Inflammasome-Related Inflammation in Diabetic Retinopathy. *Invest Ophthalmol Vis Sci* 2018, 59(2):978-985.
9. Wei W, Li L, Deng L, Wang ZJ, Dong JJ, Lyu XY, Jia T, Wang L, Wang HX, Mao H *et al*: Autologous Bone Marrow Mononuclear Cell Transplantation Therapy Improved Symptoms in Patients with Refractory Diabetic Sensorimotor Polyneuropathy via the Mechanisms of Paracrine and Immunomodulation: A Controlled Study. *CELL TRANSPLANT* 2020, 29:2138941962.
10. Lontchi-Yimagou E, Sobngwi E, Matsha TE, Kengne AP: Diabetes mellitus and inflammation. *Curr Diab Rep* 2013, 13(3):435-444.
11. Ghorpade DS, Ozcan L, Zheng Z, Nicoloso SM, Shen Y, Chen E, Bluher M, Czech MP, Tabas I: Hepatocyte-secreted DPP4 in obesity promotes adipose inflammation and insulin resistance. *NATURE*

2018, 555(7698):673-677.

12. Ying W, Fu W, Lee YS, Olefsky JM: The role of macrophages in obesity-associated islet inflammation and  $\beta$ -cell abnormalities. *Nature reviews. Endocrinology* 2020, 16(2):81-90.
13. Hara-Chikuma M, Tanaka M, Verkman AS, Yasui M: Inhibition of aquaporin-3 in macrophages by a monoclonal antibody as potential therapy for liver injury. *NAT COMMUN* 2020, 11(1).
14. DeNardo DG, Barreto JB, Andreu P, Vasquez L, Tawfik D, Kolhatkar N, Coussens LM: CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *CANCER CELL* 2009, 16(2):91-102.
15. Yuan F, Ma J, Xiang X, Lan H, Xu Y, Zhao J, Li Y, Zhang W: Improvement of Adipose Macrophage Polarization in High Fat Diet-Induced Obese GHSR Knockout Mice. *BIOMED RES INT* 2018, 2018:4924325.
16. Martin LM, Moller M, Weiss U, Russe OQ, Scholich K, Pierre S, Geisslinger G, Niederberger E: 5-Amino-1-beta-D-Ribofuranosyl-Imidazole-4-Carboxamide (AICAR) Reduces Peripheral Inflammation by Macrophage Phenotype Shift. *INT J MOL SCI* 2019, 20(13).
17. Jaggi U, Yang M, Matundan HH, Hirose S, Shah PK, Sharifi BG, Ghiasi H: Increased phagocytosis in the presence of enhanced M2-like macrophage responses correlates with increased primary and latent HSV-1 infection. *PLOS PATHOG* 2020, 16(10):e1008971.
18. Redente EF, Higgins DM, Dwyer-Nield LD, Orme IM, Gonzalez-Juarrero M, Malkinson AM: Differential polarization of alveolar macrophages and bone marrow-derived monocytes following chemically and pathogen-induced chronic lung inflammation. *J Leukoc Biol* 2010, 88(1):159-168.
19. Yang YF, Zhou YD, Hu JC, Luo FL, Xie Y, Shen YY, Bian WX, Yin ZN, Li HL, Zhang XL: Ficolin-A/2, acting as a new regulator of macrophage polarization, mediates the inflammatory response in experimental mouse colitis. *IMMUNOLOGY* 2017, 151(4):433-450.
20. Radhakrishnan RK, Thandi RS, Tripathi D, Paidipally P, McAllister MK, Mulik S, Samten B, Vankayalapati R: BCG vaccination reduces the mortality of Mycobacterium tuberculosis-infected type 2 diabetes mellitus mice. *JCI Insight* 2020, 5(5).
21. Ding S, Xu S, Ma Y, Liu G, Jang H, Fang J: Modulatory Mechanisms of the NLRP3 Inflammasomes in Diabetes. *Biomolecules* 2019, 9(12):850.
22. Jo EK, Kim JK, Shin DM, Sasakawa C: Molecular mechanisms regulating NLRP3 inflammasome activation. *CELL MOL IMMUNOL* 2016, 13(2):148-159.
23. Kelley N, Jeltema D, Duan Y, He Y: The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. *INT J MOL SCI* 2019, 20(13):3328.

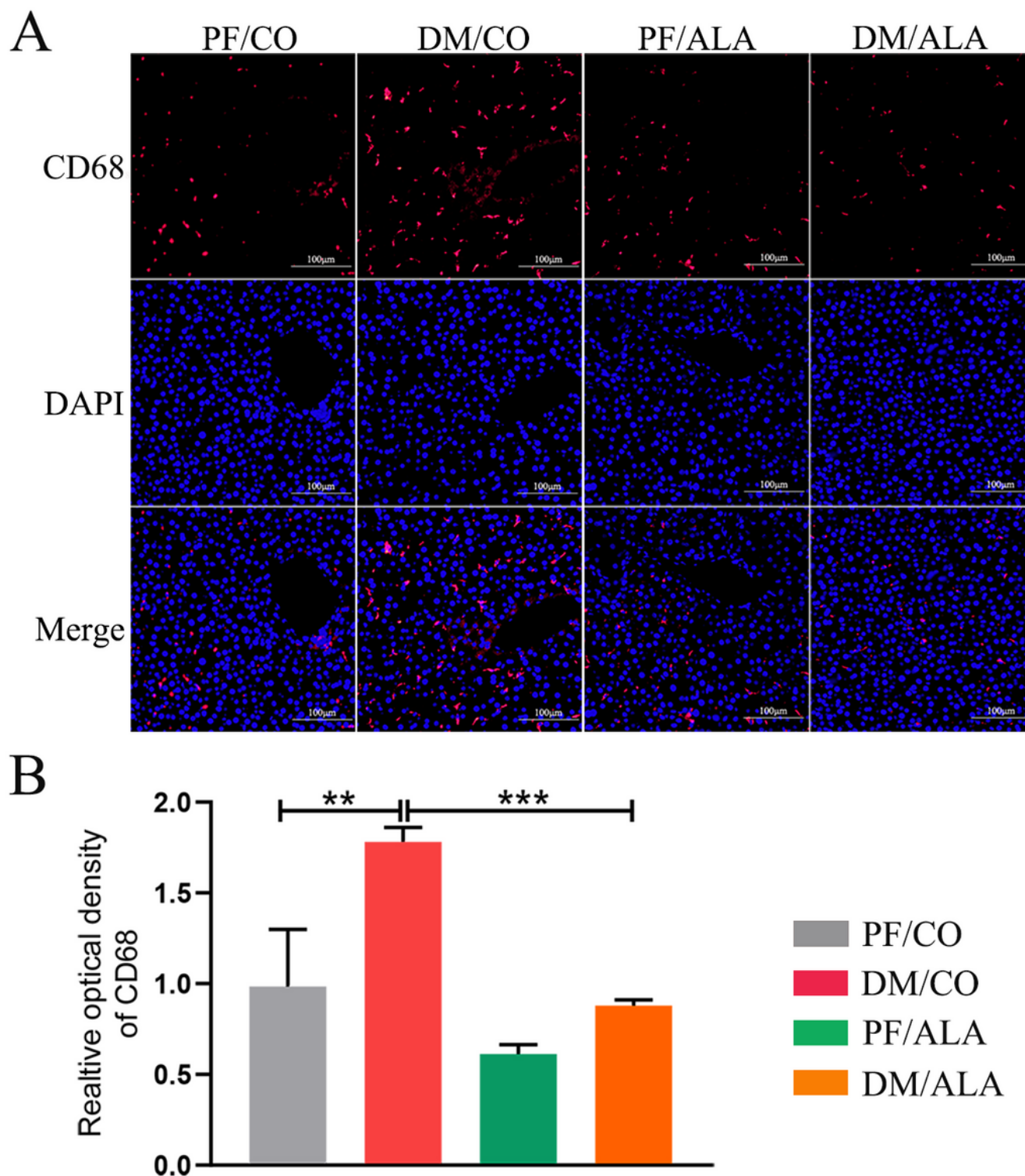
24. Lee HM, Kim JJ, Kim HJ, Shong M, Ku BJ, Jo EK: Upregulated NLRP3 inflammasome activation in patients with type 2 diabetes. *DIABETES* 2013, 62(1):194-204.
25. Dror E, Dalmas E, Meier DT, Wueest S, Thevenet J, Thienel C, Timper K, Nordmann TM, Traub S, Schulze F *et al*: Postprandial macrophage-derived IL-1beta stimulates insulin, and both synergistically promote glucose disposal and inflammation. *NAT IMMUNOL* 2017, 18(3):283-292.
26. Zhou R, Tardivel A, Thorens B, Choi I, Tschopp J: Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *NAT IMMUNOL* 2010, 11(2):136-140.
27. Burdge GC: Metabolism of  $\alpha$ -linolenic acid in humans. *Prostaglandins, Leukotrienes and Essential Fatty Acids* 2006, 75(3):161-168.
28. Kim K, Nam YA, Kim HS, Hayes AW, Lee B:  $\alpha$ -Linolenic acid: Nutraceutical, pharmacological and toxicological evaluation. *FOOD CHEM TOXICOL* 2014, 70:163-178.
29. Zhang X, Wang H, Yin P, Fan H, Sun L, Liu Y: Flaxseed oil ameliorates alcoholic liver disease via anti-inflammation and modulating gut microbiota in mice. *LIPIDS HEALTH DIS* 2017, 16(1).
30. Wang T, Sha L, Li Y, Zhu L, Wang Z, Li K, Lu H, Bao T, Guo L, Zhang X *et al*: Dietary  $\alpha$ -Linolenic Acid-Rich Flaxseed Oil Exerts Beneficial Effects on Polycystic Ovary Syndrome Through Sex Steroid Hormones—Microbiota—Inflammation Axis in Rats. *FRONT ENDOCRINOL* 2020, 11.
31. Li Y, Yu Z, Liu Y, Wang T, Liu Y, Bai Z, Ren Y, Ma H, Bao T, Lu H *et al*: Dietary  $\alpha$ -Linolenic Acid-Rich Flaxseed Oil Ameliorates High-Fat Diet-Induced Atherosclerosis via Gut Microbiota-Inflammation-Artery Axis in ApoE<sup>-/-</sup> Mice. *Frontiers in Cardiovascular Medicine* 2022, 9.
32. Kim J, Ahn M, Choi Y, Kang T, Kim J, Lee NH, Kim GO, Shin T: Alpha-Linolenic Acid Alleviates Dextran Sulfate Sodium-Induced Ulcerative Colitis in Mice. *INFLAMMATION* 2020, 43(5):1876-1883.
33. Mustad VA, Demichele S, Huang YS, Mika A, Lubbers N, Berthiaume N, Polakowski J, Zinker B: Differential effects of n-3 polyunsaturated fatty acids on metabolic control and vascular reactivity in the type 2 diabetic ob/ob mouse. *METABOLISM* 2006, 55(10):1365-1374.
34. Wang F, Zhu H, Hu M, Wang J, Xia H, Yang X, Yang L, Sun G: Perilla Oil Supplementation Improves Hypertriglyceridemia and Gut Dysbiosis in Diabetic KKAy Mice. *MOL NUTR FOOD RES* 2018, 62(24):e1800299.
35. GPR120 agonists for the treatment of diabetes: a patent review (2014-present).
36. Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, Li P, Lu WJ, Watkins SM, Olefsky JM: GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. *CELL* 2010, 142(5):687-698.



37. Lu M, Lin SC, Huang Y, Kang YJ, Rich R, Lo YC, Myszka D, Han J, Wu H: XIAP induces NF-kappaB activation via the BIR1/TAB1 interaction and BIR1 dimerization. *MOL CELL* 2007, 26(5):689-702.
38. Oh DY, Walenta E, Akiyama TE, Lagakos WS, Lackey D, Pessentheiner AR, Sasik R, Hah N, Chi TJ, Cox JM *et al*: A Gpr120-selective agonist improves insulin resistance and chronic inflammation in obese mice. *NAT MED* 2014, 20(8):942-947.
39. Kaikini AA, Dhodi D, Muke S, Peshattiwar V, Bagle S, Korde A, Sarnaik J, Kadwad V, Sachdev S, Sathaye S: Standardization of type 1 and type 2 diabetic nephropathy models in rats: Assessment and characterization of metabolic features and renal injury. *J Pharm Bioallied Sci* 2020, 12(3):295-307.
40. Zhu L, Sha L, Li K, Wang Z, Wang T, Li Y, Liu P, Dong X, Dong Y, Zhang X *et al*: Dietary flaxseed oil rich in omega-3 suppresses severity of type 2 diabetes mellitus via anti-inflammation and modulating gut microbiota in rats. *LIPIDS HEALTH DIS* 2020, 19(1):20.
41. Donath MY, Shoelson SE: Type 2 diabetes as an inflammatory disease. *NAT REV IMMUNOL* 2011, 11(2):98-107.
42. He F, Huang Y, Song Z, Zhou HJ, Zhang H, Perry RJ, Shulman GI, Min W: Mitophagy-mediated adipose inflammation contributes to type 2 diabetes with hepatic insulin resistance. *J EXP MED* 2021, 218(3).
43. Pauls SD, Rodway LA, Winter T, Taylor CG, Zahradka P, Aukema HM: Anti-inflammatory effects of alpha-linolenic acid in M1-like macrophages are associated with enhanced production of oxylipins from alpha-linolenic and linoleic acid. *J NUTR BIOCHEM* 2018, 57:121-129.
44. Lumeng CN, Saltiel AR: Inflammatory links between obesity and metabolic disease. *J CLIN INVEST* 2011, 121(6):2111-2117.
45. Ying W, Mahata S, Bandyopadhyay GK, Zhou Z, Wollam J, Vu J, Mayoral R, Chi NW, Webster N, Corti A *et al*: Catestatin Inhibits Obesity-Induced Macrophage Infiltration and Inflammation in the Liver and Suppresses Hepatic Glucose Production, Leading to Improved Insulin Sensitivity. *DIABETES* 2018, 67(5):841-848.
46. Eussen SR, Geleijnse JM, Giltay EJ, Rompelberg CJ, Klungel OH, Kromhout D: Effects of n-3 fatty acids on major cardiovascular events in statin users and non-users with a history of myocardial infarction. *EUR HEART J* 2012, 33(13):1582-1588.
47. Kim J, Ahn M, Choi Y, Kang T, Kim J, Lee NH, Kim GO, Shin T: Alpha-Linolenic Acid Alleviates Dextran Sulfate Sodium-Induced Ulcerative Colitis in Mice. *INFLAMMATION* 2020, 43(5):1876-1883.
48. Rupa EJ, Li JF, Arif MH, Yaxi H, Puja AM, Chan AJ, Hoang VA, Kaliraj L, Yang DC, Kang SC: Cordyceps militaris Fungus Extracts-Mediated Nanoemulsion for Improvement Antioxidant, Antimicrobial, and Anti-Inflammatory Activities. *MOLECULES* 2020, 25(23).

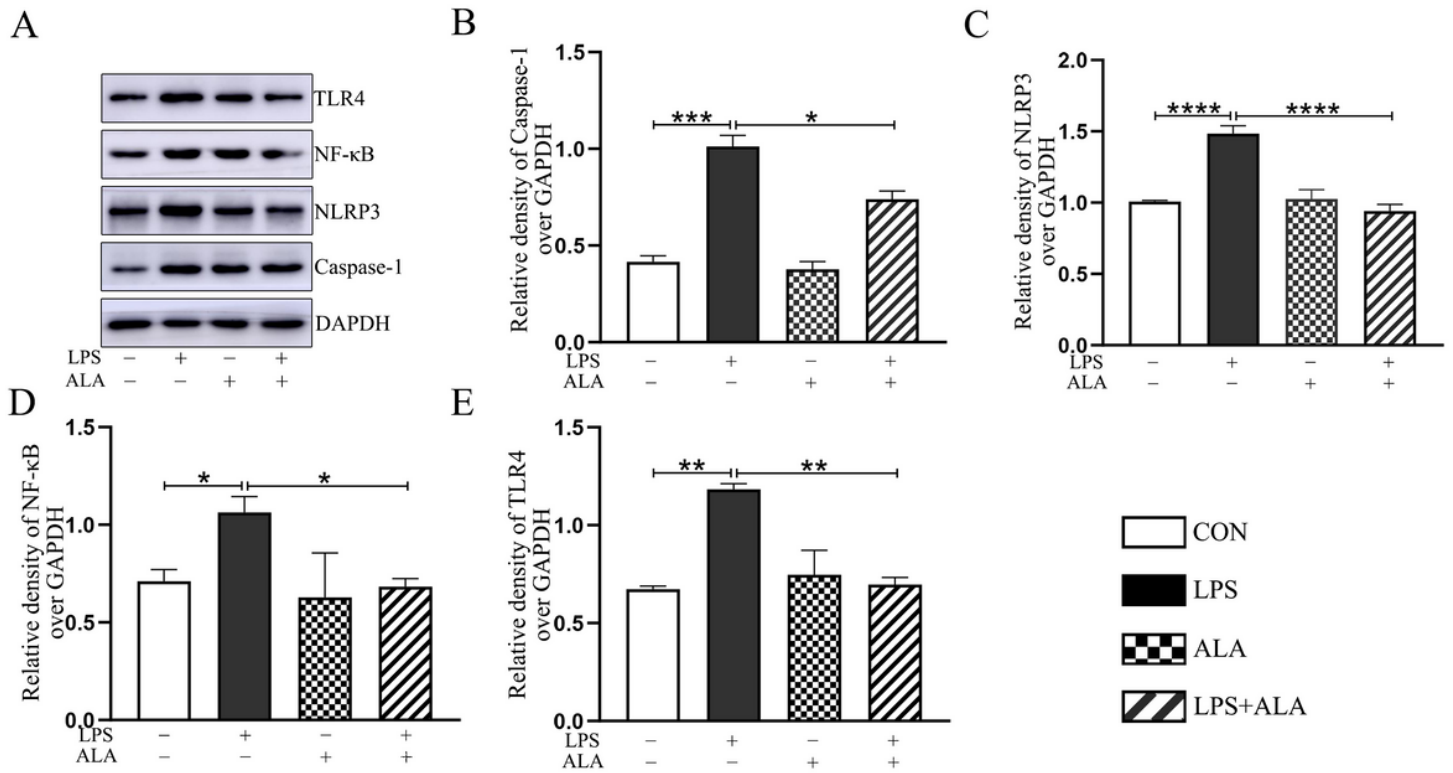
49. Hudita A, Galateanu B, Dinescu S, Costache M, Dinischiotu A, Negrei C, Stan M, Tsatsakis A, Nikitovic D, Lupuliasa D *et al*: In Vitro Effects of Cetylated Fatty Acids Mixture from Celadrin on Chondrogenesis and Inflammation with Impact on Osteoarthritis. *CARTILAGE* 2020, 11(1):88-97.
50. Lee BC, Lee J: Cellular and molecular players in adipose tissue inflammation in the development of obesity-induced insulin resistance. *Biochim Biophys Acta* 2014, 1842(3):446-462.
51. Yan Y, Jiang W, Spinetti T, Tardivel A, Castillo R, Bourquin C, Guarda G, Tian Z, Tschopp J, Zhou R: Omega-3 fatty acids prevent inflammation and metabolic disorder through inhibition of NLRP3 inflammasome activation. *IMMUNITY* 2013, 38(6):1154-1163.
52. Kelley N, Jeltema D, Duan Y, He Y: The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. *INT J MOL SCI* 2019, 20(13).
53. Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, Fernandes-Alnemri T, Wu J, Monks BG, Fitzgerald KA *et al*: Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J IMMUNOL* 2009, 183(2):787-791.
54. Olefsky JM, Glass CK: Macrophages, inflammation, and insulin resistance. *ANNU REV PHYSIOL* 2010, 72:219-246.

## Figures



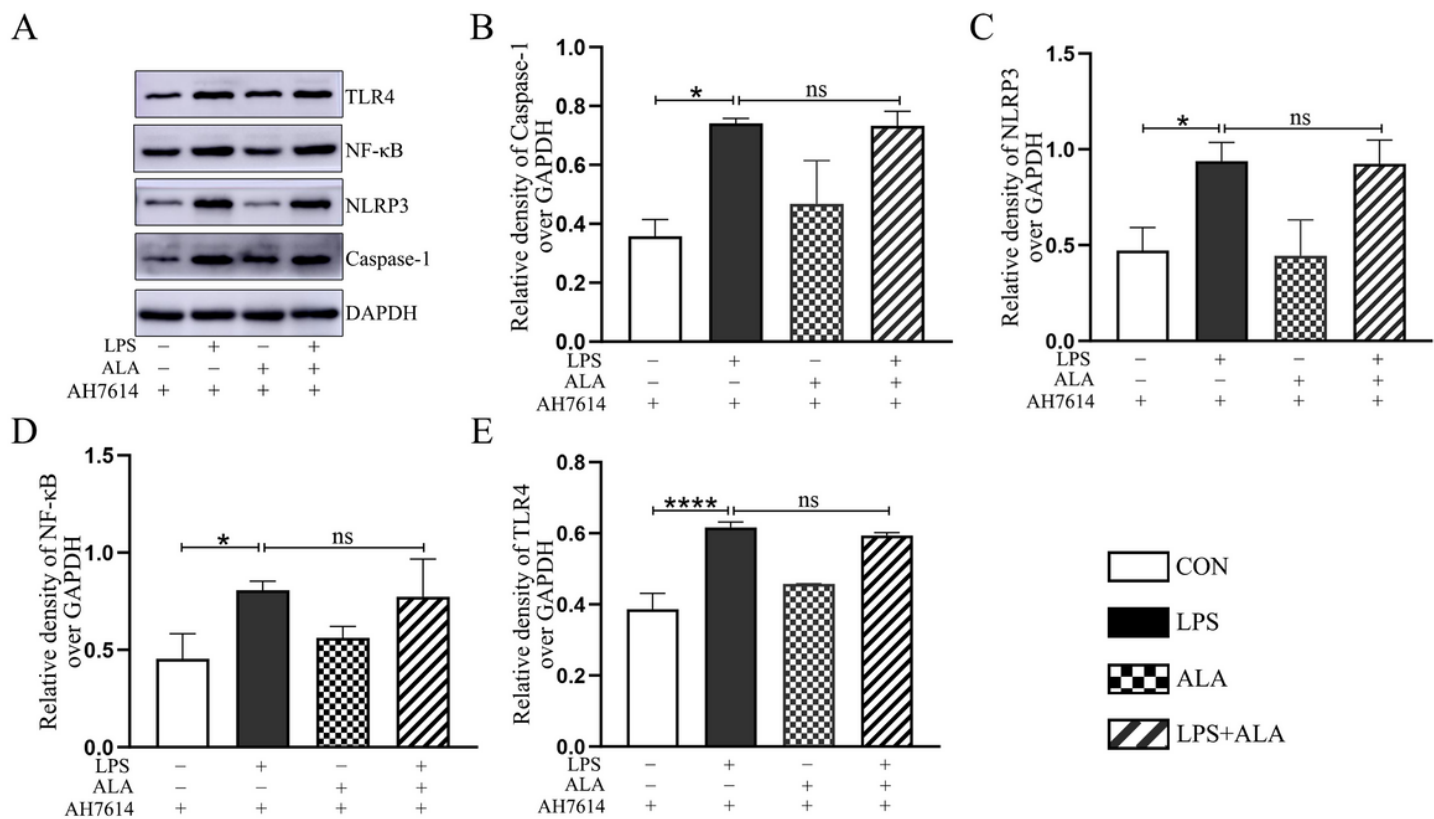
**Figure 1**

ALA significantly changed the positive expression rate of hepatic CD68 in T2DM. (A) Results of hepatic immunofluorescence (IF) were stained by rabbit anti-rat CD68 antibody. (B) The number of CD68<sup>+</sup> cells in diverse groups. Original magnification,  $\times 200$  (A). Data are expressed as mean  $\pm$  SEM. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . All experiments were performed in triplicate.



**Figure 2**

Effects of ALA treatment on NLRP3 inflammasome, NF-κB and inflammatory indications in RAW264.7 cells. (A) Western blot analysis of caspase-1, NLRP3, NF-κB and TLR4. Semi-quantitative analysis of the relative levels of caspase-1 (B), NLRP3 (C), NF-κB (D) and TLR4 (E) by densitometric analysis. Data are expressed as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ . All experiments were performed in triplicate. NLRP3: nod-like receptor protein 3; NF-κB: nuclear factor-κB; TLR4: Toll-like receptor 4; ALA:  $\alpha$ -Linolenic acid; LPS: lipopolysaccharide.

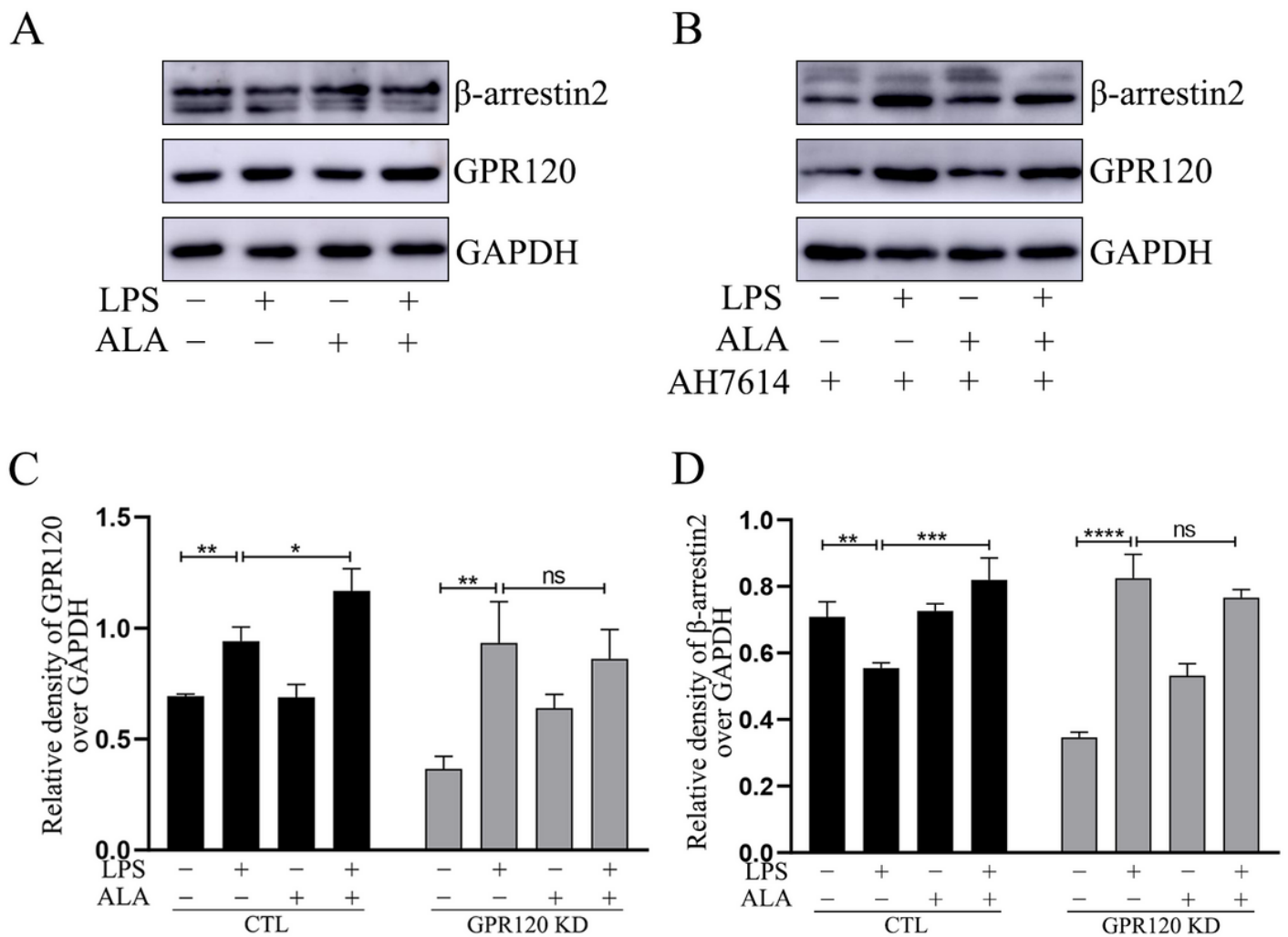


**Figure 3**

Effect of ALA on the contents of inflammatory factors in RAW264.7 from cell culture supernatants. RAW264.7 cells were divided into two groups: CTL group (normal RAW264.7 cells) and GPR120 KD group (intervention with AH7614). Each group was divided into a control group (no LPS was added), LPS group (treated with 100 ng/mL LPS for 24 h), ALA control group (treated with 20  $\mu$ M ALA for 2 h), ALA intervention group (100 ng/mL LPS+20  $\mu$ M ALA for 24h). (A) IL-1 $\beta$ , (B) IL-18, (C) TNF- $\alpha$ , and (D) IL-6 concentrations were determined by ELISA. Data are expressed as mean  $\pm$  SEM. ns: no significance,  $P>0.05$ ; \*\*\*\* $P<0.0001$ . All experiments were performed in triplicate. IL-1 $\beta$ , 18, 6: Interleukin-1 $\beta$ , 18, 6; TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ ; ALA:  $\alpha$ -Linolenic acid; LPS: lipopolysaccharide; AH7614: the GPR120 antagonist.

**Figure 4**

AH7614 reversed the inhibitory effects of ALA in LPS-induced inflammation response in RAW264.7 cells. (A) Western blot analysis of caspase-1, NLRP3, NF- $\kappa$ B and TLR4. Semi-quantitative analysis of the relative levels of caspase-1 (B), NLRP3 (C), NF- $\kappa$ B (D) and TLR4 (E) by densitometric analysis. Data are expressed as mean  $\pm$  SEM. ns: no significance,  $P>0.05$ ; \* $P<0.05$ , \*\*\*\* $P<0.0001$ . All experiments were performed in triplicate. NLRP3: nod-like receptor protein 3; NF- $\kappa$ B: nuclear factor- $\kappa$ B; TLR4: Toll-like receptor 4; ALA:  $\alpha$ -Linolenic acid; LPS: lipopolysaccharide; AH7614: the GPR120 antagonist.



**Figure 5**

ALA-mediated anti-inflammatory signaling was associated with the expression of GPR120 and  $\beta$ -arrestin2. (A, B) Representative immunoblots of GPR120 and  $\beta$ -arrestin2 in different group. (C, D) Semiquantitative analysis of relative levels of GPR120 and  $\beta$ -arrestin2 by densitometric analysis. Data are expressed as mean  $\pm$  SEM. ns: no significance,  $P>0.05$ ;  $*P<0.05$ ,  $**P<0.01$ ,  $***P<0.001$ ,  $****P<0.0001$ . All experiments were performed in triplicate. ALA:  $\alpha$ -Linolenic acid; LPS: lipopolysaccharide; AH7614: the GPR120 inhibitor.

**Figure 6**

ALA significantly suppressed M1 macrophages and TNF- $\alpha$ , increased M2 macrophages and IL-10 in RAW264.7 mouse macrophages stimulated by LPS. (A, B) Levels of TNF- $\alpha$  and IL-10 released in culture supernatants were determined by ELISA. (C, E) Representative immunoblots of iNOS and Arg-1 in different groups. (D, F) Semiquantitative analysis of relative levels of iNOS and Arg-1 by densitometric

analysis. RAW264.7 cells were pretreated with LPS (100 ng/mL) for 24 h after treatment with 20 mM concentrations of ALA for 2 h. Data are expressed as mean  $\pm$  SEM. ns: no significance,  $P>0.05$ ; \* $P<0.05$ , \*\*\* $P<0.001$ , \*\*\*\* $P<0.0001$ . All experiments were performed in triplicate. ALA:  $\alpha$ -Linolenic acid; LPS: lipopolysaccharide; AH7614: the GPR120 inhibitor. TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ ; IL-10: Interleukin-10; iNOS: Inducible nitric oxide synthase; Arg-1: Arginine-1.