

# Treatment of experimental non-alcoholic steatohepatitis by targeting $\alpha 7$ nicotinic acetylcholine receptor-mediated inflammatory responses in mice

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**Abstract.** Non-alcoholic fatty liver disease (NAFLD) is one of the most common types of liver disease, affecting up to 30% of the general population worldwide. Non-alcoholic steatohepatitis (NASH) is a severe form of NAFLD without any effective therapies available. The present study showed that activation of  $\alpha 7$ -nicotinic acetylcholine receptor ( $\alpha 7$  nAChR) may be a novel potential strategy for NASH therapy. Treatment with the  $\alpha 7$  nAChR agonist nicotine for three weeks obviously attenuated hepatic steatosis in a high-fat diet-induced mouse model of NASH. Investigation of the underlying mechanism showed that nicotine reduced the secretion of the pro-inflammatory cytokines tumor necrosis factor  $\alpha$  and interleukin 6 *in vitro* and *in vivo*. Inflammation is an integral part of NASH and is the most prevalent form of hepatic pathology found in the general population; therefore, the effect of  $\alpha 7$  nAChR activation against NASH may be ascribed to its anti-inflammatory effects. In addition, the present study showed that nicotine-stimulated  $\alpha 7$  nAChR activation led to a significant downregulation of nuclear factor kappa B (NK- $\kappa$ B) and extracellular signal-regulated kinase (ERK). It therefore appeared that activation of  $\alpha 7$  nAChR suppressed the production of pro-inflammatory cytokines through NK- $\kappa$ B and ERK pathways. In conclusion, the present study indicated that targeting  $\alpha 7$  nAChR may represent a novel treatment strategy for NASH.

## Introduction

Non-alcoholic fatty liver disease (NAFLD) is a cause of fatty liver, occurring when fat is deposited in the liver (steatosis) not due to excessive alcohol use. It is associated with insulin resistance and metabolic syndrome (1). NAFLD is currently considered to be the most common cause of chronic liver disease worldwide (2) and associated with other potentially life-threatening diseases and increased mortality from cardiovascular diseases, malignancy and hepatic complications. NAFLD has also been found to be associated with several extra-hepatic disorders, including breast cancer, polycystic ovary syndrome and renal dysfunction (3-15). NAFLD encompasses a wide spectrum of liver diseases ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) (1), which is the most extreme form of NAFLD and is regarded as a major cause of cirrhosis of the liver of unknown cause (16). NASH is a major health problem and complicated by portal hypertension and hepatic decompensation, and is occasionally accompanied with hepatocellular carcinoma (HCC) (17).

Recently, various treatment modalities have been applied in NASH, including lifestyle modification, surgical intervention and pharmacological agents (including insulin sensitizers, anti-oxidant agents, lipid-lowering agents and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) antagonists) (18-20). However, to date, there are no US Food and Drug Administration-approved medical therapies for NASH or liver fibrosis. There is an urgent requirement for novel therapeutic approaches (17,21). As inflammatory activation has a significant role in NASH progression, anti-inflammatory therapy for NASH is of increasing interest (22). For example, TNF- $\alpha$  antagonist pentoxifylline, interleukin (IL)-6 antagonist Sant7 and the TNF- $\alpha$ -specific monoclonal antibodies infliximab, adalimumab and certolizumab have been studied in a number of clinical NAFH trials (23). At present, anti-inflammatory strategies for NASH are restricted to targeting one single cytokine, e.g., IL-1 receptor, IL-6 or TNF- $\alpha$ . However, multiple cytokines are involved in the inflammatory response of NASH. Therefore, targeting an upstream signaling molecule that regulates multiple cytokine production may improve the objective

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response rates (24). Recently, a novel neural pathway termed as cholinergic anti-inflammatory reflex, has been discovered, which inhibits the production of inflammatory cytokines and may be a novel anti-inflammatory strategy for NASH.

The  $\alpha 7$ -nicotinic acetylcholine receptor ( $\alpha 7$  nAChR) is a sub-type of nicotinic acetylcholine receptor and has a crucial role in mediating the cholinergic anti-inflammatory signaling pathway (25). It is expressed on different types of cells, including neurons, macrophages, lymphocytes, monocytes and dendritic cells. Activation of the  $\alpha 7$  nAChR expressed on resident macrophages may suppress the local inflammation by reducing the production of pro-inflammatory cytokines TNF- $\alpha$  and IL-6, which are closely associated with certain inflammatory diseases, including sepsis, rheumatoid arthritis, asthma and diabetes (26). It is therefore indicated that  $\alpha 7$  nAChR is a promising target for developing novel anti-inflammatory drugs. However, to date, it has remained to be clarified whether  $\alpha 7$  nAChR is associated with NASH.

The present study assessed whether activation of  $\alpha 7$  nAChR was able to prevent the progression of NASH, and whether targeting of  $\alpha 7$  nAChR may represent a novel strategy for NASH therapy.

## Materials and methods

**Cell culture and reagents.** RAW 264.7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Life Technologies) in a humidified atmosphere of 95% air with 5% CO<sub>2</sub> at 37°C. Nicotine was purchased from Sigma-Aldrich (St. Louis, MO, USA; n=80).

**Experimental protocols and animals.** C57 male mice at four weeks of age (weight, 17-23 g) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China) and housed in the laboratory animal center of Zhejiang Chinese Medical University (Hangzhou, China) at 22°C with a 12-h light/dark cycle. Mice were randomly divided into four groups (n=10) and fed either a control diet (10% kcal as fat; Mediscience Ltd., Yangzhou, China) or a high-fat diet (HFD; 60% kcal as fat; Mediscience Ltd) for 18 weeks with or without nicotine for three weeks: 1) Control group, mice were fed a control diet and supplemented with normal saline; (2) HFD group, mice were fed a HFD and supplemented with normal saline; (3) control + nicotine 5 mg/kg group, mice were fed a control diet and supplemented with nicotine at a dose of 5 mg/kg; (4) HFD + nicotine 5 mg/kg group, mice were fed a HFD and supplemented with nicotine at the dose of 5 mg/kg. During the 18 weeks of feeding, the body weight was measured every week. At the end of the experiment, mice were sacrificed by cardiac puncture under CO<sub>2</sub> anesthesia, and livers were collected for further analysis.

All animals used in the present study were housed and cared for in accordance with the Chinese Pharmacological Society Guidelines for Animal Use. The protocols of the present study were approved by the Committee on the Ethics of Animal Experiments of the Zhejiang Chinese Medical University (Hangzhou, China; permit no. 2012-1849). All surgeries were

performed under sodium pentobarbital anesthesia (70 mg/kg; Sigma-Aldrich) and all efforts were made to minimize suffering.

**Biochemical serum analysis.** The activity levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (10) were determined using an automatic blood chemical analyzer (Dry-Chem 4000i; Fujifilm, Tokyo, Japan). TNF- $\alpha$  and IL-6 levels were measured using ELISA kits (cat. nos. EK0527 and EK0441; Boster Biological Inc., Wuhan, China).

**Histological examination and Oil Red O staining.** The fixed liver tissue was cut into 3-mm blocks, which were embedded in paraffin and cut into 4- $\mu$ m slices. After being de-paraffinized using xylene and ethanol dilutions and re-hydration, the sections were stained with hematoxylin and eosin (H&E; Bogoo, Shanghai, China) to examine the tissue structure, inflammatory cell infiltration, necrosis and lipid accumulation.

For Oil Red O staining, cryosections of optimal cutting temperature compound-embedded liver tissues (10 mm) were fixed in 10% buffered formalin for 5 min at room temperature, stained with Oil Red O (Biohao Company, Wuhan, China) for 1 h, washed with 10% isopropanol and then counterstained with hematoxylin for 30 sec. A Nikon E600 microscope (Nikon, Tokyo, Japan) and Leica Application Suite (Leica Microsystems, Inc., Buffalo Grove, IL, USA) were used to capture images of the Oil Red O-stained tissue sections at 40x magnification.

**Isolation of macrophages from liver tissue.** Forty normal, healthy mice were anesthetized and liver tissues were perfused *in situ* via the superior vena cava with a perfusion buffer (13 Hanks' balanced salt solution; Gino Biological Medical Technology, Co., Ltd., Hangzhou, China), followed by a digestion buffer [13 Hanks' balanced salt solution, supplemented with 0.05% collagenase (Type IV; Sigma-Aldrich), 1.25 mmol/l CaCl<sub>2</sub>, 4 mmol/l MgSO<sub>4</sub> and 10 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]. The resulting cell suspension was filtered through a sterile 100-mm nylon mesh (Solarbio, Beijing, China) and centrifuged at 50 xg to selectively sediment hepatocytes from non-parenchymal cells (NPCs). The pellet of hepatocytes was re-suspended and subsequently washed two more times with centrifugation at 50 xg. The NPCs in the first and second supernatants from the low-speed centrifugations were pelleted by high-speed centrifugation (1,300 x g), followed by re-suspension in a small volume prior to isopycnic sedimentation in Percoll as previously described (27). Cell viability (90%) was determined by trypan blue exclusion (Sigma-Aldrich). The Kupffer cells were treated with lipopolysaccharide (LPS; *Escherichia coli* O111:B4; Sigma-Aldrich; 100 nM) for 16-18 h, following which the culture medium was replaced with medium without serum, and in the presence or absence of nicotine (concentrations between 0 and 10  $\mu$ m) for 6 h. Treatment with  $\alpha$ -bungarotoxin ( $\alpha$ -BGT; Zhongxin Dongtai Company, Laiyang, China) was also performed.

**Western blot analysis.** Whole-cell lysates were prepared using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Nantong, China), protein concentrations were detected using a bicinchoninic acid assay kit (Beyotime Institute

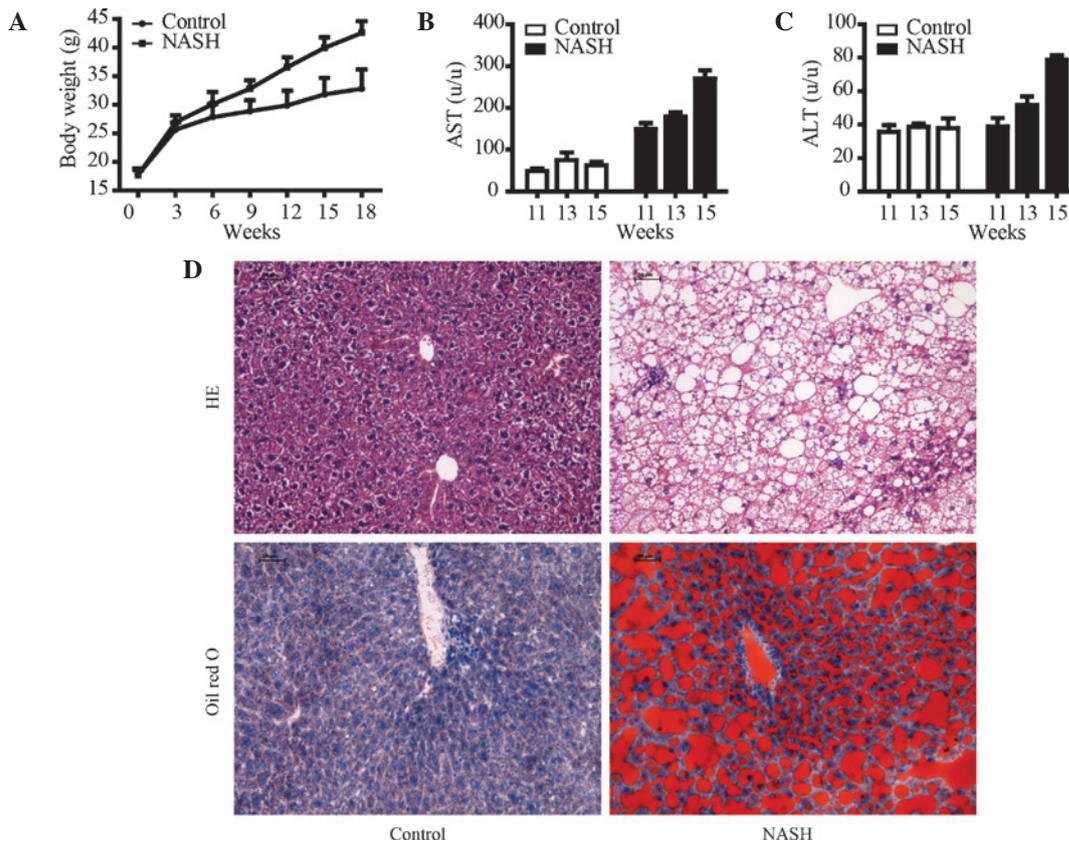


Figure 1. Physiological and biochemical parameters, and histological analysis of mice that were fed a HFD to induce NASH. (A) The body weight was monitored weekly during intake of the HFD. Values are expressed as the mean  $\pm$  standard deviation (n=20). (B and C) Activities of ALT and AST were detected at weeks 11, 13, 15 of HFD. Values are expressed as the mean  $\pm$  standard deviation (n $\geq$ 6 in each group). (D) Hepatic morphological changes were examined microscopically following HE staining and hepatic lipid accumulation was examined by Oil Red O staining at 18 weeks of intake of HFD. Representative images of each group are shown (magnification, x40). NASH, non-alcoholic steatohepatitis; HFD, high-fat diet; HE, hematoxylin and eosin; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

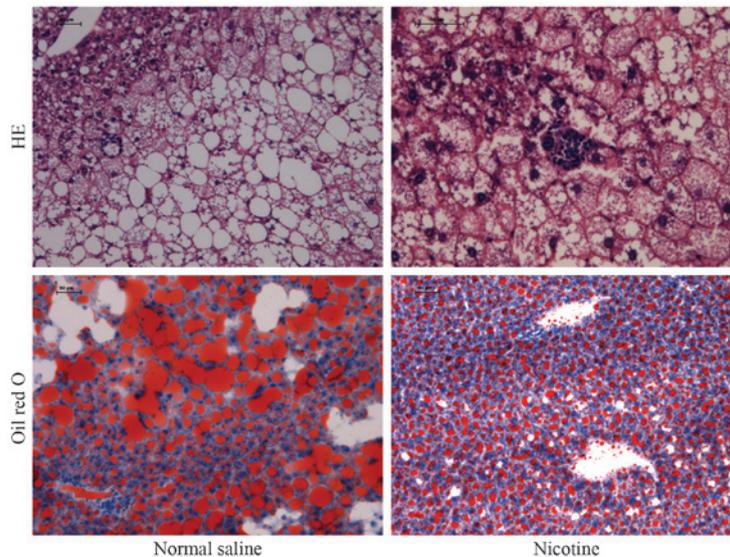


Figure 2. Activation of the  $\alpha 7$ -nicotinic acetylcholine receptor by nicotine treatment attenuated high-fat-diet-induced hepatic steatosis in mice. Representative HE-stained and Oil Red O-stained liver sections are shown (magnification, x40). HE, hematoxylin and eosin.

of Biotechnology) and western blotting was performed, as previously described (27). Briefly, equal amounts of protein were separated by SDS-PAGE. Proteins were then transferred onto nitrocellulose membranes and identified with anti- $\alpha 7$  nAChR

polyclonal antibody (cat. no. 23791-AP; Proteintech USA), anti-NF- $\kappa$ B monoclonal antibody (cat. no. 4764S; Cell Signaling Technology, Inc., Beverly, MA, USA), anti-inhibitor of NF- $\kappa$ B (I $\kappa$ B) antibody (cat. no. 4814; CST Company, Boston, MA,

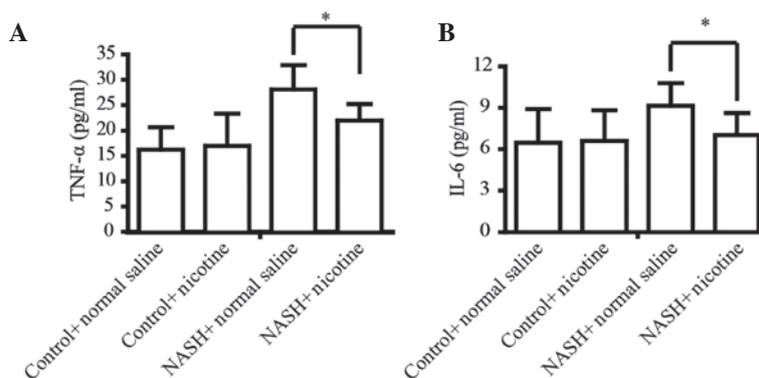


Figure 3. Nicotine induces suppression of TNF- $\alpha$  and IL-6 in mouse model of non-alcoholic fatty liver disease. Mice were treated with nicotine (5 mg/kg, intraperitoneally) or vehicle for three weeks, and the levels of (A) TNF- $\alpha$  and (B) IL-6 were measured. Values are expressed as the mean  $\pm$  standard deviation (n=9 per group). \*P<0.05. TNF, tumor necrosis factor; IL, interleukin; NASH, non-alcoholic steatohepatitis.

USA), anti-extracellular signal-regulated kinase (ERK) monoclonal antibody (cat.no. 20G11; Cell Signaling Technology, Inc.) and anti-GAPDH antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 1:1,000. Detection was performed using a horseradish peroxidase-conjugated secondary antibody and SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc., Rockford, IL, USA) according to the manufacturer's instructions. Kodak films (Kodak, Rochester, NY, USA) were used to visualize the gels.

**Statistical analysis.** Values are expressed as the mean  $\pm$  standard deviation. Statistical analyses were performed using one-way analysis of variance or the unpaired Student's *t*-test as indicated. Statistical analysis was performed using SPSS v.10.0 statistical software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference between values.

## Results

**HFD-induced NASH.** In the present study, a mouse model of NASH was generated by intake of a HFD. After 18 weeks of HFD intake, the body weight was significantly increased, which indicated the establishment of the obesity mouse model (Fig. 1A). As shown in Fig. 2B and C, activities of AST and ALT were increased in mice on an HFD compared with those in the control mice which received a normal diet. It appeared that the HFD induced liver injury. To determine whether HFD induced hepatic steatosis, liver pathological examination by H&E staining was performed (Fig. 1D). The hepatic cell structure in the control group was normal. However, the HFD increased hepatic damage with obvious hepatic necrosis. Further examination of the hepatic lipid accumulation status with Oil Red O staining revealed that the HFD significantly induced hepatic lipid accumulation compared to that in the control group.

**Activation of  $\alpha 7$  nAChR attenuates HFD-induced hepatic steatosis.** In order to identify whether activation of  $\alpha 7$  nAChR can prevent NASH and the subsequent hepatic injury,  $\alpha 7$  nAChR agonist nicotine was administered to mice receiving the HFD. As shown in Fig. 2, administration of nicotine significantly, but not completely, prevented HFD-induced hepatic necrosis and hepatic lipid accumulation.

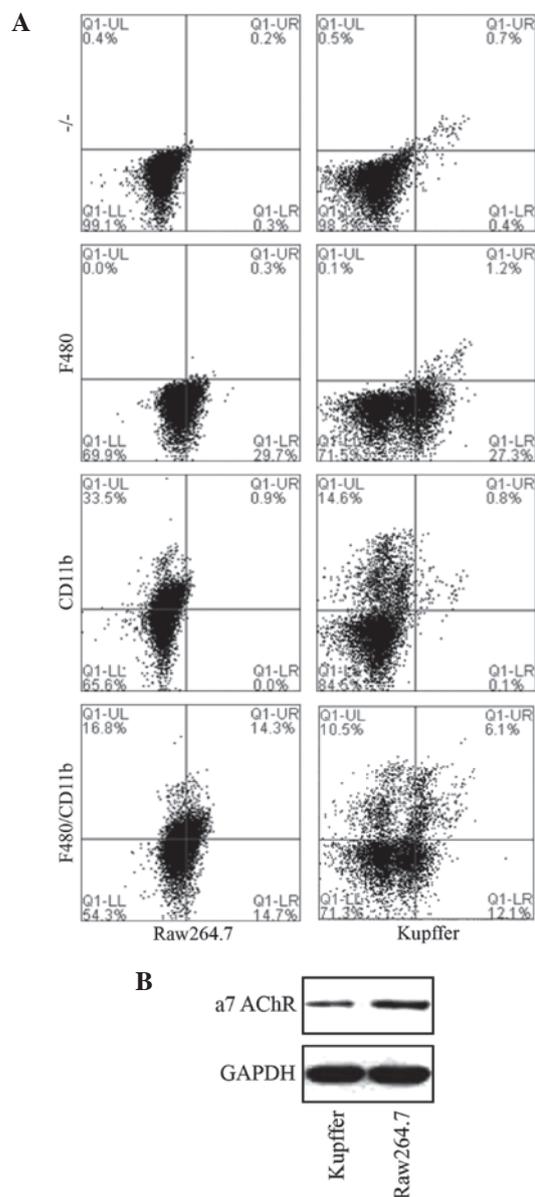


Figure 4. Isolated and identified primary liver Kupffer cells. (A) The isolated primary liver Kupffer cells were identified by using flow cytometry and compared with RAW264.7 cells. (B)  $\alpha 7$  nAChR expression profiles in primary liver Kupffer cells and RAW264.7 cells. GAPDH was employed as a loading control. Results shown are representative of at least three independent experiments.  $\alpha 7$  nAChR,  $\alpha 7$ -nicotinic acetylcholine.

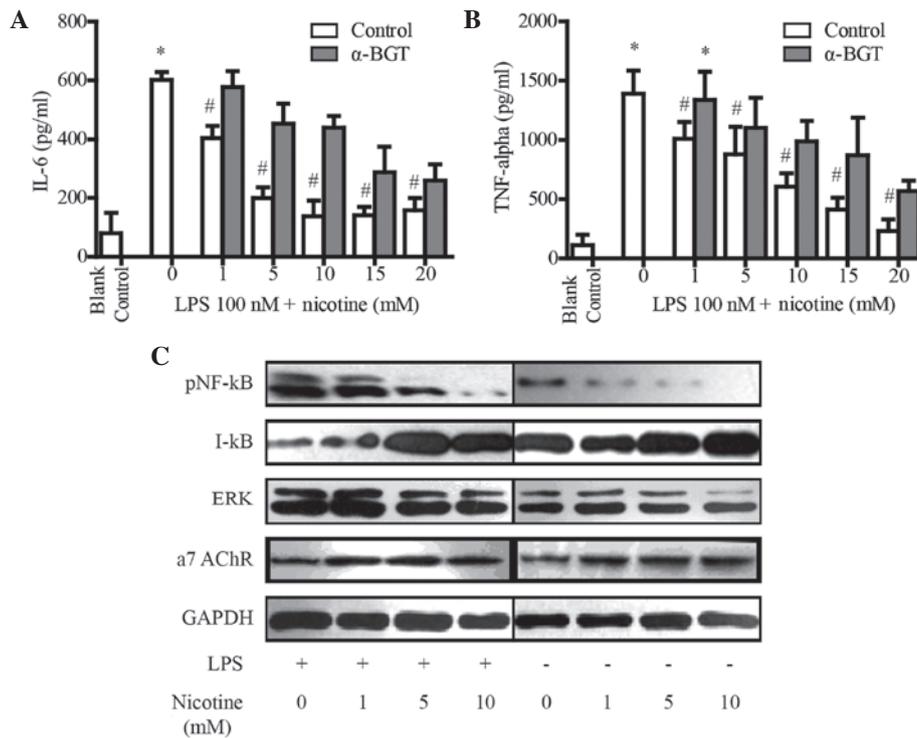


Figure 5. Nicotine reduces the secretion of TNF- $\alpha$  and IL-6 in primary Kupffer cells via  $\alpha 7$  nAChR. (A and B) The supernatant was collected from the culture of Kupffer cells after treatment with LPS and nicotine in the presence or absence of  $\alpha$ -BGT, and cytokine levels were assessed using ELISA kits. Values are expressed as the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.01$  vs. blank control group; # $P < 0.01$  vs. LPS + 0 nM nicotine group. (C) Effects of nicotine on the NF- $\kappa$ B and ERK pathway in primary Kupffer cells. GAPDH was used as a loading control. Blots shown are representative of at least three independent experiments. TNF, tumor necrosis factor; IL, interleukin; NF- $\kappa$ B, nuclear factor kappa B; p, phosphorylated, I- $\kappa$ B, inhibitor of NF- $\kappa$ B; ERK, extracellular signal-regulated kinase;  $\alpha 7$  nAChR,  $\alpha 7$ -nicotinic acetylcholine; LPS, lipopolysaccharide;  $\alpha$ -BGT,  $\alpha 7$  nAChR antagonist  $\alpha$ -bungarotoxin.

*Activation of  $\alpha 7$  nAChR attenuates HFD-induced hepatic inflammation.* Inflammation is the main pathological consequence of HFD-induced NASH and is characterized by a release of inflammatory factors, which contributes to hepatic fibrosis (28,29). Thus, the present study determined whether nicotine can prevent HFD-induced hepatic inflammation. The secretion of the classic inflammatory factors TNF- $\alpha$  and IL-6 was detected by ELISA. The HFD significantly upregulated the serum levels of TNF- $\alpha$  and IL-6 in mice. However, nicotine treatment significantly attenuated HFD-induced upregulation of serum TNF- $\alpha$  and IL-6 (Fig. 3).

*Nicotine exerts anti-inflammatory effects via targeting  $\alpha 7$  nAChR and inhibiting the NF- $\kappa$ B and ERK pathways.* To investigate the underlying mechanism of the reduction of pro-inflammatory cytokines TNF- $\alpha$  and IL-6 by  $\alpha 7$  nAChR activation, primary macrophages from the liver were isolated and assessed. Primary liver Kupffer cells were successfully isolated and identified by staining with CD11b and F480 macrophage-specific markers (Caltag Laboratories, Burlingame, CA, USA) and flow cytometric detection (BD-Accuri C6, BD Biosciences Franklin Lakes, NJ, USA) in comparison with murine macrophage RAW 264.7 cells (Fig. 4).

In the mouse model of NASH, the  $\alpha 7$  nAChR agonist nicotine significantly attenuated HFD-induced upregulation of serum TNF- $\alpha$  and IL-6. To determine the anti-inflammatory mechanisms of  $\alpha 7$  nAChR-activation, the present study assessed whether nicotine treatment blocked the production of TNF- $\alpha$  and IL-6 with or without  $\alpha 7$  nAChR antagonist  $\alpha$ -bungarotoxin

( $\alpha$ -BGT) in the primary liver Kupffer cells. The secretion of the classic inflammatory factors TNF- $\alpha$  and IL-6 was detected by ELISA. Stimulation with lipopolysaccharide (LPS) significantly upregulated the secretion of TNF- $\alpha$  (Fig. 5A) and IL-6 (Fig. 5B) in the cell culture supernatants. However, nicotine treatment significantly attenuated LPS-induced upregulation of TNF- $\alpha$  (Fig. 5A) and IL-6 (Fig. 5B) in a dose-dependent manner. Furthermore,  $\alpha 7$  nAChR antagonist  $\alpha$ -BGT blocked the nicotine-induced reduction of TNF- $\alpha$  (Fig. 5A) and IL-6 (Fig. 5B), which indicated that nicotine reduced the production of TNF- $\alpha$  and IL-6 via activating  $\alpha 7$  nAChR.

Release of inflammatory cytokines is mostly mediated via the ERK and NF- $\kappa$ B pathways, and ERK and NF- $\kappa$ B are the main downstream signaling molecules of  $\alpha 7$  nAChR (27). The present study therefore investigated whether activation of  $\alpha 7$  nAChR reduces the production of cytokines via inhibiting the ERK and NF- $\kappa$ B pathways (Fig. 5C). As shown in Fig. 5, nicotine treatment upregulated the protein levels of  $\alpha 7$  nAChR in Kupffer cells in a dose-dependent manner, which was consistent with the results of a previous study (27). Furthermore, nicotine obviously downregulated ERK and NF- $\kappa$ B levels in Kupffer cells.

## Discussion

The results present study suggested that specific interference with  $\alpha 7$  nAChR represents a novel strategy for the treatment of NASH. It was shown that treatment with the  $\alpha 7$  nAChR agonist nicotine for three weeks obviously attenuated hepatic

steatosis and reduced the production of TNF- $\alpha$  and IL-6 in an HFD-induced mouse model of NASH. To investigate the underlying mechanism, the primary macrophages from mouse livers were isolated and treated with nicotine. The results showed that nicotine reduced LPS-induced secretion of TNF- $\alpha$  and IL-6 *in vitro*, which was blocked by  $\alpha 7$  nAChR antagonist  $\alpha$ -BGT. These results indicated that nicotine suppressed TNF- $\alpha$  and IL-6 secretion by LPS-stimulated macrophages through  $\alpha 7$  nAChR activation. Furthermore, the present study showed that nicotine-stimulated  $\alpha 7$  nAChR activation significantly downregulated NK- $\kappa$ B and ERK. It appeared that the activation of  $\alpha 7$  nAChR suppressed the production of pro-inflammatory cytokines through NK- $\kappa$ B and ERK pathways.

NASH is increasingly recognized as a major epidemiological problem, linking the metabolic syndrome to liver fibrosis, cirrhosis and hepatocellular carcinoma. Currently discussed treatment options comprise drugs approved for managing the symptoms of impaired glucose metabolism, hypertension and hyperlipidemia, including angiotensin I antagonists or insulin sensitizers (30). However, the incidence of NAFLD in the human population is further increasing, affecting up to 30% of the general population worldwide, despite the availability of these drugs (31). In addition, the side effects of approved drugs preclude treatment of patient sub-populations, thus underlining the requirement for additional specific treatment options (27).

To test the effects of  $\alpha 7$  nAChR activation on NASH, the HFD-induced mouse model of NASH was employed. The model developed symptoms within a time frame of 18 weeks and was characterized by a rather mild elevation in liver enzymes, such as ALT, in the circulation as well as the presence of lobular inflammation, which is also observed in humans with NASH (32). The present study showed that the  $\alpha 7$  nAChR agonist nicotine reduced NASH-associated hepatic steatosis in mouse models. Furthermore, nicotine treatment decreased the secretion of the pro-inflammatory cytokines TNF- $\alpha$ , IL-6 in mice with NASH. This result indicated that activation of  $\alpha 7$  nAChR and the resulting anti-inflammatory effects may represent a novel therapeutic strategy for NASH.

Inflammation characterized by the release of soluble factors, including chemokines and cytokines, in addition to immune cell activation, is regarded as an integral part of NASH and several lines of evidence suggested that targeting of inflammation is a promising tool for the management of NASH (29). The results of the present study showed that  $\alpha 7$  nAChR agonist nicotine reduced the production of TNF- $\alpha$  and IL-6 in the mouse model of NASH *in vivo* and in primary Kupffer cells *in vitro*. These results were consistent with those of previous studies, which reported that activation of the  $\alpha 7$  nAChR expressed on resident macrophages may suppress the local inflammation by reducing the production of pro-inflammatory cytokines TNF- $\alpha$  and IL-6 (27). Furthermore, the present study found that nicotine-induced  $\alpha 7$  nAChR activation significantly inhibited the expression of NF- $\kappa$ B and ERK. This result indicated that activation of the  $\alpha 7$  nAChR may inhibit cytokine production by Kupffer cells via the NF- $\kappa$ B and ERK pathways.

In conclusion, the present study indicated that modulating the inflammatory response in affected livers via activating  $\alpha 7$  nAChR may represent a novel strategy for the treatment of NASH. The feasibility of this strategy requires pre-clinical and clinical validation in further studies.

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