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Original Article

Apolipoprotein A-I inhibits LPS-induced atherosclerosis in ApoE$^{-/-}$ mice possibly via activated STAT3-mediated upregulation of tristetraprolin

Kai YIN[1, 2, #], Shi-lin TANG[1, 3, #], Xiao-hua YU[1, #], Guang-hui TU[4], Rong-fang HE[5], Jin-feng LI[1], Di XIE[1, 2], Qing-jun GUI[2], Yu-chang FU[6], Zhi-sheng JIANG[1], Jian TU[7, *], Chao-ke TANG[1, *]

[1]Institute of Cardiovascular Research, Key Laboratory for Atherosclerology of Hunan Province, Life Science Research Center, University of South China, Hengyang 421001, China; [2]Department of Diagnostics, the Medical College, University of South China, Hengyang 421001, China; [3]Department of Intensive Care Unit, the First Affiliated Hospital of University of South China, Hengyang 421001, China; [4]Department of Pathology, Nan Xian Peoples Hospital, Yiyang 413200, China; [5]Department of Pathology, the First Affiliated Hospital, University of South China, Hengyang 421001, China; [6]Department of Nutrition Sciences, University of Alabama at Birmingham, Birmingham, AL 35294-0012, USA; [7]Institute of Pharmacy and Pharmacology, University of South China, Hengyang 421001, China

Aim: To investigate the effects of the major component of high-density lipoprotein apolipoprotein A-I (apoA-I) on the development of atherosclerosis in LPS-challenged ApoE$^{-/-}$ mice and the underlying mechanisms.
Methods: Male ApoE-KO mice were daily injected with LPS (25 μg, sc) or PBS for 4 weeks. The LPS-challenged mice were intravenously injected with rAAV-apoA-I-GFP or rAAV-GFP. After the animals were killed, blood, livers and aortas were collected for biochemical and histological analyses. For *ex vivo* experiments, the abdominal cavity macrophages were harvested from each treatment group of mice, and cultured with autologous serum, then treated with LPS.
Results: Chronic administration of LPS in ApoE$^{-/-}$ mice significantly increased the expression of inflammatory cytokines (TNF-α, IL-1β, IL-6, and MCP-1), increased infiltration of inflammatory cells, and enhanced the development of atherosclerosis. In LPS-challenged mice injected with rAAV-apoA-I-GFP, viral particles and human apoA-I were detected in the livers, total plasma human apoA-I levels were grammatically increased; HDL-cholesterol level was significantly increased, TG and TC were slightly increased. Furthermore, overexpression of apoA-I significantly suppressed the expression of proinflammatory cytokines, reduced the infiltration of inflammatory cells, and decreased the extent of atherosclerotic lesions. Moreover, overexpression of apoA-I significantly increased the expression of the cytokine mRNA-destabilizing protein tristetraprolin (TTP), and phosphorylation of JAK2 and STAT3 in aortas. In *ex vivo* mouse macrophages, the serum from mice overexpressing apoA-I significantly increased the expression of TTP, accompanied by accelerated decay of mRNAs of the inflammatory cytokines.
Conclusion: ApoA-I potently suppresses LPS-induced atherosclerosis by inhibiting the inflammatory response possibly via activation of STAT3 and upregulation of TTP.

Keywords: apolipoprotein A-I; heart; atherosclerosis; inflammation; cytokines; JAK2/STAT3 signaling pathway; tristetraprolin; lipopolysaccharide; ApoE$^{-/-}$ mice; macrophage

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Introduction

Over the past few decades, our understanding of the pathogenesis of atherosclerosis has undergone a major revolution, the conceptual basis of which is that inflammation plays a key role in the development of atherosclerosis[1, 2]. Although chronic inflammation triggered by metabolic mediators such as cholesterol and ceramide has been reported to promote the development of atherosclerosis[3, 4], persistent bacterial infections resulting in a chronic inflammatory condition have clearly been associated with an increased incidence of atherosclerosis[5]. Several studies have shown that antigens of chlamydia pneumoniae are persistently present within coronary atheroma from heart disease patients[6]. In addition, the serum levels of lipopolysaccharide (LPS), a common bacteria-

# These authors contributed equally to this work.
* To whom correspondence should be addressed.
E-mail tchaoke@yahoo.com.cn (Chao-ke TANG);
tujian0734@yahoo.com.cn (Jian TU)
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derived product, is associated with a greater risk of atherosclerosis in humans[7]. Therefore, inhibition of LPS-induced inflammation might represent a useful treatment for chronic inflammatory diseases such as atherosclerosis.

Apolipoprotein A-I (apoA-I), the major protein of high-density lipoprotein (HDL) that promotes the intercellular cholesterol efflux, inhibits the progression of atherogenic lesions[8]. Overexpression of human apoA-I in a mouse model of familial hypercholesterolemia has been found to inhibit the progression of atherosclerosis[9, 10]. Although the most comprehensively studied antiatherogenic function of apoA-I is reverse cholesterol transport (RCT), the antiatherogenic activity of apoA-I has recently been attributed to its anti-inflammatory and antioxidant properties[11, 12]. However, whether the anti-inflammatory effect of apoA-I independently contributes to a reduction in atherosclerosis has not yet been revealed.

Precisely what role apoA-I plays in modulating the inflammatory response remains to be elucidated. Several studies have shown that the effect of LPS binding could account for the potent antiinflammatory properties of apoA-I[13, 14]. Nevertheless, pretreating with apoA-I and then washing it out still reduced the LPS-stimulated inflammatory response in multiple cell lines[15, 16]. In addition, Marta *et al* have recently provided direct evidence that apoA-I does not directly interact with the bacteria[17], suggesting that apoA-I-induced intercellular signaling may play a significant role in initiating its antiinflammatory function. We have previously reported that apoA-I inhibits the mRNA expression of various proinflammatory cytokines via activation of STAT3 and that apoA-I upregulates the cytokine mRNA-destabilizing protein tristetraprolin (TTP) in both THP-1 macrophages and human primary macrophages[18]. TTP has been reported to be expressed in human atherosclerotic lesions[19], and it has been implicated in the prevention of atherosclerosis plaque formation in ApoE$^{-/-}$ mice[20]. However, the potential role of apoA-I-mediated antiinflammatory activity and modulation via either STAT3 or TTP signaling during LPS-induced atherosclerosis has not yet been investigated. In this study, we evaluated whether the elevation of human apoA-I could protect against LPS-induced inflammation and atherosclerosis in ApoE$^{-/-}$ mice. We also reported on the signaling mechanisms that regulate the apoA-I-mediated effects on LPS-induced atherosclerosis.

Materials and methods

Generation of recombinant adeno-associated virus

Recombinant adeno-associated virus (rAAV) was generated as previously described[21]. Briefly, the gene fragment encoding human apoA-I was generated by RT-PCR using the sense primers 5′-GGCCGGATCCCGGCATTTCTGGCAGAGATCT-3′ and the anti-sense primers 5′-GGCCGTCGACGCCTCACTGGGTGTTGAGCTTCTT-3′ from the mRNA of the human liver cDNA library. rAAV-apoA-I-GFP was constructed by cloning the human apoA-I cDNA in a rAAV-IRES-GFP plasmid (Cell Biolabs, San Diego, CA, USA). DH5α was transformed by rAAV-apoA-I-GFP and then grown to saturation in LB medium. Positive recombinants were transfected into 293 cells using the rAAV helper-free system (Stratagene, La Jolla, CA, USA) for virus packaging and propagation. rAAV-apoA-I-GFP and rAAV-GFP were purified by CsCl banding.

Animal studies

Male 20-week-old ApoE$^{-/-}$ mice (C57BL/6 background, Laboratory Animal Center of Peking University, China) were housed under barrier conditions, fed a normal chow diet, and randomly assigned to daily subcutaneous injections of LPS (25 μg) or PBS as a control. LPS-challenged mice were injected via the tail vein with 1×10^{11} viral particles of rAAV-apoA-I-GFP or rAAV-GFP at the beginning (n=20). After 4 weeks, animals were killed to allow for localization of the viral particles and detection of human apoA-I expression in the liver. Blood was collected for the detecting plasma levels of apoA-I, lipid, and inflammatory cytokines. Aortas were separated for evaluating the extent of the lesion area, for detecting the infiltration of inflammatory cells, and for determining the expression of proinflammatory cytokines. All animal experiments were conducted in accordance with the Institutional Animal Ethics Committee and the University of South China Animal Care Guidelines for the Use of Experimental Animals.

Immunohistochemistry and histological analysis of atherosclerosis

Immunohistochemical analyses were performed on fresh-frozen, OCT-embedded proximal aortic sections (10 μm), as previously described[22]. Slides were fixed in cold acetone and incubated with monoclonal anti-mouse CD68 as a macrophage marker (Santa Cruz, CA, USA). The DAB kit (Boster, Wuhan, China) was used for detecting peroxidase activity. Immunohistochemical quantitative analyses were performed as described previously[23]. Three sections at the level of the aortic sinus were examined. To count CD68-positive cells in the aortic sinus, four squared counting boxes (75 μm per side) were taken per section. CD68 staining in the plaque was measured in 20 sampling windows per section, and the values (grey levels) obtained were divided by the background value measured in the blood vessels. Statistical analyses were performed by a one-way ANOVA followed by the appropriate *post-hoc* test. H&E and Oil-Red O staining were performed on fresh-frozen, OCT-embedded proximal aortic sections (10 μm). The total lesion area and the percentage of vessel occlusion were measured with a microscope connected to a computer-linked imaging analysis system (Wuhan Qianping Ltd, Wuhan, China). The percentage of vessel occlusion was measured as the ratio of the vessel intima area (without plaque) to the vessel lumen area (with plaque). All calculations analyzed used the mean of eight samples, each at 50 μm apart, spanning the aortic sinus.

Lipid and lipoprotein analysis

Serum total cholesterol (TC), triglyceride (TG), HDL-C, and LDL-C were measured in the mice after an overnight fast with a clinical chemistry analyzer (Hitachi, Ltd, Tokyo, Japan) by

fully enzymatic methods and using the Friedewald formula. The serum apolipoprotein A-I (apoA-I) content was quantitated by immunoturbidimetric assay.

Ex vivo peritoneal macrophage experiment

Mice were given an intraperitoneal injection of 2 mL of 4% thioglycollate broth 3 d prior to harvesting macrophage cells. Abdominal cavity macrophages were obtained by injecting 5 mL of RPMI-1640 into the peritoneal cavity and withdrawing the cell suspension after 2 min of gentle massaging. The macrophages were centrifuged and resuspended in RPMI-1640 with 10% FBS. The cell suspension was dispensed into a 96-well plate and incubated for 4 h at 37 °C. After rinsing away the unattached cells, the macrophages (4×10^4/per well) were incubated in the presence of serum obtained from a different treatment group of mice and then treated with LPS.

Real-time polymerase chain reaction

Total RNA from tissues and cells were extracted using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. Relative changes in gene expression were measured by quantitative real-time PCR (RT-PCR), using SYBR Green detection chemistry, on the Quantitative PCR System (Applied Biosystems, Foster City, CA, USA). Melting curve analysis of all real-time PCR products was performed and shown to produce a single DNA duplex. Quantitative measurements were determined using the ΔΔCt method, and the expression of β-actin was used as the internal control. The primers used for RT-PCR were the mouse ABCA1 sense primer: 5′-GCCGTCTTTCCAGGACAGTATG-3′ and the anti-sense primer: 5′-CAGGGTGGCTCTTCTCATCAAT-3′; the mouse TTP sense primer: 5′-GGTACCCCAGGCTGGCTTT-3′ and the anti-sense primer: 5′-ACCTGTAACCCCAGAACTTGGA-3′; the mouse TNF-α sense primer: 5′-TCTCATCAGTTCTATGGCCC-3′ and the anti-sense primer: 5′-GGGAGTAGACAAGGTACAAC-3′; the mouse IL-1β sense primer: 5′-TTAGACAACTGCACTACAGGCTC-3′ and the anti-sense primer: 5′-GCTCTGCTTGTGAGGTGCTGATG-3′; the mouse β-actin sense primer: 5′-TCCTTCGTTGCCGGTCCACA-3′ and the anti-sense primer: 5′-CGTCTCCGGAGTCCATCACA-3′.

mRNA decay assay

The mRNA decay assay was performed as previously described[18]. LPS-stimulated mouse macrophages were treated with serum obtained from the different treatment groups for 3 h and then exposed to actinomycin D (act D) to inhibit transcription. Total RNA was harvested at different time points. Inflammatory cytokine mRNA levels at each time point were quantified using RT-PCR and were normalized against β-actin. Remnant inflammatory cytokine mRNAs relative to the amount at time point 0 of act D exposure were depicted.

Western blot analysis

Proteins were fractionated by SDS-PAGE and electroblotted onto immobilon-P transfer membranes. The membranes were then incubated with antibodies against human apoA-I (Calbiochem), mouse apoA-I (Abcam), mouse TTP (Abcam), mouse ABCA1 (Abcam), mouse p-STAT3 (Santa Cruz), and mouse p-JAK2 (Santa Cruz) in blocking solution at 4 °C overnight. The membranes were washed and incubated with HRP-conjugated secondary antibodies. The proteins were detected using ECL Plus Detection Reagent (Amerisham Biosciences, Foster City, CA, USA).

Measurement of inflammatory cytokines by ELISA

Serum was separated by centrifugation of the blood samples from the mice. Culture supernatants in cells were collected as described previously[18]. Freshly isolated aorta was homogenized in Tris buffer (20 mmol/L) with 0.35 mol/L sucrose and centrifuged at 12000×*g* for 5 min. The supernatant was analyzed for protein concentration. All samples were stored at -20 °C until analysis. The levels of inflammatory cytokines in the samples were measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, UK). The cytokine standards were used to generate standard curves.

Statistical analysis

Data are expressed as mean±SD. Results were analyzed by a one-way ANOVA and student's *t* test using SPSS 13.0 software. Statistical significance was obtained when *P*-values were less than 0.05.

Results

Influence of rAAV-apoA-I-GFP on apoA-I and plasma lipids in ApoE$^{-/-}$ mice

To determine the effect of injected rAAV2-apoA-I-GFP on human apoA-I expression in ApoE$^{-/-}$ mice, we examined the location of viral particles and human apoA-I expression in the liver and plasma of the mice after systemic injection of rAAV2 for 4 weeks. As shown in Figure S1 and Figure 1A, viral particles and human apoA-I were detected in the livers of mice injected with rAAV-apoA-I-GFP (LPS+apoA-I group). Total plasma human apoA-I levels were also increased in the mice injected with rAAV-apoA-I-GFP (Figure 1B). Other groups have not detected the expression of human apoA-I (Figure 1A, B). Except for HDL-cholesterol, the total plasma lipids did not noticeably change in LPS-treated mice (LPS group) compared with the PBS-treated mice (control group). ApoA-I expression (LPS+apoA-I group), but not GFP expression (LPS+GFP group), resulted in significantly increased HDL-cholesterol levels as well as slightly increased TG and TC in the LPS-challenged ApoE$^{-/-}$ mice (Table S1).

ApoA-I has anti-inflammatory effects in LPS-challenged ApoE$^{-/-}$ mice

To investigate the effect of apoA-I expression on inflammation, plasma and aortic concentrations of a set of inflammatory cytokines were measured. After daily injections of LPS for 4 weeks, concentrations of inflammatory cytokines, including TNF-α, IL-1β, IL-6, and MCP-1, were increased in both the plasma and aortic tissue. Expression of human apoA-I signifi-

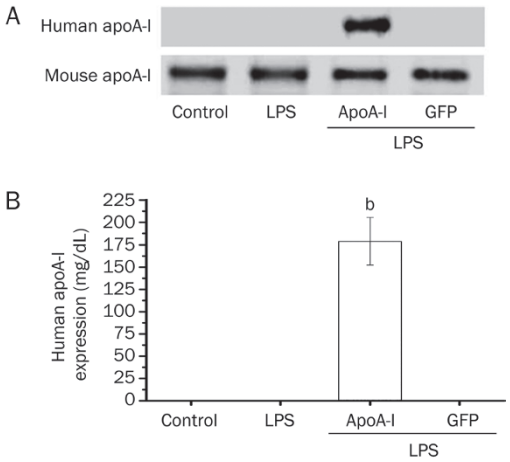

Figure 1. Influence of rAAV-apoA-I-GFP on apoA-I and plasma lipids in ApoE$^{-/-}$ mice. (A) Western blots of apoA-I from the livers of ApoE$^{-/-}$ mice. (B) Human apoA-I levels in the plasma of ApoE$^{-/-}$ mice were determined by immunoturbidimetry. Control (PBS injection); LPS (LPS injection); ApoA-I (injection with rAAV-apoA-I-GFP and LPS); and GFP (injection with rAAV-GFP and LPS). Mean±SEM. n=3. ^{b}P<0.05 *vs* control groups.

cantly reduced the plasma and aortic concentration of inflammatory cytokines in LPS-challenged ApoE$^{-/-}$ mice (P<0.05 versus LPS; Table S2A, S2B). We then examined the mRNA expression of various proinflammatory cytokines (TNF-α, IL-1β, IL-6, and MCP-1) in the aorta by quantitative RT-PCR. Human apoA-I-expressing mice showed significant reduction in the mRNA expression levels of proinflammatory cytokines compared to those from the control LPS groups (P<0.05 versus LPS; Figure 2).

Because macrophages are the main source of inflammatory cytokines in atherosclerotic lesions, we next investigated the effect of apoA-I expression on the macrophage infiltration in the aortic sinus of LPS-challenged mice. The results showed that the staining intensities of CD68, a macrophage marker, in human apoA-I-expressing mice were clearly reduced in comparison to those from LPS-challenged ApoE$^{-/-}$ mice (Figure 3).

ApoA-I inhibits atherosclerosis in LPS-challenged ApoE$^{-/-}$ mice

To investigate the effects of apoA-I expression on atheroma progression in LPS-challenged ApoE$^{-/-}$ mice, the histological analyses of atherosclerotic lesions of mice from different treatment groups were compared. Representative examples of H&E-stained aortic sinuses are shown in Figure 4A. Compared with PBS-infused (control) mice, LPS infusion significantly increased the extent of the lesion in the aortic sinus (P<0.01 versus control group), whereas human apoA-I expression, but not GFP expression, significantly reduced the development of atherosclerosis in LPS-challenged ApoE$^{-/-}$ mice compared with LPS-infused mice. The aortic sinus lesion area was 0.138±0.051 mm^2 in the control group, 0.399±0.088 mm^2 in the LPS group, 0.250±0.078 mm^2 in the LPS+apoA-I group, and 0.415±0.068 mm^2 in the LPS+GFP group. The mice expressing human apoA-I had a 50% regression in lesion area

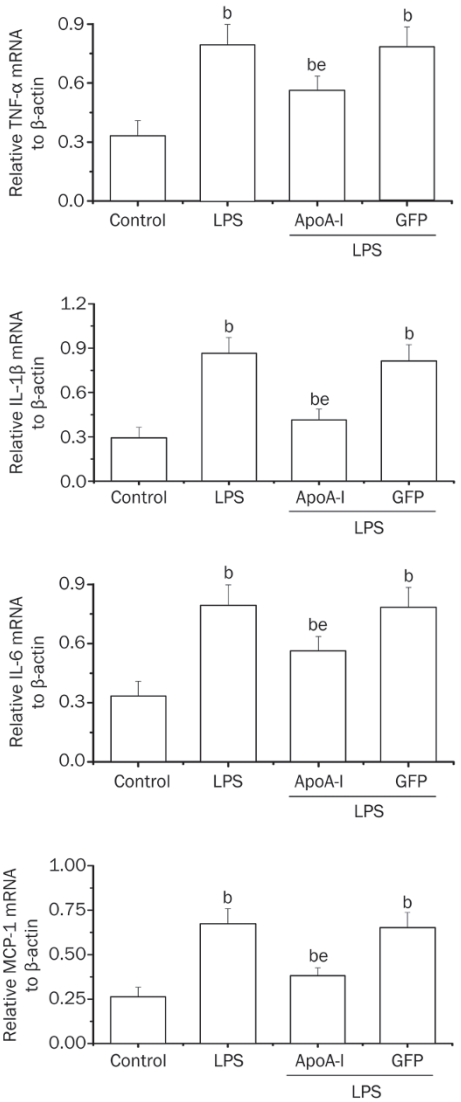

Figure 2. Effect of apoA-I on mRNA expression of inflammatory cytokines in LPS-challenged ApoE$^{-/-}$ mice. RNA from the aortas of different treatment groups of ApoE$^{-/-}$ mice were isolated and analyzed to determine the relative mRNA levels of several different inflammatory cytokines, including TNF-α, IL-1β, IL-6, and MCP-1; measurements were normalized to the levels of β-actin. Data are mean±SD. The analysis was performed in at least 8 separate samples, ^{b}P<0.05 *vs* control group and ^{e}P<0.05 *vs* LPS group.

as compared with control LPS mice (P<0.05 versus LPS group) (Figure 4A).

Oil Red O-stained aortic sinuses also demonstrated the difference in lipid deposition in the atherosclerotic lesions of ApoE$^{-/-}$ mice. Lesions in the control mice showed mild lipid accumulation, while LPS infusion clearly increased the lipid accumulation in the lesions. In contrast, LPS-challenged mice treated with human apoA-I displayed decreased lipid deposition in the aortic roof when compared with LPS and control GFP mice (P<0.05 versus LPS group) (Figure 4B).

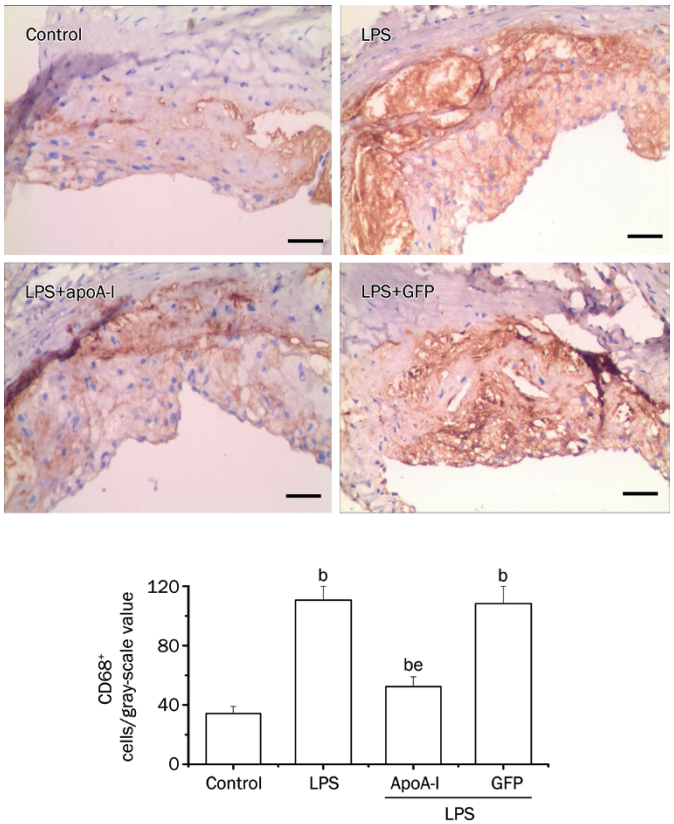

Figure 3. Effect of apoA-I on the infiltration of inflammatory cells in LPS-challenged ApoE$^{-/-}$ mice. Immunohistochemical staining of macrophages (CD68-positive, brown DAB reaction) in aortic sinuses of ApoE$^{-/-}$ mice (n=8). Scale bars: 200 μm. Bar graph shows quantitative analysis of gray-scale values of CD68-positive cells. ^{b}P<0.05 *vs* control group and ^{e}P<0.05 *vs* LPS group.

ApoA-I upregulated the expression of TTP and the activation of STAT3

Because TTP plays a crucial role in the anti-inflammatory response associated with apoA-I in human macrophages *in vitro*[18], we next tested whether TTP could be the mediator of the antiinflammatory and antiatherosclerotic effects of apoA-I *in vivo*. In the aortas of LPS-challenged mice, human apoA-I expression significantly induced TTP mRNA and protein expression, while control GFP had no effect on TTP expression (Figure 5A, 5B). The JAK2/STAT3 pathway, which usually promotes the transcription of TTP, has been shown to be activated by apoA-I in mouse macrophages[15]. We detected the phosphorylation of JAK2 and STAT3 in mouse aortas and found human apoA-I-induced phosphorylation of JAK2 and STAT3 in LPS-challenged mice (Figure 5C), suggesting that the effects of apoA-I on TTP expression and activation of STAT3 are not restricted to *in vitro* culture conditions but also occur *in vivo*.

The ATP-binding membrane cassette transporter AI (ABCA1), a major receptor for apoA-I, plays a central role in mediating the effect of apoA-I[24]; thus, we next measured the mRNA and protein levels of ABCA1 in mouse aortas. The results demonstrated that ABCA1 was significantly decreased after LPS treatment; however, human apoA-I significantly increased the protein expression of ABCA1 (Figure 5D, 5E).

ApoA-I-mediated attenuation of the expression of inflammatory cytokines is associated with increased mRNA decay and expression of TTP in *ex vivo* mouse macrophages

To further investigate the molecular mechanisms involved in apoA-I-mediated anti-inflammatory effects in LPS-challenged mice, we harvested primary macrophages from the abdominal cavities of different treatment groups of mice. Mouse macrophages were treated with LPS (10 ng/mL) for 3 h after being cultured with autologous serum in order to closely mimic the *in vivo* environment. As shown in Figure 6A, 6B, serum from human apoA-I-expressing mice substantially increased the expression of TTP in LPS-treated mouse macrophages, suggesting that TTP may be involved in the antiinflammatory effect of apoA-I in these mice.

We then investigated the role of TTP in antiinflammation of apoA-I in mouse macrophages. First, mouse macrophages were treated with LPS and/or autologous serum followed by exposure to actinomycin D (act D, 5 μg/mL) to stop transcription. The RT-PCR results indicated that serum from human apoA-I-expressing mice induced a marked increase in TNF-α and IL-1β mRNA degradation (Figure 6C), suggesting that mRNA decay plays a crucial role in the apoA-I-mediated decrease of inflammatory cytokines, which is consistent with previous findings in human macrophages[18]. To investigate the effect of TTP on the inflammatory cytokine mRNA decay induced by apoA-I, an efficient siRNA targeting TTP was used (Figure 6D). Compared with cells transfected with control siRNA, the LPS-induced expression of TNF-α and IL-1β were slightly increased in TTP-silenced macrophages. However, the apoA-I-mediated inhibition of the production of cytokines induced by LPS was significantly impaired in TTP-silenced macrophages. Serum from human apoA-I expressing mice caused a reduction of TNF-α and IL-1β to 65.41% and 63.5% in LPS-stimulated control cells, respectively, whereas apoA-I caused a reduction of TNF-α and IL-1β to 87.5% and 83.2% in TTP-silenced cells, respectively (Figure 6E) (87.5% *vs* 65.41%, and 83.2% *vs* 63.5%, respectively; n=3; P<0.05 *vs* control cells). In addition, the apoA-I-mediated mRNA decay of inflammatory cytokines, such as TNF-α and IL-1β, was also abolished in TTP-silenced cells (62.8% and 60.3%) compared with control cells (41.6% and 47.8%) (Figure 6F) (41.6% *vs* 62.8%, and 47.8% *vs* 60.3%, respectively; n=3; P<0.05 *vs* TTP-silenced cells).

Discussion

Overexpression of apoA-I, which is mainly synthesized and secreted by the liver[25], has been associated with the inhibition of the progression of atherosclerosis in various animal models[9, 10, 26, 27]. One mechanism is believed to be by the promotion of RCT[28]. Recently, various studies have shown that apoA-I modulates the immune-inflammatory response[29–31], suggesting that the antiatherogenic effect of apoA-I may also be due to its anti-inflammatory activity. The direct proof of

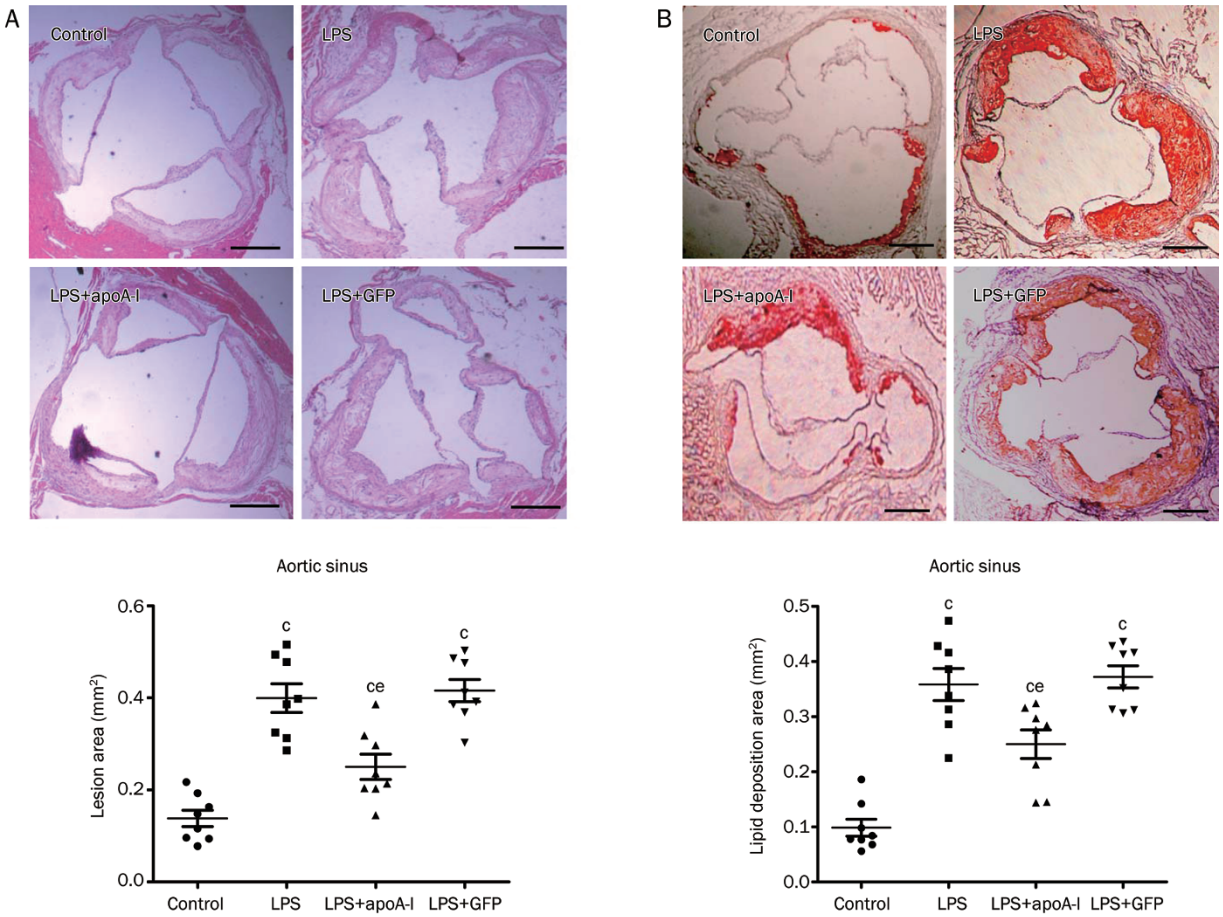

Figure 4. Effect of apoA-I on the development of atherosclerosis in LPS-challenged ApoE$^{-/-}$ mice. (A) Representative cross-sections of H&E-staining of the aortic sinuses of ApoE$^{-/-}$ mice, magnification 40× (Left); Quantification of lesion area (mean±SEM, *n*=8). (B) Representative cross-sections of Oil Red O-staining of aortic sinuses of ApoE$^{-/-}$ mice (*n*=8). Bar graph shows quantitative analysis of lesion area (A) and lipid deposition (B) in aortic sinuses. $^{c}P<0.01$ *vs* control group and $^{e}P<0.05$ *vs* LPS group. Scale bars: 200 μm.

this concept, however, has not been irrefutably verified. In this study, we demonstrated for the first time that apoA-I inhibits the expression of LPS-induced inflammatory cytokines, infiltration by inflammatory cells, and the development of atherosclerosis *in vivo*. In addition, we established that these antiinflammatory and antiatherogenic effects may be associated with apoA-I-mediated upregulation of the cytokine mRNA-destabilizing protein TTP and activation of STAT3; however, complimentary *in vivo* experiments remain to be conducted, such as the use of TTP/STAT3 knockout mice to establish direct, causal relationships between the antiinflammatory effects and antiatherogenic effects of apoA-I.

The activation of the innate immune system has been found to play a key role in accelerating atherosclerosis in animals and increasing the risk of cardiovascular disease (CVD) in human[32, 33]. LPS, a well-known component of gram-negative bacteria, is a major contributor to the activation of the innate immune system[34]. Increased serum levels of LPS have been reported to promote the progression of CVD in humans[7, 35]. In this study, we observed that subcutaneous injections of LPS alone resulted in enhanced atherosclerotic lesion size in ApoE$^{-/-}$ mice. As the major cell type for the innate immune response and cholesterol metabolism, macrophages are considered to be crucial regulatory targets to inhibit the development of atherosclerosis[36]. After activation, the infiltration of macrophages into the vessel wall is a key step in the inflammatory response contributing to the initiation of atherosclerosis[37]. Our study has demonstrated that the infiltration of CD68-positive macrophages in the atherosclerotic lesions of LPS-treated mice was clearly increased. Moreover, an increase in the infiltration of inflammatory cells resulted in increased expression in the aortas of LPS-treated mice of various proinflammatory cytokines and chemokines, which may play crucial roles in LPS-induced atherogenesis.

ApoA-I has been reported to inhibit neutrophil activation and leukocyte recruitment to the endothelium in *in vivo* models of inflammation[29]. In the present study, we demonstrated that overexpression of human apoA-I for 4 weeks results in a significant decrease in macrophage infiltration in the aortas of LPS-treated mice. In addition, LPS-induced proinflammatory cytokines, including TNF-α, IL-1β, IL-6, and MCP-1, in the sera and aortas were also decreased by apoA-I. Increased

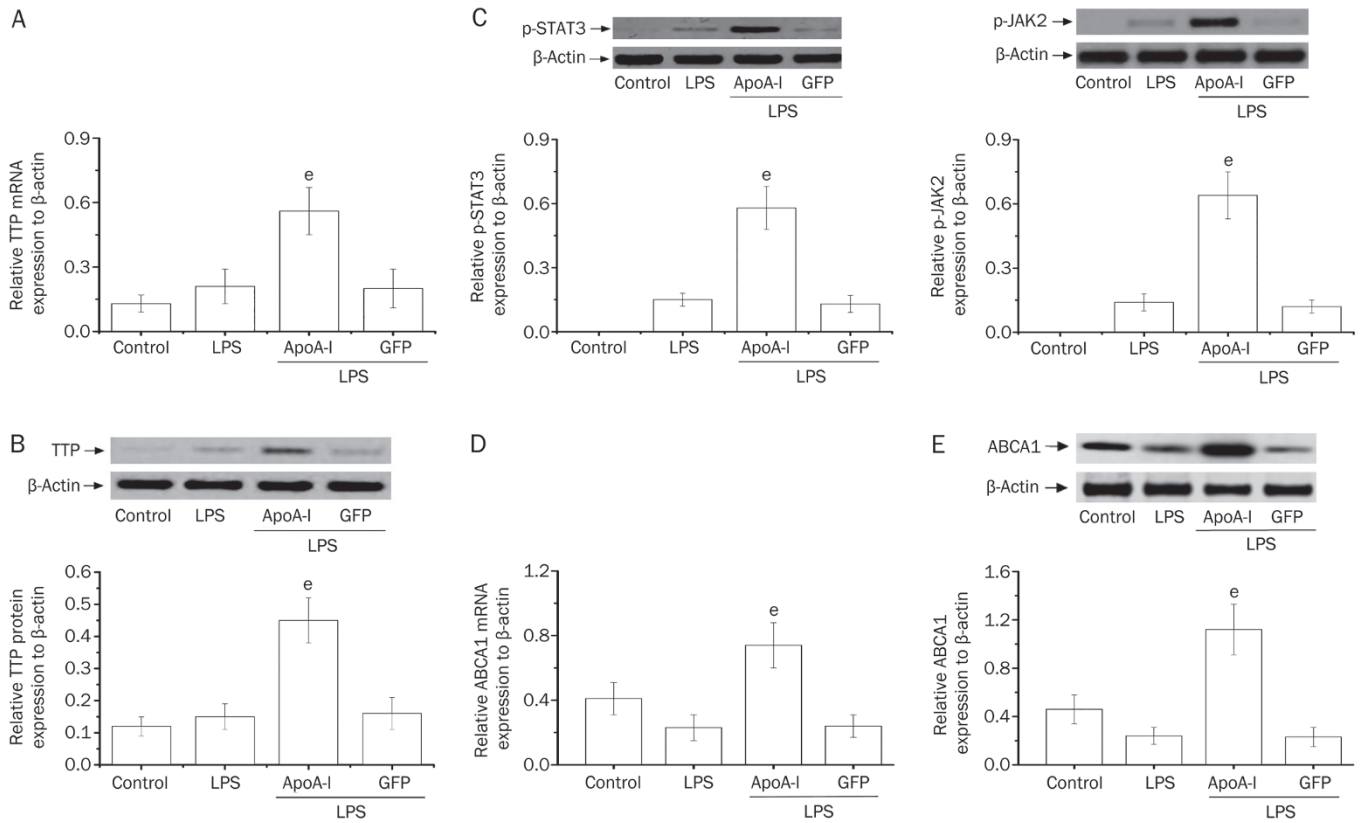

Figure 5. Effect of apoA-I on TTP expression and STAT3 activation in LPS-challenged ApoE$^{-/-}$ mice. (A) TTP mRNA levels in freshly isolated aorta were measured by RT-PCR and normalized to the levels of β-actin. (B) TTP protein levels in freshly isolated aorta were measured by Western blotting and normalized to the levels of β-actin. (C) Phosphorylation levels of JAK2 (p-JAK2) and STAT3 (p-STAT3) in freshly isolated aorta were measured by Western blotting and normalized to the levels of β-actin. (D) ABCA1 mRNA levels in freshly isolated aorta were measured by RT-PCR and normalized to the levels of β-actin. (E) ABCA1 protein levels in freshly isolated aorta were measured by Western blotting and normalized to the levels of β-actin. Mean±SEM. *n*=3. $^{e}P<0.05$ *vs* LPS group.

cholesterol levels, especially LDL-cholesterol levels, have been shown to promote the initiation of vascular inflammation[3, 38]. However, cholesterol levels in transgenic apoA-I mice are not decreased. By contrast, the concentrations of TC and TG are increased in apoA-I-overexpressing mice, and the exact mechanism of this process is unknown. These results suggest that the cytokines that are inhibited by apoA-I differ from those that are responsible for decreasing cholesterol levels. Recently, apoA-I was found to influence cell functions through diverse receptors and multiple pathways[39–41]. Our group has previously reported that apoA-I, or its mimetic peptides, dramatically increased the activation of the Rho GTPase CDC42 via an ABCA1-dependent mechanism[42]. Recently, we and Oram *et al* found that apoA-I could exhibit antiinflammatory properties via an ABCA1-dependent STAT3 activation in macrophages[15, 18]. STAT3 activation has been suggested to correlate with a lower state of inflammation[43, 44]. Khan *et al* recently revealed that AAV/hSTAT3-gene delivery lowers aortic inflammatory cell infiltration and atherogenesis in LDLR-KO mice with high cholesterol[44]. Yoshioka *et al* have also reported that AAV5-mediated interleukin-10 gene transfer inhibits atherogenesis in ApoE-deficient mice through a STAT3-dependent antiinflammatory pathway[43]. In the present study, we demonstrated that apoA-I expression significantly increased the phosphorylation of STAT3 as well as its upstream kinase, JAK2. ABCA1, a major receptor for apoA-I in macrophages, plays a central role in regulating the function of apoA-I and inflammation has been shown to inhibit the expression of ABCA1[45]. In the current study, our results demonstrate that apoA-I upregulates the expression of ABCA1 in LPS-challenged ApoE$^{-/-}$ mice. Taken together, these data strongly suggest that apoA-I-mediated inhibition of LPS-induced inflammation and atherosclerosis might occur via ABCA1-dependent STAT3 activation.

TTP, an intracellular protein also called ZFP36, is known to have antiinflammatory activity via binding and destabilizing inflammatory cytokine mRNAs[46]. In human atherosclerotic plaques and peripheral blood monocytes, TTP has been identified as one of the most highly expressed macrophage transcriptional regulators[19]. Knockout of TTP in the ApoE$^{-/-}$ mouse model of atherosclerosis resulted in marked exacerbation of aortic plaque formation[20], suggesting that TTP is a novel therapeutic target for atherosclerosis-related cardiovascular disease in addition to its effect on immune-

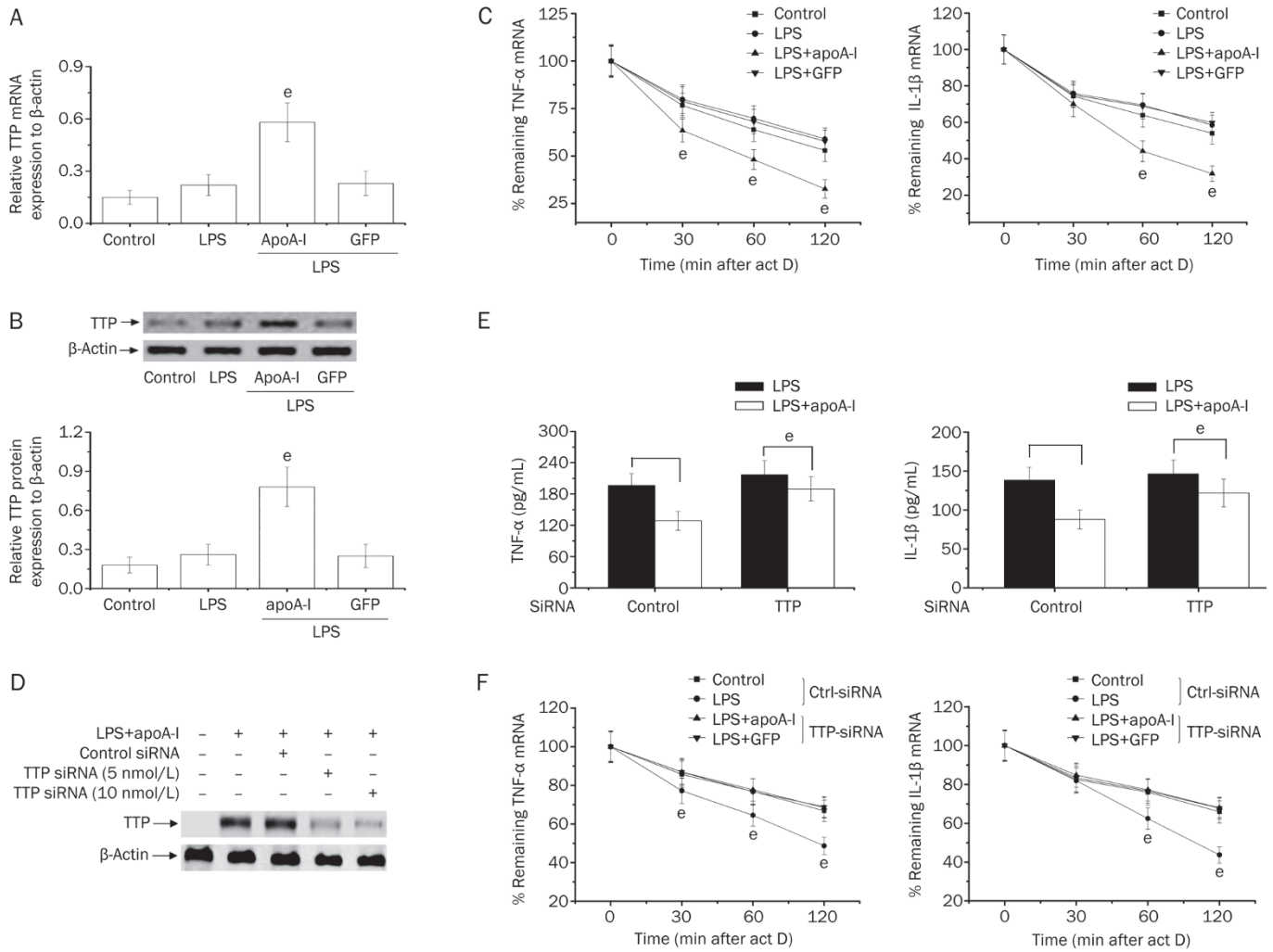

Figure 6. Effect of apoA-I on TTP expression and inflammatory cytokine mRNA decay in mouse macrophages. After being cultured with autologous serum and then treated with LPS (10 ng/mL) for 3 h, TTP mRNA (A) and protein (B) levels in mouse macrophages were measured by RT-PCR and normalized to the levels of β-actin, $^{e}P<0.05$ *vs* LPS group. (C) After being cultured with autologous serum and treated with LPS for 2 h, actinomycin D (act D; 5 μg/mL) was then added to the mouse macrophages to stop transcription. At the indicated time points, mRNA expression of cytokines (TNF-α and IL-1β) was quantified using RT-PCR and normalized to β-actin expression. Remnant cytokine mRNA levels relative to the amount at the time point 0 of act D exposure are depicted, $^{e}P<0.05$ *vs* LPS group. (D) Mouse macrophages were transfected with control (WT) or TTP siRNA (5 or 10 nmol/L) for 48 h. Protein samples were immunoblotted with TTP and β-actin antibodies. (E) After being cultured with serum from rAAV-apoA-I-injected mice, mouse macrophages transfected with control or TTP siRNA were incubated with LPS for 3 h. The levels of TNF-α and IL-1β were measured by ELISA. The inhibition rates of apoA-I on inflammatory cytokines were compared, $^{e}P<0.05$ *vs* control cells (WT). (F) Mouse macrophages transfected with control (WT) or TTP siRNA were injected with LPS alone or pretreated with serum from rAAV-apoA-I-treated mice for 2 h followed by the addition of act D (5 μg/mL) to stop transcription. At the indicated time points, TNF-α and IL-1β mRNA were quantified using RT-PCR. Values were normalized against β-actin. Remnant cytokine mRNA levels relative to the amount at the time point 0 of act D exposure are depicted, $^{e}P<0.05$ compared to control cells (WT). Mean±SD. $n=3$.

mediated inflammatory disease[47]. The expression of TTP was regulated by activated STATs in macrophages[18, 48]. The present study revealed that STAT3 activation in human apoA-I-expressing mice was associated with increased expression of TTP in aortas. In addition, serum from the apoA-I-expressing mice has been found to upregulate the expression of TTP in LPS-stimulated mouse peritoneal macrophages. Furthermore, inhibition of TTP with siRNA in the mouse peritoneal macrophages resulted in impairment of the antiinflammation effect of apoA-I. These results suggest that apoA-I-mediated down-regulation of inflammation and atherosclerosis might be result from the antiinflammatory effect of TTP. Therefore, we investigated the effects of apoA-I overexpression on inflammatory cytokine mRNA decay. Our results showed that apoA-I significantly promoted the mRNA decay of TNFα and IL-1β and that knockdown of TTP resulted in an increase in mRNA levels of cytokines in apoA-I-treated macrophages. These results indicate that TTP-mediated inflammatory cytokine

mRNA decay is a novel mechanism that is responsible for the anti-atherosclerotic effect of apoA-I.

In conclusion, the present study demonstrates that apoA-I reduces LPS-induced inflammatory responses, both *in vitro* and *in vivo*, and inhibits the development of atherosclerosis. The mechanism underlying the antiinflammation and anti-atherogenic effect of apoA-I may be related to the upregulation of TTP and the activation of the STAT3 signaling pathway.

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Author contribution

Kai YIN and Chao-ke TANG designed the research; Kai YIN, Shi-lin TANG, Xiao-hua YU, Guang-hui TU, Rong-fang HE, Jin-feng LI, and Jian TU performed the experiments; Qing-jun GUI and Yu-chang FU contributed to the analysis of the data; and Kai YIN, Zhi-sheng JIANG, and Chao-ke TANG wrote the paper.

Supplementary information

Supplementary figure and tables are available at website of Acta Pharmacologica Sinica on NPG.

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