Research Article

Effect of lipid-bound apolipoprotein A-I cysteine mutant on ATF3 in RAW264.7 cells

Yunlong Wang1,*, Yanhui Wang2,*, Shaoyou Jia3, Qingzhe Dong1, Yuanbin Chen1, Shulai Lu4 and Lin Hou2

1Biological Specimen Bank, The Affiliated Hospital of Qingdao University, Qingdao, China; 2Department of Biochemistry, Medical College, Qingdao University, Qingdao, Shandong, China; 3Department of Ophthalmology, The Affiliated Hospital of Medical College Qingdao University, 16 Jiangsu Road, Qingdao, China; 4Department of Stomatology, Qingdao Municipal Hospital, Qingdao, China

Correspondence: Shulai Lu (wylapollo@163.com; lshl97@163.com) or Lin Hou (qingyi001@126.com)

Activating transcription factor 3 (ATF3) is a TLR-induced repressor that plays an important role in the inhibition of specific inflammatory signals. We previously constructed recombinant high density lipoproteins (rHDL) (including rHDLWT, rHDLM, rHDL228 and rHDL74) and found that rHDL74 had a strong anti-inflammatory ability. In the present study, we investigate the roles of recombinant apolipoprotein A-I (ApoA-I) (rHDLWT) and its cysteine mutant HDLs (rHDLM, rHDL228 and rHDL74) on ATF3 function in RAW264.7 cells stimulated by lipopolysaccharide. Our results showed that compared with the LPS group, rHDL74 can decrease the level of TNF-α and IL-6, whereas rHDL228 increases their expression levels. RT-PCR and Western blotting results showed that compared with the LPS group, rHDL74, rHDLWT and rHDLM can markedly increase the expression level of ATF3, whereas the level of ATF3 decreases in the rHDL228 group. In summary, the different anti-inflammatory mechanisms of the ApoA-I cysteine mutants might be associated with the regulation of ATF3 level.

Introduction

As a basic and most common indicator of the disease process, the inflammatory response can be defined as the penetration of infectious agents, the enhancement of antigen and the response to cellular injury [1]. In most cases, the inflammatory response is eventually controlled by the release of endogenous anti-inflammatory mediators or by accumulating negative regulatory factors within the immune cell. Such mechanisms allow these inflammatory cells to be cleared at the appropriate time [2]. However, these mechanisms of negative regulation, including the persistent accumulation of negative regulatory factors and activation of white blood cells, may become dysfunctional and, thus, uncontrollable [3]. Epidemiological studies have shown that HDL or its component apolipoprotein A-I (ApoA-I), plays a significant role in anti-inflammatory and antioxidative activities [4,5] and is vital in reverse cholesterol transport [6].

Macrophages are one of the most important immune cells in vivo [7]. Macrophages play a crucial role in the immune response because they can kill a pathogen either directly through phagocytosis or indirectly through the secretion of a series of anti-inflammatory mediators [8]. Inflammatory mediators generated by activation of macrophages are associated with several pathophysiological diseases [9], such as rheumatoid arthritis and pulmonary fibrosis [8]. LPS is the main component of the cell wall of Gram-negative bacilli and is a main component of the inflammatory reaction [10]. The inflammatory reaction induced by LPS could induce the expression of inflammatory cytokines through a series of signal transduction, involving transcription factor activation after combining with the LPS receptor CD14 and TLR4 [11]. LPS-stimulated macrophages can serve as a model for studying inflammation and anti-inflammatory diseases [12,13]. In recent years, many animal studies have found that HDL can chelate LPS to inhibit the inflammatory response by preventing activation of intracellular TLR4 [14]. Activating transcription factor 3 (ATF3) is a negative regulator of a particular set of TLR4-induced pro-inflammatory cytokine genes (e.g. TNF, IL-6 and IL-12p40).
ATF3 expression is induced by TLR stimulation or various other stimuli [16] and operates by a negative feedback system to limit the overproduction of pro-inflammatory cytokines such as IL-6, TNF-α and CD14 [17,18]. Furthermore, studies have shown that HDL mediates the anti-inflammatory transcriptional reprogramming of macrophages via the transcriptional repressor ATF3 [14]. HDL can antagonize TLR responses by regulating ATF3 expression both in vitro and in vivo, which demonstrates that HDL plays a critical role in anti-inflammatory activity [14]. Furthermore, our current understanding of the role of ATF3 in innate immune cells is that ATF3 is a vital inducible repressor of specific transcriptional networks within the innate inflammatory response [19].

In previous studies, we constructed rHDLs (rHDLWT, rHDLM, rHDL74 and rHDL228) by combining wild-type or mutant ApoA-I in a solution of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and found that they have different anti-inflammatory properties [20,21]. In the present study, we used an LPS-induced inflammatory response in RAW264.7 cells to investigate whether the anti-inflammatory properties of these rHDLs are related to the regulation of ATF3 expression.

Materials and methods
Reagents and antibodies
LPS and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were purchased from Sigma. ELISA kits were purchased from BlueGene. The Pierce BCA Protein Assay kit and Detoxi-Gel™ Endotoxin Removing Gel were purchased from Thermo. TRizol Reagent was purchased from Life Technologies. Ni-NTA His Bind resin was purchased from Novagen. PrimeScript™ RT reagent Kit with gDNA Eraser and SYBR Premix Ex Taq were purchased from Takara. High-glucose DMEM was purchased from HyClone. FBS was purchased from Gibco. The following antibodies were used: anti-ATF3 and secondary antibodies (anti-rabbit IgG (whole molecule)-peroxidase produced in goat (Sigma; 1:8000), monoclonal GAPDH (Sigma; 1:5000) and secondary antibodies (anti-mouse IgG (Fc specific)-peroxidase produced in goat (Sigma; 1:14000).

Cells
RAW264.7 mouse macrophages were purchased from the Shanghai Cell Bank. Recombinant Escherichia coli containing the coding region for human ApoA-I and cysteine mutant was preserved in the Laboratory of The Affiliated Hospital of Qingdao University. All animal experiments were approved by the animal care committee of the Affiliated Hospital of Qingdao University.

Extraction and purification of recombinant apolipoproteins
The expression and purification of recombinant ApoA-I and cysteine mutants were based on previous methods, except for the following alterations. The recombinant ApoA-I and cysteine mutants were purified by nickel column chromatography and concentrated using enrichment centrifuge tubes. Proteins were then resolved by SDS/PAGE and quantified using BCA kit. Removal of endotoxin and construction of rHDLs were carried out as described previously [18,19]. Purified proteins were stored at −20°C.

Cell culture and pretreatment
RAW264.7 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100-units/ml penicillin and 100 μg/ml streptomycin at 37°C in an incubator with humidified air and 5% CO2. Cells were treated with trypsin and centrifuged at 1500 rev/min for 5 min for passaging. Cells were cultured in six-well plates (5 × 10⁵ cells per well).

The RAW264.7 cells used in our study were divided into a total of six groups (control, LPS groups and four test groups: rHDL228, rHDLWT, rHDLM and rHDL74). All groups were cultured in 2-ml complete medium for 20 h; the medium was replaced and 1 μg/ml LPS was added to all groups, except the control group, followed by culture for 24 h. Then, 500 μg/ml of the different rHDLs were added into the corresponding test groups for 10 h. Morphological changes were observed by microscopy.

To investigate the cytotoxic effect of different rHDLs, cell viability was measured with the Cell Counting Kit-8 according to the manufacturer’s protocol. Briefly, 1 × 10⁵ cells/well were seeded in 96-well plates, then treated with 3.125, 6.25, 12.5, 25 or 50 μg/ml different rHDLs for 24 h, after that the culture medium was replaced with 100 μl of medium containing 10 μl of CCK-8 per well and the cells were incubated for one more hour. Then, the cell viability was measured.
Measurement of cytokines expression by ELISA kits
The production of cytokines IL-6 and TNF-α in cell-culture supernatants were measured by ELISA using the manufacturer’s instructions.

RNA isolation and SYBR Green qPCR
Total RNA was isolated using TRIzol (Life Technologies) according to the manufacturer’s instructions, and the concentration of total RNA was assessed using NanoDrop 2000C (Thermo, U.S.A.). Approximately, 1 μg of extracted RNA from each sample was transcribed to cDNA using a PrimeScript™ RT reagent Kit with gDNA Eraser. cDNA amplification was measured by quantitative real-time PCR using SYBR® Premix Ex Taq II (Tli RNaseH Plus). Primers were purchased from Invitrogen. Murine HPRT: forward 5’-TGAAGTACTCATTATAGTCAAGGGCA-3’ and reverse 5’-CTGGTGAAAAGGACCTCTCG-3’. Murine ATF3: forward 5’-GACTGAGATTCGCCATCCA-3’ and reverse 5’-CCGCCTCCTTTTCCTCTCAT-3’. Primer specificity was checked by melt curve analysis. Each treatment includes three replicates with the SYBR green mix in the CFX96 (Bio–Rad Laboratories, Hercules, CA). The expression of the target gene was normalized to the house-keeping gene HPRT. Relative quantification of gene expression was performed using the difference in threshold cycle (C_T) method (ΔΔC_T = C_T target – C_T control) and the relative expression equaled 2−ΔΔC_T (ΔΔC_T = ΔC_T target – ΔC_T control). All data are presented as fold change relative to the control.

Western blotting for ATF3
To confirm ATF3 mRNA translation in cells, we used Western blotting analysis. Proteins were extracted from the cells, and the protein concentration was measured using a BCA assay kit. Equal amounts of protein per sample were applied to pre-cast SDS/PAGE (12% gels) with MOP buffer and proteins were transferred to PVDF membranes. Membranes were incubated with 5% BSA in Tris buffer for 2 h at room temperature and overnight at 4°C with specific primary antibody. Then, membranes were washed with TBST, incubated with secondary antibodies for 1 h and washed for more than three times. Immunoreactivity was visualized, and blots were scanned for analysis using the Image J2x software.

Statistical analysis
Values were shown as mean ± S.D., and differences between groups were analysed using one-way ANOVA analysis using SPSS 21.0 software for Windows (SPSS Inc., U.S.A.). P<0.05 was considered statistically significant. Additional analyses and making graphs were performed with Prism 6.0 (GraphPad Software Inc.).

Results
Expression and purification of rHDLs
The recombinant plasmid was transferred into E. coli and cultured in LB medium. Then, E. coli was collected and disrupted for purification of proteins on an Ni2+ affinity column, followed by SDS/PAGE (12% protein gel). Coomassie Brilliant Blue staining was used to visualize the 28-kDa protein of interest present in the lysate (Figure 1). These data implied that the protein of interest can be constructed following DPPC embedded experiments to make rHDL.

rHDLs at low concentrations had no cytotoxic effect on RAW264.7 cells
To investigate the effect of rHDLs on cell proliferation and viability, the cells were treated with 3.125, 6.25, 12.5, 25 or 50 μg/ml of different rHDLs for 24 h. The CCK-8 assay showed that rHDLs displayed no cytotoxic effect at a low concentration on RAW264.7 cells (Figure 2).

Effect of rHDL on the morphological changes of RAW264.7 cells stimulated by LPS
Compared with the control group, the morphology of LPS-treated cells changed obviously (Figure 3): they became diamond-shaped and had pseudopodia and degradation appeared in most cells. The cells in the HDL74 group were significantly restored to a wild-type appearance and were characterized by a spindle or circular shape and showed less degradation compared with LPS group. rHDL74 and rHDLWT also had obvious inhibition against inflammation, whereas cells treated with rHDL228 had no differences compared with the LPS group.
Figure 1. Recombinant purified ApoA-I and its mutants were examined by SDS/PAGE (12% gel)
Lanes 1–5 represent wild-type ApoA-I, A-I (N74C), low molecular mass marker, A-I (R173C) and A-I (S228C).

Figure 2. CCK-8 assay after treating with different rHDLs
The different columns represent the viability of cells treated with corresponding concentrations of rHDL as shown on the right (μg/ml).

Figure 3. Photomicrographs of representative morphological changes of LPS-induced RAW264.7 cells
(SP ×100) Control: without any treatment, LPS, rHDL_WT, rHDL_74, rHDL_228 and rHDL_M represent the groups that were treated by LPS, LPS + rHDL_WT, LPS + rHDL_74, LPS + rHDL_228 and rHDL_M respectively.
Figure 4. TNF-α and IL-6 levels at 24 h after LPS treatment
Compared with the LPS group, the rHDL74-treated group showed significantly reduced levels of TNF-α and IL-6. However, rHDL228 exhibited an increase for these factors. *P < 0.05, compared with control group; #P < 0.05, compared with LPS group; &P < 0.05, compared with rHDL74 group. (A) TNF-α level at 24 h after LPS treatment. (B) IL-6 level at 24 h after LPS treatment.

Effect of rHDL on the expression of TNF-α and IL-6
To detect the effect of the rHDLs on LPS-treated cells, we determined the cell culture fluid levels of TNF-α and IL-6 by ELISA. Figure 4A shows that rHDLWT and rHDLM can directly lower the supernatant inflammatory cytokine TNF-α (rHDLWT: 291.86 ± 12.77 ng/ml, rHDLM: 251.319 ± 7.22 ng/ml, P < 0.001) compared with the LPS group (377.43 ± 8.09 ng/ml). Compared with the rHDLWT, the supernatant level of TNF-α in the rHDL74 group (rHDL74: 214.77 ± 14.68 ng/ml, P < 0.01 compared with rHDLWT) was significantly reduced. However, the rHDL228 (399.366 ± 2.23 ng/ml, P < 0.001 compared with control) had a much higher level of TNF-α compared with the control group. As shown in Figure 4B, rHDLWT and rHDLM can directly lower the supernatant inflammatory cytokine IL-6 (rHDLWT: 303.15 ± 9.70 ng/ml, rHDLM: 269.81 ± 6.37 ng/ml, P < 0.001 compared with LPS) compared with the LPS group (657.83 ± 10.3 ng/ml). Compared with rHDLWT, the supernatant level of IL-6 in the rHDL74 group (rHDL74: 260.264 ± 10.07 ng/ml, P = 0.0104 compared with rHDLWT) was significantly decreased. The level of IL-6 with rHDL74 was close to that of the control group (control: 234.647 ± 9.27, P = 0.0669).

Effects of rHDLs on the expression of ATF3
The anti-inflammatory function of HDLs is closely related to ATF3 expression levels. In animal experiments, HDL injection induced high levels of ATF3 expression [14]. To determine whether ATF3 is responsible for the anti-inflammatory effects of rHDLs, we detected the mRNA levels of ATF3 using quantitative real-time RT-PCR (Figure 5A) and examined the protein levels through Western blotting (Figure 5B, C). Fluorescence RT-PCR showed that compared with the LPS group, the expression level of ATF3 in the rHDL74 group was the highest; rHDLWT and rHDLM groups also had increased ATF3 expression levels (P < 0.05). In contrast with other rHDLs, the ATF3 expression level of rHDL228 group was lower than the LPS group. The mRNA expression results were consistent with the results of ELISA and cell morphology analysis, which showed that rHDL74, rHDLM and rHDLWT can reduce the level of inflammation and enhance the expression level of ATF3. To verify whether enhancement occurred at the translation level to increase protein expression, Western blotting was conducted. Compared with the control group, the expression levels of ATF3 in the LPS group and rHDL228 group were significantly reduced and that of the rHDL228 group was lower than the LPS group. The expression levels of rHDLWT and rHDLM were increased, and this difference was significant, with the highest expression level was of rHDL74. The anti-inflammatory effect of the mutant was most significant for rHDL74.

Discussion
Inflammation is a specific mechanism underlying multiple physiological and disease processes [22]. Studies have shown that HDL can inhibit the development and progression of inflammation and antioxidant effects [23]. The key characteristic in the early phase of inflammation in plaques is the involvement of the innate immune system with respect to the macrophage. These cells are activated in the blood vessel wall in response to lipoproteins containing apolipoprotein B, such as low-density lipoproteins (LDL and VLDL) [24]. The cholesterol carried by HDL causes the macrophages to become ‘foam cells’ and inflammatory cytokines are then secreted by macrophage foam cells [14,25,26]. Studies have shown that HDL in macrophages may reduce inflammation, dependent on the transcription
factor ATF3, to activate the anti-inflammatory pathway [27]. In our study, we assessed the anti-inflammatory function of ApoA-I cysteine mutants, and our results showed that the rHDL74 mutant can increase the ATF3 level and exhibit a high anti-inflammatory ability.

ApoA-I is the main component of the HDL, and studies have shown that it plays a major role in cholesterol efflux and anti-atherosclerosis [6,28]. SRC–HDL, CSL-111, CSL-112 and ETC-216 whose main component is ApoA-I or its natural cysteine mutant ApoA-I_M had been used in clinical trials. Previous studies have shown that these HDLs can quickly transport cholesterol and eliminate atherosclerotic plaques [6,29-32]. The drug ETC-216 based on ApoA-I_M was developed by the Esperion Pharmaceutical Company, and clinical research has shown that the drug has a significant anti-atherosclerosis effect [32]. Previously, we have designed and constructed seven cysteine mutants of ApoA-I containing a natural mutant ApoA-I_M i.e. A-I (S52C), A-I (N74C), A-I (K107C), A-I (G129C), A-I (R173C), A-I (K195C), A-I (S228C), and their functions were studied. The 74 and 52 mutants A-I (N74C) and A-I (S52C) and rHDLs: rHDL74 and rHDL52 had significantly increased anti-inflammatory functions compared with wild-type, whereas the 228 mutant rHDL228 exacerbated inflammation [14,20,21,33]. The mechanism of anti-inflammatory action is not clear.

We, therefore, investigated the effects of rHDLs on LPS-stimulated RAW264.7 macrophages and studied the anti-inflammatory abilities of the rHDL. Our study suggested that high-density lipoprotein (HDL) exerted different effects on inflammatory cytokine expression. Cell morphology and ELISA detection showed that rHDL74 had the strongest inhibition of inflammation. Wild-type rHDLWT and rHDLM also inhibited inflammation, but the effect was less than that of rHDL74. Correspondingly, to verify the relationship between expression levels of inflammatory factors and rHDL and ATF3 expression, we observed that the anti-inflammatory effect of rHDL was proportional to the expression level of ATF3. rHDL74 had the strongest inhibition of inflammation and the expression level of ATF3 was also the highest. At the same time, we observed that rHDL228 might have pro-inflammatory effects and we found that the expression level of ATF3 protein and mRNA was the lowest.

ATF3 is the key transcriptional repressor. It can be induced by TLR stimulation and acts by a negative-feedback mechanism to limit excessive production of pro-inflammatory cytokines including TNF, IL-6 and IL-12p40 [17,18]. Although, it is known that HDL can sequester LPS and thereby prevent cellular activation through TLR4 [34], recent evidence indicates that HDL could promote ATF3 expression, leading to down-regulation of TLR-induced inflammatory responses [14]. In the present study, we found that the rHDL74, rHDLWT and rHDLM mutants could enhance
the expression levels of the TLR ATF3 response to negatively regulate inflammation, whereas rHDL-228 may inhibit ATF3 activity and aggravate inflammation. As amino acid mutations may affect protein conformation and cysteine residues can be engaged in disulfide bridges, these artificial single amino acid mutations may play their unique roles in the formation of ApoA-I homodimers, thereby influencing the HDL protein function and HDL-ATF3-TLR pathway. In conclusion, the anti-inflammatory function of rHDL that we constructed was positively correlated with ATF3 expression. How these mechanisms develop in different cell types in vivo and in different microenvironments is the next major focus of our research and for discerning the relationship between HDL and ATF3 and innate immune research in general.

Author contribution
Y.W. designed the experiments. Y.W., Y.W., S.L. and L.H. performed the experiments. S.J., Q.D., S.L., Y.C. and L.H. analysed the data. Y.W. and Y.W. wrote the paper.

Funding
This work was supported by the Key Research and Development Program of Shandong Province [grant number 2016GSF201404]; Shandong Medical and Health Science and Technology Development Plan [grant number 2016WS0264]; National Nature Science [grant number 30900238]; and Qingdao Innovation and Entrepreneurship Leading Talent Project [grant number 13-CX-3].

Competing interests
The authors declare that there are no competing interests associated with the manuscript.

Abbreviations
ApoA-I, apolipoprotein A-I; ATF3, activating transcription factor 3; CCK-8, cell counting kit-8; HDL, high-density lipoprotein; rHDL, recombinant HDL; LPS, lipopolysaccharide; TLR, toll-like receptor; VLDL, very low density lipoprotein.

References
1 Kulinsky, V.I. (2007) Biochemical aspects of inflammation. Biochem. (Mosc.) 72, 595–607
2 Lawrence, T., Willoughby, D.A. and Gilroy, D.W. (2002) Anti-inflammatory lipid mediators and insights into the resolution of inflammation. Nat. Rev. Immunol. 2, 787–795
3 Hanada, T. and Yoshimura, A. (2002) Regulation of cytokine signaling and inflammation. Cytokine Growth Factor Rev. 13, 413–421
4 Madahian, S., Navab, K.D., Pourtabatabaei, N., Seyedali, S., Safar, S., Vazirian, S. et al. (2014) Inflammation, high density lipoprotein and endothelium. Curr. Med. Chem. 21, 2902–2909
5 Baker, J.V., Neuhaus, J., Duprez, D., Cooper, D.A., Hoy, J., Kulzer, L. et al. (2011) Inflammation predicts changes in high-density lipoprotein particles and apolipoprotein A1 following initiation of antiretroviral therapy. AIDS 25, 2133–2142
6 Krause, B.R. and Remaley, A.T. (2013) Reconstituted HDL for the acute treatment of acute coronary syndrome. Curr. Opin. Lipidol. 24, 480–486
7 Fujisawa, N. and Kobayashi, K. (2005) Macrophages in inflammation. Curr. Drug Targets Inflamm. Allergy 4, 281–286
8 Bain, C.C. and Mowat, A.M. (2014) Macrophages in intestinal homeostasis and inflammation. Immunol. Rev. 260, 102–117
9 Kaperonis, E.A., Liapis, C.D., Kakisis, J.D., Dimitroulis, D. and Papavassiliou, V.G. (2006) Inflammation and atherosclerosis. Eur. J. Vasc. Endovasc. Surg. 31, 386–393
10 Li, L., Sapkota, M., Kim, S.W. and Soh, Y. (2015) Herbacetin inhibits inducible nitric oxide synthase via JNK and nuclear factor-κB in LPS-stimulated RAW264.7 cells. Eur. J. Pharmacol. 765, 115–123
11 Mogensen, T.H. (2009) Pathogen recognition and inflammatory signaling in innate immune defenses. Clin. Microbiol. Rev. 22, 240–27
12 Caro, E., Rosenberg, I.M., Brandwein, S.L., Beck, P.L., Reinecker, H.C. and Podolsky, D.K. (2000) Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. J. Immunol. 164, 966–972
13 Ahn, C.B., Jung, W.K., Park, S.J., Kim, Y.T., Kim, W.S. and Je, J.Y. (2016) Gallic acid-g-Chitosan modulates inflammatory responses in LPS-stimulated RAW264.7 cells via NF-κB, AP-1, and MAPK pathways. Inflammation 39, 366–374
14 De Nardo, D., Labzin, L.I., Kono, H., Seki, R., Schmidt, S.V., Beyer, M. et al. (2014) High-density lipoprotein mediates anti-inflammatory reprogramming of macrophages via the transcriptional regulator ATF3. Nat. Immunol. 15, 152–160
15 O’Neill, L.A., Sheedy, F.J. and McCoy, C.E. (2011) MicroRNAs: the fine-tuners of Toll-like receptor signalling. Nat. Rev. Immunol. 11, 163–175
16 Hai, T., Wolford, C.C. and Chang, Y.S. (2010) ATF3, a hub of the cellular adaptive-response network, in the pathogenesis of diseases: is modulation of inflammation a unifying component? Gene Expr. 15, 1–11
17 Whitmore, M.M., Ippraguirre, A., Kubelka, L., Weninger, W., Hai, T. and Williams, B.R. (2007) Negative regulation of TLR-signaling pathways by activating transcription factor-3. J. Immunol. 179, 3622–3630
18 Gilchrist, M., Thorsson, V., Li, B., Rust, A.G., Korb, M., Roach, J.C. et al. (2006) Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. Nature 441, 173–178
19 De Nardo, D. (2015) Toll-like receptors: activation, signalling and transcriptional modulation. *Cytokine* **74**, 181–189

20 Wang, Y., Zhu, X., Wu, G., Shen, L. and Chen, B. (2008) Effect of lipid-bound apoA-I cysteine mutants on lipopolysaccharide-induced endotoxemia in mice. *J. Lipid Res.* **49**, 1640–1645

21 Zhu, X., Wu, G., Zeng, W., Xue, H. and Chen, B. (2005) Cysteine mutants of human apolipoprotein A-I: a study of secondary structural and functional properties. *J. Lipid Res.* **46**, 1303–1311

22 Medzhitov, R. (2008) Origin and physiological roles of inflammation. *Nature* **454**, 428–435

23 Kamari, Y., Shaish, A., Shemesh, S., Vax, E., Grosskopf, I., Dotan, S. et al. (2011) Reduced atherosclerosis and inflammatory cytokines in apolipoprotein-E-deficient mice lacking bone marrow-derived interleukin-1alpha. *Biochem. Biophys. Res. Commun.* **405**, 197–203

24 Tabas, I., Williams, K.J. and Boren, J. (2007) Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. *Circulation* **116**, 1832–1844

25 Nguyen, C.T., Kim, E.H., Luong, T.T., Pyo, S. and Rhee, D.K. (2015) TLR4 mediates pneumolysin-induced ATF3 expression through the JNK/p38 pathway in *Streptococcus pneumoniae*-infected RAW264.7 cells. *Mol. Cells* **38**, 58–64

26 Medzhitov, R. and Horng, T. (2009) Transcriptional control of the inflammatory response. *Nat. Rev. Immunol.* **9**, 692–703

27 Lai, P.F., Cheng, C.F., Lin, H., Tseng, T.L., Chen, H.H. and Chen, S.H. (2013) ATF3 protects against LPS-Induced inflammation in mice via inhibiting HMGB1 expression. *Evid. Based Complement. Alternat. Med.* **2013**, 716481

28 Sirtori, C.R. (2004) ApoA-I Milano and regression of atherosclerosis. *JAMA* **291**, 1319; author reply 1320

29 Gursky, O., Jones, M.K., Mei, X., Segrest, J.P. and Atkinson, D. (2013) Structural basis for distinct functions of the naturally occurring Cys mutants of human apolipoprotein A-I. *J. Lipid Res.* **54**, 3244–3257

30 Ibanez, B., Vilahur, G., Cimmino, G., Speidl, W.S., Piner, A., Choi, B.G. et al. (2008) Rapid change in plaque size, composition, and molecular footprint after recombinant apolipoprotein A-I Milano (ETC-216) administration: magnetic resonance imaging study in an experimental model of atherosclerosis. *J. Am. Coll. Cardiol.* **51**, 1104–1109

31 Parolini, C., Marchesi, M., Lorenzon, P., Castano, M., Balconi, E., Miragoli, L. et al. (2008) Dose-related effects of repeated ETC-216 (recombinant apolipoprotein A-I Milano/1-palmitoyl-2-oleoyl phosphatidylcholine complexes) administrations on rabbit lipid-rich soft plaques: in vivo assessment by intravascular ultrasound and magnetic resonance imaging. *J. Am. Coll. Cardiol.* **51**, 1098–1103

32 Nicholls, S.J., Uno, K., Kataoka, Y. and Nissen, S.E. (2011) ETC-216 for coronary artery disease. *Expert. Opin. Biol. Ther.* **11**, 387–394

33 Zhang, X., Zhu, X. and Chen, B. (2010) Inhibition of collar-induced carotid atherosclerosis by recombinant apoA-I cysteine mutants in apoE-deficient mice. *J. Lipid Res.* **51**, 3434–3442

34 Levine, D.M., Parker, T.S., Donnelly, T.M., Walsh, A. and Rubin, A.L. (1993) *In vivo* protection against endotoxin by plasma high density lipoprotein. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 12040–12044