RESEARCH ARTICLE

TNFα alter cholesterol metabolism in human macrophages via PKC-θ-dependent pathway

A Zhi Sha Ma1,2†, Qian Zhang1† and Zhi Yuan Song1*

Abstract

Background: Studies have shown that inflammation promoted atherosclerotic progression; however, it remains unclear whether inflammation promoted atherosclerotic progression properties by altering cholesterol metabolism in human macrophages. In the present study, we evaluated a potential mechanism of inflammation on atherogenic effects. We evaluated the ability of TNFα to affect Reverse cholesterol transport (RCT) and cholesterol uptake and its mechanism(s) of action in human macrophages.

Results: We initially determined the potential effects of TNFα on cholesterol efflux in the human macrophages. We also determined alterations in mRNA and protein levels of ABCA1, ABCG1, LXRα, CD-36, SR-A in human macrophages using quantitative real-time polymerase chain reaction (PCR) and Western immunoblot analyses. The cholesterol efflux rate and protein expression of ABCA1, ABCG1, LXRα, CD-36, SR-A were quantified in human macrophages under PKC-θ inhibition using PKC-θ siRNA. Our results showed that TNFα inhibited the rate of cholesterol efflux and down-regulation the expression levels of ABCA1, ABCG1 and LXRα and up-regulation the expression levels of CD-36, SR-A in human macrophages; PKC-θ inhibition by PKC-θ siRNA attenuated the effect of TNFα on ABCA1, ABCG1, LXRα, SR-A, CD-36 expression.

Conclusions: Our results suggest TNFα alter cholesterol metabolism in human macrophages through the inhibition of Reverse cholesterol transport and enhancing cholesterol uptake via PKC-θ-dependent pathway, implicating a potential mechanism of inflammation on atherogenic effects.

Keywords: Reverse cholesterol transport, Cholesterol efflux, TNFα

Background

Atherosclerosis is a chronic inflammatory disease characterized by early and prolonged presence of macrophages within the innermost layer of the arterial wall [1]. Arterial-wall macrophages uncontrolled uptake of modified low-density lipoprotein (LDL) and caused foam-cell formation, resulting in the development of atherosclerosis [2]. Three main mechanisms are known to be anti-atherosclerotic: Endothelial progenitor cells, plaque neovascularization and reverse cholesterol transport (RCT). Recently, RCT has received special attention in the literature. The entire macrophage-specific RCT, regulated predominantly by the macrophage transporters ABCA1, ABCG1 [3], is a process to remove excess cholesterol from peripheral macrophages to HDL and transport them to the liver for subsequent elimination as bile acids and neutral steroids [4].

Tumour necrosis factor alpha (TNFα) is a pro-inflammatory cytokine involved in initiating inflammatory responses [5]. Human studies and animal models implicate TNFα in atherosclerotic plaque formation [6]. TNFα is produced primarily by macrophages, but also by a broad variety of cell types including lymphoid cells, mast cells, endothelial cells; it promotes macrophage activation, lymphocyte trafficking and homeostasis, cellular infiltration of the plaque and stimulates production of other cytokines which increase plaque instability leading to thrombus formation [7]. Cross-sectional data indicate positive associations between TNFα and degree of atherosclerosis or level of prevalent CVD [8]. However, it is still unclear that TNFα promote atherosclerotic plaque formation whether related to alter cholesterol metabolism in human macrophages. Therefore, we set out to investigate the effect of TNFα treatment on Reverse cholesterol
transport function and the expression of CD-36, SR-A in human macrophages.

Methods
Materials
TNFa (Hoffmann-La Roche Ltd, Basel, Switzerland), Total RNA extraction reagent RNAiso Plus, PrimeScript RT reagent kit, SYBR-Green PCR kit (Takara, Japan). Western immunoblot reagents were purchased from the Beyotime Institute of Biotechnology (China). All other chemicals were of the best grade available from commercial sources.

Cell culture
Human peripheral blood monocytes were isolated from three healthy volunteers (Lin Wang, Peng Zhou, Rui Ma, Department of Cardiology, Southwest Hospital, The Third Military Medical University, Chongqing, China) using Ficoll/Hypaque gradient centrifugation. The pooled monocytes from the volunteers were incubated in DMEM supplemented with 10% autologous serum for 10 days so that they would differentiate into macrophages. This research carried out on humans was in compliance with the Helsinki Declaration.

Cellular cholesterol efflux experiments
Human macrophages were cultured as indicated above. Human macrophages were then labeled with [3H]-cholesterol (0.3 μCi/mL) in serum-free DMEM medium containing 50 μg/ml ox-LDL and 0.2% bovine serum albumin (BSA) for 24 h. The cells were washed twice with phosphate buffered saline (PBS) and incubated in 2 mL of DMEM media containing 0.2% BSA without or with TNFa at 5 ng/ml, 10 ng/ml for 48 h. The media were then replaced with DMEM containing 0.2% BSA in the presence of lipid-free apoA-I (10 μg/mL) or HDL (50 μg/mL) for 24 h. Efflux media was obtained at the times designated and centrifuged to remove floating cells. Monolayers were washed twice with media was obtained at the times designated and cultured for 96 hours. Cells were harvested and protein extracts prepared in accordance with manufacturer’s instructions. Western immunoblot analyses [12% SDS-PAGE; 30 μg protein per lane] were then performed using rabbit anti-ABCA1, anti-ABCG1, anti-LXRα, anti-CX36, anti-SR-A and anti-GAPDH (Abcam, USA)-specific antibodies. Proteins were visualized using enhanced chemiluminescence.

Transfections for LXRα silencing and screening effective PKC-θ siRNA fragments
The PKC-θ-siRNA specific for mouse PKC-θ and non-silencing (control) siRNAs were synthesised by Shanghai Genechem (Shanghai, China). Human macrophages (1×10⁹ cells/well) were transfected using Lipofectamine 2000 (Invitrogen). Following 48 h transfection, the second siRNA fragment of PKC-θ suppressed expression of the PKC-θ gene by 75% according to RT-PCR analyses. The oligonucleotide sequences used to construct siRNA used in this study were: 5'-ATC TCA ATG AGT ATG T-3' for PKC-θ (PKC-θ-siRNA).

LXRα siRNA transfection and western immunoblot analyses
Human macrophages were grown in culture flasks at a density of 1×10⁵/ml for 12 hours, washed with PBS, and TNFa at 10 ng/ml, 10 ng/ml was added to the DMEM culture media containing 10% human serum. The negative control was added to the first culture flask and PKC-θ-siRNA was added to the remaining flasks and cultured for 96 hours. Cells were harvested and protein extracts were prepared in accordance with the...
manufacturer's instructions. The proteins were then subjected to Western immunoblot analyses [12% SDS-PAGE; 60 μg protein per lane] using rabbit anti-ABCA1, anti-ABCG1, anti-LXRx, anti-CD36, anti-SR-A and anti-GAPDH (Abcam, USA)-specific antibodies. Proteins were visualized using enhanced chemiluminescence methods.

Statistical analyses
Data are expressed as mean ± standard error of the mean (SEM). Results were analyzed using ANOVA with SPSS 13.0 software. P < 0.05 was considered statistically significant. All experiments were performed in at least triplicate.

Results

TNFa inhibited cholesterol efflux in human macrophages
We initially examined the effect of TNFa on cholesterol efflux in human macrophages using liquid scintillation counting assays. Figure 1(A, B) shows that TNFa at 5 ng/ml, 10 ng/ml obvious inhibited apoA-I- and HDL-mediated cholesterol efflux in human macrophages compared with the negative control.

TNFa down-regulated the mRNA and protein expression of ABCA1, ABCG1, LXRx in human macrophages
To further explore the effects of TNFa on human macrophage-mediated RCT, we examined the effects of TNFa on ABCA1, ABCG1 and LXRx expression in human macrophages using real-time quantitative PCR and Western immunoblot analyses. Our results showed that TNFa at 5 ng/ml, 10 ng/ml significantly decreased the mRNA (Figure 2) and protein (Figure 3A, B) expression levels of ABCA1, ABCG1, LXRx.

Figure 1 TNFa inhibit cholesterol efflux from human macrophages. After human macrophages were labeled with 3H-cholesterol, the indicated doses of either TNFa or the vehicle were added to the cultures, which were then incubated in the presence of either 10 μg/mL human apoA-I (A) or 50 μg/mL of human HDL (B) for 24 h. Cholesterol efflux was determined as described in Methods. Data are presented as mean ± SEM, *P < 0.05 vs control.

TNFa up-regulated the mRNA and protein expression of CD-36, SR-a in human macrophages
We then examined the effects of TNFa on the expression levels of CD-36, SR-A expression in human macrophages. As demonstrated in Figure 2 that the mRNA expression levels of CD-36, SR-A were significantly up-regulated in human macrophages treated with TNFa at 5 ng/ml, 10 ng/ml compared with the negative control. The protein expression of CD-36, SR-A were also significantly up-regulated (Figure 3A, C) in human macrophages treated with TNFa at 5 ng/ml, 10 ng/ml compared with the negative control.

PKC-θ inhibition by siRNA significantly attenuated the effect of TNFa on ABCA1, ABCG1, LXRx, CD-36, SR-a expression
We next investigated the effect of treatment with PKC-θ siRNA on TNFa (10 ng/ml)-induced expression of ABCA1, ABCG1, LXRx, CD-36, SR-A. PKC-θ siRNA suppressed the expression of PKC-θ gene (Figure 4D) by 76% according to Western immunoblot analyses. As showed in Figure 4(A, B, C ) that PKC-θ siRNA significantly attenuated the effect of TNFa on ABCA1, ABCG1, LXRx, CD-36, SR-A expression.

Discussion

TNFa plays a central role in inflammation; it induces the expression of other proinflammatory molecules, chemotactic cytokines and adhesion factors [9]. In vivo and in vitro studies have shown that high levels of TNFa lead to exacerbation of the inflammatory response [10]. This, together with its potent immunomodulator activities, has been suggested as important to the pathogenesis of diseases such as asthma and Atherosclerosis [11]. In this study, we evaluated a potential mechanism of TNFa on atherogenic effects. As shown in Figure 1A and B, TNFa at 5 ng/ml, 10 ng/ml significantly inhibited both apoA-I- and HDL-mediated cholesterol efflux from human macrophages. Next, we determined the mRNA and protein levels of ABCA1, ABCG1, LXRx in order to elucidate the molecular mechanisms underlying the inhibited cholesterol efflux in human macrophages due to TNFa.

A previous study noted that ATP binding cassette transporters (ABCA1, ABCG1) and LXRx are the best characterized cellular transporters/receptor involved in macrophage RCT [12]. The initial comparison of macrophage-specific RCT between ABCA1-deficient and wild-type mice showed direct evidence that ABCA1 deficiency reduces RCT from macrophages to feces in vivo [13]. ABCG1 is another ABC transporter that is able to load more cholesterol onto mature HDLs from the peripheral tissues and is important in allowing...
macrophages to efflux arterial wall cholesterol, eventually preventing atherosclerotic vascular disease [14].

Our results showed that TNFα at 5 ng/ml, 10 ng/ml significantly decreased the mRNA expression levels of ABCA1, ABCG1 and LXRα compared with the control group (Figure 2), resulting in significantly down-regulated protein expression levels in human macrophages (Figure 3A, B). This indicates that TNFα inhibit reverse cholesterol transport in human macrophages via ABCA1 and ABCG1-mediated pathways.

Interestingly, we also found that the mRNA (Figure 2) and protein (Figure 3A, C) expression levels of pattern-recognition scavenger receptors CD-36 and SR-A, were significantly up-regulated in human macrophages treated with TNFα at 5 ng/ml, 10 ng/ml. SR-A belongs to scavenger receptor A family, and has been implicated in atherosclerosis [15]. CD36 is a member of the class B scavenger receptor family and its activation has been implicated in foam cell formation. It appears that SR-A and CD36 account for greater than 90% of the lipid accumulation in macrophages exposed to oxidized LDL [16]. So it suggested that TNFα can promote cholesterol uptake by enhancing the expression of CD-36 and SR-A in human macrophages.

Protein kinase C (PKC) comprises a family of serine/threonine kinases that are involved in the regulation of many cellular responses, including proliferation, differentiation, stress responses, and lipid metabolism [17]. PKC isozymes have been classified in three subfamilies: conventional (cPKCs α, βI, βII and γ), novel (nPKCs δ, ε, θ and η) and atypical (aPKCs λ, μ, ζ). Conventional PKCs are regulated by diacylglycerol (DAG), phosphatidylserine and calcium, whereas novel PKCs are calcium-independent, but regulated by DAG and phosphatidylserine, and atypical PKC isoforms are regulated by phosphatidylserine, but calcium, DAG- and TPA-independent [18]. Previous study showed that PKC activation mediates production of granulocyte/macrophage colony-stimulating factor (GM-CSF), which plays a priming role in Ox-LDL-induced macrophage proliferation [19]. Protein kinase C-β and δ mediate cholesterol accumulation in PMA-activated macrophages and another report shows that stably overexpressed a dominant-negative of PKC-α inhibits LPS-induced iNOS expression.

**Figure 2** Human macrophages were treated either with or without TNFα at 5 ng/ml, 10 ng/ml for 24 h. (A) ABCA1, (B) ABCG1, (C) LXRα, (D) CD-36, (E) SR-A gene expression were measured using real-time quantitative PCR. Similar results were obtained in three independent experiments. Data are mean ± SEM. *P < 0.05 vs control.
in RAW 264.7 macrophages [20,21]. HIV-1 Tat protein induces TNF-α and IL-10 production via a PKC-βII and δ isozymes dependent way in human macrophages [22]. 6-Gingerol inhibits ROS and iNOS in lipopolysaccharide-stimulated mouse macrophages, through the suppression of PKC-α and NF-κB pathways, [23].

PKC-θ mediates the critical T cell receptor (TCR) signals selectively required for T cell activation in vivo [24]. PKC-θ plays a crucial role in the activation of various transcription factors such as NF-kB, AP-1 and nuclear factor of activated T cells (NFAT) [25,26]. Mature PKC-θ−/− T cells failed to proliferate and produce interleukin 2 (IL-2) upon TCR stimulation due to defective activation of NF-kB and AP1 [27]. Mice deficient in other isoforms of PKC do not have a defect in T cell activation, thereby reinforcing the importance of PKC-θ in T cell activation [28]. Considering the multiple functions of PKC-θ in T cell activation, it would be interesting to examine whether PKC-θ plays a role in the effect of TNFa on ABCA1, ABCG1, LXRα and SR-A protein expression were measured using Western immunoblotting techniques. Similar results were obtained in three independent experiments. Data are mean ± SEM. *P < 0.05 vs control.
ABCG1, LXRα and up-regulation the expression of CD-36, SR-A in human macrophages, we utilized PKC-θ-siRNA to inhibit PKC-θ. The results show that PKC-θ-siRNA significantly attenuated the effect of TNFα on ABCA1, ABCG1, LXRα, SR-A, CD-36 expression compared with the negative control (Figure 4A, B, C). This phenomenon confirmed that PKC-θ is involved in the effect of TNFα on ABCA1, ABCG1, LXRα, SR-A, CD-36 expression in human macrophages. These data suggest that PKC-θ activity is involved in the effect of TNFα on ABCA1, ABCG1, LXRα, SR-A, CD-36 expression in human macrophages, and the isotype-selective inhibitor to PKC-θ may suppress atherosclerotic progression.

**Conclusion**

These results suggest that TNFα promote atherosclerotic progression by altering cholesterol metabolism though inhibiting Reverse cholesterol transport and enhancing cholesterol uptake in human macrophages via PKC-θ-dependent pathway.
Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
AZSM designed the experiments and wrote the manuscript; AZSM carried out cell culture, the molecular genetic studies, the immunosays and PKC-B inhibition using PKC-B siRNA. QZ performed cellular cholesterol efflux experiments and statistical analyses. ZYS participated in study design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Author details
1Department of Cardiology, Southwest Hospital, The Third Military Medical University, Chongqing, China. 2Department of Cardiology, The Fifth Hospital of PLA, Yinchuan, China.

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References
1. Luos AJ. Atherosclerosis. Nature 2000, 407:233–241.
2. Bobryshev YV. Monocyte recruitment and foam cell formation in atherosclerosis. Micron 2006, 37:209–222.
3. Joan Carles EG, Noemí R, Josep J, Francisco B-V: Monocyte recruitment and foam cell formation in atherosclerosis. Circ Res 2005, 96:1221–1233.
4. Lewis GF, Rader DJ: Tumour necrosis factor-alpha: the role of this multifunctional cytokine in asthma. Immunol Cell Biol 2003, 81:132–140.
5. Mckellar GE, McCarey DW, Sattar N, McNibrnes IE: Role for TNF in atherosclerosis lessons from autoimmune disease. Nat Rev Cardiol 2009, 6:410–417.
6. Jessup W, Gelissen IC, Gaus K, Kriethardis L: Roles of ATP binding cassette transporters A1 and G1, scavenger receptor Bl and membrane lipid domains in cholesterol export from macrophages. Curr Opin Lipidol 2006, 17:247–257.
7. Calpe-Berdiel L, Rottan N, Palomer X, Ribas V, Blanco-Yaca F, Escolà-Gil JC: Direct evidence in vivo of impaired macrophage-specific reverse cholesterol transport in ATP-binding cassette transporter A1-deficient mice. Biochim Biophys Acta 2005, 1738:5–9.
8. Michael L: Fitzgerald, zahedhi mujawar, Norimasa tamehiro. ABC transporters, atherosclerosis and inflammation. Atherosclerosis 2010, 211:361–370.
9. Petera M, Barazszezkia A, Dudasak J, et: Highly upregulated expression of CD36 and MSR1 in circulating monocytes of patients with acute coronary syndromes. Protein J 2012, 31:511–518.
10. Kunjathoor W, Febbraio M, Podrez EA, et: Scavenger receptors class a-II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. J Biol Chem 2002, 277:49962–49988.
11. Stapleton CM, Joung Hyuck J, Yong-Sik K, Grace L, Reynolds A, Panettieri J, Anton M: Jetteana, induction of ANGPTL4 expression in human airway smooth muscle cells by PMA through activation of PKC and MAPK pathways. Exp Cell Res 2010, 316:507–516.
12. Way KJ, Eva C, George L. King. Identification of PKC-isofrom-specific biological actions using pharmacological approaches. TIPS 2001, 21:181–187.
13. Biwa T, Hakamata H, Sakai M, et al: Induction of murine macrophage growth by oxidized low-density lipoprotein is mediated by granulocyte macrophage colony-stimulating factor. J Biol Chem 1998, 273:2830–13.
14. Ma H-T, Lin W-W, Zhao B, Wen-Tung W, Huang W, Li Y: Nancy L, Jones, Howard S. Kruth : Protein kinase C-β and -δ isoforms mediate cholesterol accumulation in PMA-activated macrophages. Biochem Biophys Res Commun 2006, 349:214–220.
15. Denis A, Chano F, Tremblay P, St-Pierre Y, Descoteaux: Protein kinase C-alpha modulates lipopolysaccharide-induced functions in a murine macrophage cell line. Journal of Biological Chemistry 1998, 273:32787–32792.
16. Leghmari K, Contreiras X, Moureaux C, Bahraoui E: HIV-1 Tat protein induces TNF-α and IL-10 production by human macrophages: Differential implication of PKC-bil and -d isoforms and MAP kinases ERK1/2 and p38. Cell Immunol 2008, 254:46–55.
17. T. Y, Lee K-C, Chen S-Y, Chang H-H: 6-Gingerol inhibits ROS and iNOS through the suppression of PKC-α and NF-κB pathways in lipopolysaccharide-stimulated mouse macrophages. Biochem Biophys Res Commun 2009, 382:134–139.
18. Attman A, Isakov N, Baier G: Protein kinase c-teta: a new essential superstar on the T-cell stage. Immunol Today 2000, 21:567–573.
19. Sun Z, Arendt CW, Ellmeier W, Schaeffer EM, Sunshine MJ, Gandhi L, et al: PKC-theta is required for TCR-induced NF-κB activation in mature but not immature T lymphocytes. Nature 2003, 404:402–407.
20. Pellestor C, Koller K, Gruber T, Tabrizi NG, Lutz C, Haly K, et: Protein kinase C, theta affects Ca2C mobilization and NFAT cell activation in primary mouse T cells. J. Exp. Med. 2003, 197:1525–1535.
21. Marland B, Kopf M: Toll-like receptors: paving the path to T cell-driven autoimmunity? Curr Opin Immunol 2007, 19:611–614.
22. Baier G: The PKC gene module: molecular biosystematics to resolve its T cell functions. Immunol Rev 2003, 192:64–79.

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