ATF3 Inhibits Tenascin-C-induced Foam Cell Formation in LPS-Stimulated THP-1 Macrophages by Suppressing TLR-4

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Aim: Efficiently inhibiting the formation of macrophage foam cells is indispensable for mitigating and treating atherosclerosis. Tenascin-C (TN-C) plays an important role in promoting atherosclerosis; therefore, it is essential to inhibit foam cell formation associated with TN-C for controlling atherosclerosis. Activating transcription factor 3 (ATF3) is one of the factors involved in regulating the complex process of foam cell formation. This study aimed to explore the role of TN-C and ATF3 in LPS-stimulated THP-1-derived macrophages.

Methods: RT-PCR was used for evaluating the expression of TN-C in LPS-stimulated THP-1 macrophages. Further, exogenous TN-C was introduced and incubated with cultured THP-1 macrophages to confirm the effect of TN-C on LPS-stimulated THP-1 macrophages. ATF3-modified THP-1 macrophages were constructed and verified by western blot. High performance liquid chromatography (HPLC) assay and Oil red O staining were applied for detecting cholesteryl ester/total cholesterol (CE/TC) and lipid formation in THP-1 macrophages.

Results: The expression of TN-C was determined to be upregulated in LPS-stimulated THP-1 macrophages in a dose- and time-dependent manner. HPLC assay and Oil red O staining confirmed that TN-C can enhance LPS-induced THP-1 macrophage foam cell formation. Moreover, ATF3 can act as a negative regulatory factor for inhibiting TN-C-induced foam cell formation by suppressing TLR-4 in LPS-stimulated THP-1 macrophages.

Conclusion: ATF3 can inhibit TN-C-induced foam cell formation in LPS-stimulated THP-1 macrophages by suppressing TLR-4. It may be a useful molecular target to control TN-C-induced foam cell formation in atherosclerosis.


Key words: ATF3, Tenascin-C, Foam cell formation, LPS, THP-1 Macrophages

Introduction

Atherosclerosis is a chronic inflammatory disease of the arterial wall characterized by the formation of fibrotic plaques in the major arteries and accumulation of lipid-loaded macrophages in arterial walls¹, ². It is the primary cause of coronary heart disease (CHD) and stroke and ultimately a major contributing factor to death worldwide³. Foam cells are an important component of atherosclerotic plaque and believed to be a key driver of atherogenesis⁴. Plaque macrophages are key innate immune cells, without the ability to properly process modified lipoproteins involved in the pathogenesis of atherosclerosis⁴, ⁵. An oxidizing environment developed by the activation of macrophages that become loaded with oxidized low-density lipoprotein (LDL) and accumulate lipid bodies in macrophages in the arterial intima results in foam cell formation⁷, ⁸. Hence, efficiently inhibiting the formation

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of macrophage foam cells is indispensable for mitigating and treating atherosclerosis.

Tenascin-C (TN-C) is a glycoprotein belonging to the tenascin family of four multimeric extracellular matrix (ECM) proteins. It has been demonstrated that TN-C plays a vital role in many diseases, such as tumor angiogenesis and metastasis. Specifically, TN-C expression in adult arteries is coincident with sites of vascular disease, which has been linked to the development and complications of intimal hyperplasia, myocardial infarction, pulmonary artery hypertension, heart failure, cardiac and arterial injuries, and atherosclerosis. TN-C is an endogenous ligand of toll-like receptor-4 (TLR-4), a key signaling molecule associated with chronic inflammatory conditions, and the levels of both molecules increase in unstable human atheroma. Further, TN-C and OX40 ligand (OX40L) have been shown to be involved in the pathogenesis of atherosclerosis. Altogether, numerous findings have revealed that TN-C may play a role in promoting atherosclerosis, such as its expression at sites of plaque rupture, its involvement in neovascularization, and its ability to influence vascular smooth muscle cell (VSMC) phenotype and pro-inflammatory cytokine/matrix metalloproteinase (MMP) production. Further, TN-C produced by oxidized low density lipoprotein (oxLDL)-pretreated macrophages increases foam cell formation through TLR-4 and the scavenger receptor CD36. For atherosclerosis control, it is essential to inhibit foam cell formation.

Activating transcription factor 3 (ATF3) is a member of the ATF/cyclic AMP response element-binding (ATF/CREB) family of transcription factors. ATF3 participates in cellular processes for adapting to extracellular or intracellular changes, in which it transduces signals from various receptors to activate or repress gene expression. Previous studies have suggested that ATF3 is able to negatively regulate the transcription of pro-inflammatory cytokines and pro-inflammatory cytokine production. ATF3 functions in a negative feedback loop response for suppressing TLR-mediated cytokine expression, particularly as a negative regulator of TLR-4. Research also indicates that ATF3 is one of the factors involved in regulating the complex process of foam cell formation.

According to the abovementioned findings, both TN-C and ATF3 are associated with foam cell formation during atherosclerosis. However, the association between these two in foam cell formation remains unclear. In this study, we explored the role of TN-C in LPS-stimulated THP-1 macrophages and found that TN-C can enhance lipopolysaccharide (LPS)-induced THP-1 macrophage foam cell formation. Further, ATF3-modified THP-1 macrophages were constructed and used for exploring the role of ATF3 in TN-C-induced foam cell formation in LPS-stimulated THP-1 macrophages. We found that ATF3 is a negative regulatory factor in this process, just like in its negative transcriptional regulation of pro-inflammatory cytokines and pro-inflammatory cytokine production. This is possibly another way by which ATF3 reveals its negative regulatory function against disease or inflammatory responses. ATF3 may be a target molecule for inhibiting TN-C-induced foam cell formation and can be further researched toward therapy for or control of the progress of atherosclerosis.

Aim

The present study aimed to identify the role of ATF3 in TN-C-induced foam cell formation in THP-1 macrophages.

Materials and Methods

Materials

RIPA lysis buffer, polyvinylidene difluoride (PVDF) membranes, Tween20, hematoxylin, paraformaldehyde, RNase inhibitor, skimmed milk powder, Oil red O solution, BCA kit, trichloroacetic acid, stigmasterol, isopropanol, acetonitrile, IFN- and lipopolysaccharide (LPS) were purchased from Sigma (St. Louis, MO, USA). OxLDL was purchased from Bio-Medical Technologies (Stoughton, MA, USA). DNase, the RT-PCR kit, and the Qiagen RNeasy kit were purchased from Qiagen (Hilden, Germany). Rabbit anti-human ATF3, TN-C, IL-6, IL-8, TLR-4 antibodies, anti- therapies, anti-influenza IgM-horse radish peroxidase (HRP) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Pyrobest DNA polymerase and avian myeloblastosis virus (AMV) reverse transcriptase were purchased from Takara Biotechnology (Stoughton, CA, USA). The SYBR® Green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA, USA).

Human THP-1 Macrophage Cultures and Treatment

The human monocytic leukemia cell line THP-1
was purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured at 37°C with 5% CO2 in RPMI 1640 medium supplemented with 10% FBS, 2-mM L-glutamine, 100-U/ mL penicillin, and 100-μg/mL streptomycin in an incubator. Cells were routinely passaged every 2–3 days. For differentiation to adherent THP-1-derived macrophages, cells were plated at 1×10^6 cells/well in 24-well plates in 100-nM phorbol 12-myristate 13-acetate (PMA) for 48 h. Before stimulation with the indicated dose (0, 30, 60, 90 ng/mL) for 12 h and times (0, 6, 12, 18, 24 h) with 60 ng/mL of LPS, cells were incubated with oxLDL (50 μg/mL) for 4 h.

Expression of TN-C mRNA in LPS-stimulated THP-1 Macrophages Assessed by qRT-PCR

After stimulation with LPS for the indicated times (0, 6, 12, 18, 24 h) with 60 ng/mL LPS and dose (0, 30, 60, 90 ng/mL) for 12 h, total RNA from the THP-1 macrophages was extracted with TRIzol and purified with the Qiagen RNeasy kit according to the manufacturer’s instructions, followed by treatment with DNase. TN-C mRNA was analyzed by one-step quantitative RT-PCR using the SYBR® Green PCR Master Mix and RT-PCR kit, according to the manufacturer’s instructions, on an ABI Prism 7700 Sequence Detection System. Primers were synthesized by the Shanghai Sangon Biological Engineering and Technology Service (Shanghai, China). The sequences of the primers were 5′-GAG ACA TCT GTG GAA GTG GA-3′ (sense) and 5′-GCT GTC ACC TTC ACC GGT CC-3′ (antisense) for TN-C. TN-C expression was quantified in association with β-actin expression using the following primers: 5′-CTC CAT CCT GGC CTC GCT GT-3′ (sense) and 5′-GCT ACT CAG TGT CAG GCT TC-3′ (antisense) for TN-C. TN-C expression was quantified in association with β-actin expression using the following primers: 5′-CTC CAT CCT GGC CTC GCT GT-3′ (sense) and 5′-GCT ACT CAG TGT CAG GCT TC-3′ (antisense).

Western Blot

ATF3-transfected THP-1 macrophages were lysed with RIPA buffer. The protein concentration was determined by the BCA assay. The protein lysates (50 μg/lane) were separated by SDS-PAGE on 10% gels and transferred onto PVDF membranes. After blocking with 0.5% skimmed milk powder in 1× PBS–Tween20, the target proteins were probed with 1:2000 rabbit anti-human ATF3 or anti-β-actin antibody overnight at 4°C. After washing for three times (15 min each time) with 1× PBS–Tween20, the membranes were incubated with goat anti-rabbit IgG-HRP antibodies (1:4000 in PBS) at room temperature for 1 h and washed again. The reactive bands were detected by ECL according to the manufacturer’s protocol, and the relative levels of ATF3 to β-actin were analyzed. Same experiments were used for detecting the expression of TN-C, IL-6, IL-8, and TLR-4 in corresponding THP-1 cells; rabbit anti-human TN-C, IL-6, IL-8, TLR-4, respectively, antibodies were used as primary antibody.
Expression and Purification of Endotoxin-free Recombinant TN-C

Total RNA was extracted from cultured THP-1 macrophages with TRIzol reagent. Further, 5 μg of total RNA was reverse-transcribed into cDNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase. The TN-C gene was amplified by PCR with 5'-AGA CAC CTA GCC AAT CCA ACC-3' as the forward primer and 5'-AAA TCC TGT TTT CTC TGG GC -3' as the reverse primer, which contained BamH I and Hind III restriction sites, respectively. The TN-C DNA fragment was verified by gene sequencing (BGI, Beijing, China), and the purified TN-C was then subcloned into pEGFP-N1, and subsequently, their recombinant plasmid pEGFP-N1-TN-C was transfected into HEK-293A cells (ATCC, Rockville, MD, USA). Further, the cells were harvested and lysed in PBS containing 0.2% Triton X-100. The complexes were sonicated and then centrifuged. The supernatant was collected, and the fusion protein was purified using His–Bind resin (Ni²⁺-resin) according to standard protocols. The purified proteins were analyzed by SDS-PAGE and further verified by western blot analysis.

Effect of rTN-C on THP-1 Macrophages and Oil Red O Staining

Cultured THP-1 macrophages (2.5 × 10⁵ cells/well) were seeded onto 6-well plates in RPMI 1640 medium with 10% FBS. Then, these cells were treated with or without recombinant human TN-C (rTN-C, 1 μM). After incubation for 24 h, the LPS-stimulated THP-1 macrophages and LPS-stimulated ATF3-transfected THP-1 macrophages (LPS: 60 ng/mL for 12 h) were washed with PBS three times, prior to incubation with oxLDL (50 μg/mL) for 4 h, followed by fixation with 4% paraformaldehyde/PBS for 15 min. Further, the cells were rinsed with ddH₂O. The neutral lipids were stained using 0.5% Oil red O solution for 10 min at 37°C. Hematoxylin was introduced to label the cell nuclei. Following this, the Oil red O-stained lipids in THP-1 macrophage-derived foam cells were morphologically evaluated by microscopy.

Lipid Assay by High Performance Liquid Chromatography (HPLC)

The cellular lipids (total cholesterol, TC; cholesterol ester, CE) were analyzed as previously described. Briefly, cells were rinsed with PBS three times and then lysed with 0.9% NaOH solution followed by homogenization in an ice bath for 10 s. The protein concentration was determined with a BCA assay kit, following which, an equal volume of trichloroacetic acid was introduced and centrifuged for 10 min. First, stigmasterol was used to construct a standard curve, and the extraction procedure was repeated. Following this, the samples were re-suspended in 100 μL of isopropanol-acetonitrile (v/v, 20:80) for 5 min. Finally, all samples were placed on the Agilent 1100 series HPLC (Wilmington, DE).

Statistical Analysis

Data were analyzed by Student's t test. For RT-PCR results, *p < 0.05 and **p < 0.01 versus the control group (0 h or 0 ng/mL LPS). For western blot results, *p indicates p < 0.05 versus normal and pCMV THP-1 macrophages. For the HPLC assay, difference between groups was considered statistically significant if **p < 0.01 or *p < 0.05.

Results

LPS-induced TN-C Expression in THP-1 Macrophages

For assessing the expression levels of TN-C in LPS-stimulated THP-1 macrophages, qRT-PCR and western blot were performed. As shown in Fig. 1A, an obvious increase in TN-C mRNA levels was observed at 12 h post-LPS stimulation. Simultaneously, after treatment with increased times of 60 ng/mL LPS stimulation, TN-C mRNA was gradually upregulated, indicating that LPS triggered a time-dependent increase in the expression of TN-C mRNA in THP-1 macrophages. After exposure to various doses of LPS for 12 h, TN-C mRNA levels increased by approximately 1.6-, 2.0-, and 2.7-fold over control in the 30 ng/mL-, 60 ng/mL-, and 90 ng/mL-treated groups, respectively (Fig. 1B).

By western blot, the protein level of TN-C in 60 ng/mL LPS-treated THP-1 macrophages was significantly higher than naive and LPS-untreated THP-1 macrophages (p < 0.05, Figs. 1C and D). These results confirmed that LPS could induce the expression of TN-C in THP-1 macrophages in a dose- and time-dependent manner.

Expression of ATF3 in THP-1 Macrophages

In transfection experiments, western blot and intracellular flow cytometry were performed for confirming the transfection of ATF3 into THP-1 macrophages. As shown in Figs. 2A and 2B, levels of ATF3 were significantly higher in ATF3-modified THP-1 macrophages compared with those in control THP-1 macrophages and empty pCMV vector-treated THP-1 macrophages. Moreover, there was no difference in ATF3 expression between control THP-1 macrophages
Exogenous TN-C Stimulates Foam Cell Formation

Previous reports have confirmed that LPS can enhance the ability of THP-1 macrophages to become foam cells\(^7\), and the abovementioned result indicates that LPS could induce the expression of TN-C in THP-1 macrophages in a dose- and time-dependent manner. In order to explore the function of TN-C in LPS-stimulated THP-1 macrophages, exogenous TN-C (rTN-C) was added to the cultured THP-1 macrophages. HPLC assay and Oil red O staining were then performed. As shown in Fig. 3A, exogenous TN-C prominently enhanced LPS-induced lipid deposition, and the ratio of CE/TC increased from 42.64% to 67.29%. The absolute contents of CE and TC for each group were shown in Fig. 3C. Furthermore, Oil
In Fig. 4A, we summarize that ATF3 remarkably decreased the TN-C-induced CE/TC ratio in LPS-stimulated THP-1 macrophages. The absolute contents of CE and TC for each group were shown in Fig. 4C. The same result was observed for foam cell formation (Fig. 4B). Together, these results suggest that exogenous TN-C enhanced LPS-induced foam cell formation, which was reverse inhibited by ATF3.

Inhibition of ATF3 on TN-C-induced Foam Cell Formation is Mediated by TLR-4

As TN-C accelerates foam cell formation by increasing TLR-4[12] and ATF3 negatively regulates TLR-4[14], we speculated that ATF3 inhibited TN-C-induced foam cell formation by negative regulating TLR-4. Through western blot, Fig. 5A reveals that TLR-4 was upregulated in rTN-C-treated LPS-stimu-
inflammatory disease, starting with the accumulation of lipoproteins, lipids, and immune cells in the arterial wall. Atherosclerosis and its complications rank as the leading cause of death, representing nearly 29% of mortalities worldwide. It has been widely demonstrated that lipid-laden foam cell formation and inflammation in vessel walls are key characteristics of early stage atherosclerosis. Hence, it is crucial to investigate the underlying mechanism of foam cell formation for atherosclerosis control or therapy. This research has identified a novel role of ATF3 in TN-C-induced foam cell formation of LPS-stimulated THP-1 macrophages and has demonstrated that ATF3 inhibits this process.

TN-C is a matricellular glycoprotein expressed during embryonic development, regeneration, wound lated THP-1 macrophages, while TLR-4 reversely decreased in rTN-C-treated ATF3 transfected LPS-stimulated THP-1 macrophages. However, the levels of CD36, the major scavenger receptor for oxidized LDL, displayed no significant change among the groups. When anti-TLR-4 antibody (5 μg/mL) was added into the medium, the inhibition of ATF3 on TN-C-induced foam cell formation was rescued (Fig. 5B). The abovementioned results indicated that ATF3 inhibited TN-C-induced foam cell formation by suppressing TLR-4.

Discussion

Atherosclerosis constitutes the single most crucial contributor of cardiovascular diseases. It is a chronic inflammatory disease, starting with the accumulation of lipoproteins, lipids, and immune cells in the arterial wall. Atherosclerosis and its complications rank as the leading cause of death, representing nearly 29% of mortalities worldwide. It has been widely demonstrated that lipid-laden foam cell formation and inflammation in vessel walls are key characteristics of early stage atherosclerosis. Hence, it is crucial to investigate the underlying mechanism of foam cell formation for atherosclerosis control or therapy. This research has identified a novel role of ATF3 in TN-C-induced foam cell formation of LPS-stimulated THP-1 macrophages and has demonstrated that ATF3 inhibits this process.

TN-C is a matricellular glycoprotein expressed during embryonic development, regeneration, wound
TN-C was upregulated in LPS-stimulated THP-1 macrophages in a dose- and time-dependent manner. In order to confirm the effect of elevated TN-C on LPS-stimulated THP-1 macrophages (LPS: 60 ng/mL for 12 h), the authors incubated THP-1 macrophages with rTN-C (1 μM). For each group, cells were prior exposed to oxLDL (50 μg/mL) for 4 h.

The exact role of TN-C in foam cell formation must be elucidated in order to collate evidence of it being associated with atherosclerosis progression. Through RT-PCR, we found that the expression of TN-C was upregulated in LPS-stimulated THP-1 macrophages in a dose- and time-dependent manner. In order to confirm the effect of elevated TN-C on LPS-stimulated THP-1 macrophages, exogenous TN-C was incubated with cultured THP-1 macrophages. Further, foam cell formation was detected by HPLC assay and Oil red O staining. We found that TN-C can enhance LPS-stimulated THP-1 macrophage foam cell formation, which promotes the progression of atherosclerosis and suppresses its control.

ATF3 functions in a negative feedback loop response to suppress TLR-mediated cytokine expression, particularly as a negative regulator of TLR-4. Meanwhile, TLR-4 has been found to bind to a domain of TN-C in order to transmit multiple signals. This aroused our interest in the role of ATF3 in TN-C-induced foam cell formation in LPS-stimulated macrophages. Therefore, ATF3-modified THP-1 mac-
cell formation through TLR-4 and scavenger receptor CD36. According to the negative regulation role of ATF3 in TLR-4 and the above findings, we inferred that ATF3 inhibits TN-C-induced foam cell formation in LPS-stimulated THP-1 macrophages by negatively regulating TLR-4. Through western blot and Oil red O staining, we verified our assumption. We conclude that ATF3 inhibits TN-C-induced foam cell formation in LPS-stimulated THP-1 macrophages by suppressing TLR-4. ATF3 may be a valuable molecular target for therapy for atherosclerosis, which needs to be confirmed by additional studies.

A previous study has identified a novel role of TN-C in platelet physiology and suggested that it plays an important role in atherothrombosis. Further, Liu R et al. has demonstrated that TN-C can stimulate foam cell formation, and this can be inhibited by a TLR-4-blocking antibody or CD36 gene silencing. They identified that TN-C increases foam cell formation through TLR-4 and scavenger receptor CD36. According to the negative regulation role of ATF3 in TLR-4 and the above findings, we inferred that ATF3 inhibits TN-C-induced foam cell formation in LPS-stimulated THP-1 macrophages by negatively regulating TLR-4. Through western blot and Oil red O staining, we verified our assumption. We conclude that ATF3 inhibits TN-C-induced foam cell formation in LPS-stimulated THP-1 macrophages by suppressing TLR-4. ATF3 may be a valuable molecular target for therapy for atherosclerosis, which needs to be confirmed by additional studies.
Conclusion

ATF3 can act as a negative regulatory factor for inhibiting TN-C-induced foam cell formation in LPS-stimulated THP-1 macrophages by suppressing TLR-4.

Acknowledgments

None.

Conflicts of Interest

The authors declare no conflicts of interest or disclosures.

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