ABCA1 Knockdown Bidirectionally Regulates Pam3CSK4-Induced Inflammatory Response in Mouse Macrophages Postprint

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Abstract

Objective: To investigate the effect of ABCA1 knockdown on Pam3CSK4-induced inflammatory response in macrophages. Methods Stable ABCA1 knockdown cell lines were established in murine monocytic macrophage RAW264.7 cells, and an inflammatory cell model was constructed by stimulating these cells with the TLR2 ligand Pam3CSK4. Changes in the transcriptional expression of related pro-inflammatory and anti-inflammatory cytokines were examined in this model. Results Upon Pam3CSK4 stimulation, the stable ABCA1 knockdown RAW264.7 cell line exhibited significant upregulation of IL-1 , TNF- , and IL-6 expression ($P<0.01$), while the transcriptional repressor cAMP-dependent transcription factor 3 (ATF3) was also significantly upregulated ($P<0.01$). Concurrently, other members of the ATF protein family, including ATF1, ATF2, ATF4, and ATF5, showed no significant changes at the transcriptional level. Conclusion In RAW264.7 macrophages, ABCA1 knockdown significantly upregulated the expression of both Pam3CSK4-induced pro-inflammatory cytokines (IL-1 , TNF- , and IL-6) and the anti-inflammatory factor ATF3. This suggests that its effect on inflammatory response may not be a unidirectional pro-inflammatory action, but rather involves bidirectional regulation. Furthermore, the mechanism by which ABCA1 participates in upregulating ATF3 expression may differ from the upstream regulatory mechanisms of other ATF protein family members.

Full Text

Bidirectional Regulation of Pam3CSK4-Induced Inflammatory Response by ATP-Binding Cassette Transporter A1 Knockdown in Mouse Mononuclear Macrophages In Vitro

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Abstract

Objective: To investigate the regulatory effect of ATP-binding cassette transporter A1 (ABCA1) knockdown on inflammatory response induced by Pam3CSK4 in mouse mononuclear macrophage RAW264.7 cells.

Methods: A mouse mononuclear macrophage RAW264.7 cell line with stable ABCA1 knockdown was constructed and stimulated with Toll-like receptor 2 (TLR2) ligand Pam3CSK4 to establish an inflammatory cell model. Changes in the transcriptional levels of relevant pro-inflammatory and anti-inflammatory cytokines were then examined in this model.

Results: In ABCA1-knockdown RAW264.7 cells, Pam3CSK4 stimulation significantly upregulated the expression of IL-1 , TNF- , and IL-6 ($P<0.01$), while also significantly increasing the expression of the transcriptional repressor cAMP-dependent transcription factor 3 (ATF3) ($P<0.01$). Meanwhile, the transcription levels of other ATF family members—ATF1, ATF2, ATF4, and ATF5—showed no significant changes.

Conclusion: In macrophage RAW264.7 cells, ABCA1 knockdown not only significantly upregulates Pam3CSK4-induced expression of pro-inflammatory factors IL-1 , TNF- , and IL-6, but also markedly enhances the expression of the anti-inflammatory factor ATF3. This suggests that ABCA1' s effect on inflammatory responses may not be unidirectional pro-inflammatory, but rather involves bidirectional regulation. The mechanism by which ABCA1 participates in upregulating ATF3 expression may also differ from the upstream regulatory mechanisms of other ATF protein family members.

Keywords: ATP-binding cassette transporter A1; Toll-like receptor 2; activating transcription factor 3; Pam3CSK4

Introduction

ATP-binding cassette transporter A1 (ABCA1) is a transmembrane protein that plays a central role in mediating reverse cholesterol transport. By directly binding to apolipoproteins ApoA-I and ApoE, ABCA1 facilitates cholesterol transfer to apolipoprotein components, and its deficiency can block most reverse cholesterol transport pathways. Recent studies have shown that ABCA1 can suppress inflammatory responses through HDL-mediated reverse cholesterol transport and participates in inhibitory regulation of inflammation via multiple mechanisms involving cellular cholesterol and phospholipid transport and formation of cell surface lipid microdomains. Under ABCA1-deficient conditions, stimulation of mouse peritoneal macrophages with ligands for TLR2, TLR3, and TLR4 significantly upregulates the expression of related inflammatory cytokines and chemokines.

Activating transcription factor 3 (ATF3) is a member of the activating transcription factor/cAMP response element-binding protein (ATF/CREB) transcription factor family. Through its leucine zipper domain, ATF3 forms homodimers or heterodimers with other transcription factors containing the same domain, binding to promoter regions of inflammation-related factors to inhibit their transcriptional activation and regulate the expression of multiple inflammation-related genes, making it an important inflammatory suppressor. ATF3 maintains low expression levels during quiescent periods but is upregulated under conditions such as endoplasmic reticulum stress, where it exerts anti-inflammatory effects. Some studies have proposed that high-cholesterol feeding can upregulate ATF3 expression in mice and activate ATF3-dependent immune suppression in T helper cells. However, whether ABCA1, which suppresses inflammation through multiple mechanisms including cholesterol transport and cell surface lipid microdomain formation, participates in the regulation of the inhibitory transcription factor ATF3 remains unclear. To examine whether ABCA1 is involved in the transcriptional regulation of ATF3 during inflammatory modulation, we constructed an ABCA1 knockdown cell line in mouse mononuclear macrophage RAW264.7 cells, established an inflammatory response cell model using the TLR2 ligand Pam3CSK4, and examined expression changes of pro-inflammatory cytokines IL-1 , TNF- , and IL-6, as well as ATF3 and its family members, in the ABCA1-knockdown RAW264.7 recombinant cell line.

Materials and Methods

1.1 Materials

The MSH030800-7-CU6 shRNA plasmid was constructed by GeneCopoeia. Fugene HD transfection reagent was purchased from Promega. RPMI 1640 medium was from Hyclone, and fetal bovine serum was from BI. The RAW264.7 mouse mononuclear macrophage cell line was obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences. Mouse monoclonal anti-ABCA1 antibody was from Abcam, rabbit polyclonal anti-ATF3 antibody from Santa

Cruz Biotechnology, and rabbit monoclonal anti- -actin antibody from Cell Signaling Technology. Goat anti-mouse IgG and goat anti-rabbit IgG were from Xintai Biotechnology. Western and IP cell lysis buffer and SDS-PAGE gel preparation kits were from Beyotime. TriPure Isolation Reagent and FastStart Universal SYBR Green Master (ROX) were from Roche, and the First Strand cDNA Synthesis Kit was from Thermo Fisher Scientific. All other reagents were analytical grade.

1.2 Methods

1.2.1 Construction of ABCA1 Knockdown Recombinant Cell Line We queried the NCBI database and selected siRNA sequence Pr006106100: sense sequence (21 bp) GGATGTATAATGAGCAGTATT and antisense sequence (21 bp) TACTGCTCATTATACATCCTT. Additionally, we commissioned GeneCopoeia to predict three ABCA1 knockdown sites and design three pairs of backup siRNA sequences. Using these four siRNA sequences and the psi-U6™ shRNA vector from GeneCopoeia as the backbone, we commissioned GeneCopoeia to construct four shRNA vectors, designated ABCA1-sh1, ABCA1-sh2, ABCA1-sh3, and ABCA1-sh4. A control shRNA vector was designated NC.

A vial of RAW264.7 cells was recovered and cultured in 10 mL of RPMI 1640 medium supplemented with 10% fetal bovine serum in a 10 cm diameter dish at 37°C with 5% CO , with passage every other day. Passage 3 RAW264.7 cells were seeded at 2×10 cells per well in a 6-well plate (6 wells total) and cultured overnight. Five plasmids were transfected into five wells: 3.3 g of each plasmid was mixed with 153 L serum-free RPMI 1640 medium, then 13.5 L Fugene HD transfection reagent was added, mixed gently, and incubated for 10-15 minutes. The plasmid mixture was slowly added dropwise to each well (150 L per well), swirled gently on a clean bench, and incubated at 37°C. After 24 hours, the medium was replaced with puromycin (final concentration 3 g/mL) to select for stable ABCA1 knockdown cell lines. One well of untransfected cells served as a blank control. Cells were cultured at 37°C with 5% CO , with medium changes every other day using RPMI 1640 containing 3 g/mL puromycin. After all control well cells died within 15 days, proliferating cells from transfected wells were expanded and frozen. The resulting stable resistant cell lines were designated RAW264.7-ABCA1-NC, RAW264.7-ABCA1-sh1, RAW264.7-ABCA1-sh2, RAW264.7-ABCA1-sh3, and RAW264.7-ABCA1-sh4.

1.2.2 Validation of ABCA1 Knockdown Stable Cell Lines Total RNA was extracted from RAW264.7-ABCA1-NC, RAW264.7-ABCA1-sh1, RAW264.7-ABCA1-sh2, RAW264.7-ABCA1-sh3, and RAW264.7-ABCA1-sh4 stable cell lines using TriPure Isolation Reagent. The five stable cell lines were seeded at 2×10 cells per well in a 6-well plate (3 parallel wells per line, 15 wells total) and cultured in RPMI 1640 medium containing 3 g/mL puromycin for 24 hours. After removing the supernatant, total RNA was extracted to

examine ABCA1 transcription levels.

RAW264.7-ABCA1-NC and RAW264.7-ABCA1-sh1 stable cell lines were seeded at 2×10 cells per well in a 6-well plate (3 parallel wells per line, 6 wells total) and cultured in RPMI 1640 medium containing 3 g/mL puromycin for 24 hours. After removing the supernatant, total protein was extracted to examine ABCA1 protein expression levels.

1.2.3 Pam3CSK4 Stimulation of ABCA1 Knockdown RAW264.7 Cell Lines RAW264.7-ABCA1-NC and RAW264.7-ABCA1-sh1 cells were seeded in 24-well plates at 5×10 cells per well (6 wells per line) and cultured overnight, designated as NC and sh1 groups. Cells were stimulated with Pam3CSK4 at a final concentration of 100 ng/mL (3 wells per group), while control groups received an equal volume of PBS (3 wells per group). After 6 hours of stimulation, total RNA was extracted using the TriPure method, and real-time quantitative PCR was performed to detect transcriptional expression differences of IL-1 , IL-6, TNF- , ATF1, ATF2, ATF3, ATF4, and ATF5.

RAW264.7-ABCA1-NC and RAW264.7-ABCA1-sh1 cells were seeded in 6-well plates at 2×10 cells per well (6 wells per line) and cultured overnight, designated as NC and sh1 groups. Cells were stimulated with Pam3CSK4 at 100 ng/mL (3 wells per group) or PBS control (3 wells per group). After 24 hours, total protein was extracted and Western blotting was performed to detect ATF3 protein expression differences.

1.2.4 Total RNA Extraction Total RNA was extracted using TriPure Isolation Reagent. One milliliter of TriPure was added to each well to lyse cells, which were then transferred to 1.5 mL Eppendorf tubes. Two hundred microliters of chloroform were added to each tube, inverted and mixed thoroughly, incubated for 5 minutes, then centrifuged at 12,000 g for 15 minutes at 4°C. Four hundred fifty microliters of the upper aqueous phase were transferred to new tubes, mixed with 450 L isopropanol, inverted and mixed, then incubated overnight. After centrifugation at 12,000 g for 15 minutes, the supernatant was removed, and the pellet was washed with 1 mL of 75% ethanol, centrifuged again at 12,000 g for 15 minutes. The supernatant was carefully removed, and the pellet was air-dried until ethanol completely evaporated. Ten microliters of DEPC-treated water were added to dissolve the RNA pellet.

1.2.5 cDNA Reverse Transcription After quantification, 5 g of total RNA was used for reverse transcription. The reaction mixture contained 5 g total RNA, 1 L Oligo(dT) primer, and DEPC water to a final volume of 12 L. This mixture was incubated at 65°C for 5 minutes, then placed on ice. Two microliters of dNTP Mix (10 mmol/L), 4 L of 5× Reaction Buffer, 1 L RiboLock RNase Inhibitor (20 U/ L), and 1 L RevertAid M-MuLV RT (200 U/ L) were added on ice for a total reaction volume of 20 L. After gentle mixing and brief

centrifugation, the reaction was incubated at 42°C for 60 minutes, followed by 70°C for 5 minutes to terminate the reaction and obtain total cDNA.

1.2.6 Real-Time Quantitative PCR Real-time quantitative PCR was performed to detect target gene transcription levels. Total cDNA was diluted 20-fold as the PCR template. The amplification mixture contained 4 L cDNA template, 1 L each of forward and reverse primers, and 5 L FastStart Universal SYBR Green Master (ROX). Amplification was performed on a Bio-Rad iQ5 system for 40 cycles to detect ABCA1 transcription levels.

1.2.7 Total Protein Extraction Culture medium supernatant was removed from cell culture wells. Each well was washed with 1 mL PBS, which was carefully aspirated, then another 1 mL PBS was added. Cells were scraped off with a cell scraper, and the cell suspension was transferred to clean Eppendorf tubes. After centrifugation at 3,000 rpm for 15 minutes at 4°C, the supernatant was removed. One hundred microliters of Western and IP cell lysis buffer containing 1 mmol/L PMSF were added to each tube, mixed, and incubated on ice for 30 minutes. After centrifugation at 13,000 rpm for 15 minutes at 4°C, the supernatant was transferred to new tubes. Protein concentration was quantified and diluted to a final concentration of 1.5 g/ L. Protein loading buffer was added, and samples were denatured at 95°C for 10 minutes.

1.2.8 Western Blotting (1) ABCA1 Western Blotting
SDS-PAGE gels were prepared with 5% stacking gel and 6% separating gel. Thirty micrograms of total protein were loaded per well. Electrophoresis was performed at 80 V for 30 minutes, then 120 V for 2.5 hours (total 3 hours). A 0.45 m NC membrane was cut to size, and a "sandwich"structure was assembled in the order: filter paper-NC membrane-gel-filter paper from anode to cathode. Transfer was performed at 320 mA constant current for 2.5 hours in an ice bath. After transfer, the NC membrane was blocked in 5% skim milk-TBST solution with shaking at 80 rpm for 3 hours at room temperature.

Two microliters of mouse anti-ABCA1 monoclonal antibody were diluted 1:1000 in 2 mL of 5% skim milk-TBST and incubated with the membrane overnight. The membrane was washed three times with TBST for 15 minutes each with shaking. One microliter of goat anti-mouse IgG was diluted 1:5000 in 5 mL of 5% skim milk-TBST and incubated with the membrane for 2 hours at room temperature with shaking at 80 rpm. After three 15-minute washes with TBST and one 15-minute wash with TBS, 500 L ECL detection reagent and 500 L reaction buffer were mixed to prepare 1 mL of chemiluminescent working solution, which was applied to the membrane. Images were captured using a gel imaging system with an 8-minute exposure.

(2) Western Blotting for Other Proteins
SDS-PAGE gels were prepared with 5% stacking gel and 10% separating gel. Thirty micrograms of total protein were loaded per well. Electrophoresis was

performed at 80 V for 30 minutes, then 120 V for 2.5 hours (total 3 hours). A 0.22 m PVDF membrane was cut to size, and a "sandwich" structure was assembled: filter paper-PVDF membrane-gel-filter paper. Transfer was performed at 15 V constant voltage for 45 minutes. The membrane was blocked in 5% skim milk-TBST with shaking at 80 rpm for 3 hours at room temperature.

Twenty microliters of rabbit anti-ATF3 polyclonal antibody were diluted 1:150 in 3 mL of 5% skim milk-TBST, and 2 L of rabbit anti- -actin monoclonal antibody were diluted 1:1000 in 2 mL of 5% skim milk-TBST. Membranes were incubated overnight at 4°C, then washed three times with TBST for 15 minutes each with shaking. One microliter of goat anti-rabbit IgG was diluted 1:5000 in 5 mL of 5% skim milk-TBST and incubated with the membrane for 2 hours at room temperature with shaking at 80 rpm. After three 15-minute washes with TBST and one 15-minute wash with TBS, chemiluminescent working solution was applied and images were captured with 2-8 minute exposures.

Results

2.1 Identification of Stable ABCA1 Knockdown Cell Lines

Real-time quantitative PCR results showed that among the four shRNA stable cell lines, RAW264.7-ABCA1-sh1 had the lowest relative ABCA1 expression at the RNA level compared to RAW264.7-ABCA1-NC, meeting the requirements for ABCA1 knockdown [Figure 1: see original paper]A, $P<0.01$). RAW264.7-ABCA1-sh2 also showed significantly reduced ABCA1 expression at the RNA level compared to RAW264.7-ABCA1-NC [Figure 1: see original paper]A, $P<0.05$). We selected the RAW264.7-ABCA1-sh1 stable cell line for further validation of ABCA1 knockdown at the protein level.

Western blotting showed that ABCA1 protein expression was downregulated in RAW264.7-ABCA1-sh1 compared to RAW264.7-ABCA1-NC [Figure 1: see original paper]B. Validation at both RNA and protein levels confirmed that among the four ABCA1 knockdown stable cell lines, RAW264.7-ABCA1-sh1 exhibited effective ABCA1 knockdown and was selected for subsequent experiments.

2.2 Expression Changes of Inflammation-Related Genes After Pam3CSK4 Stimulation in ABCA1 Knockdown Cell Lines

Real-time quantitative PCR analysis revealed that after 6 hours of Pam3CSK4 stimulation, expression of TNF- , IL-1 , and IL-6 was upregulated in RAW264.7-ABCA1-NC cells compared to the PBS-treated group, while the transcriptional repressor ATF3 showed no significant change [Figure 2: see original paper]A-2D). Similarly, after Pam3CSK4 stimulation, RAW264.7-ABCA1-sh1 cells showed significantly greater upregulation of TNF- , IL-1 , and IL-6 compared to RAW264.7-ABCA1-NC cells, and ATF3 expression was also significantly upregulated [Figure 2: see original paper]A-2D, $P<0.05$). Western blotting results showed that ATF3 protein expression was upregulated in RAW264.7-

ABCA1-sh1 cells after 24 hours of Pam3CSK4 stimulation, whereas no change was observed in RAW264.7-ABCA1-NC cells [Figure 2: see original paper]E).

2.3 Expression of Other ATF Family Members After Pam3CSK4 Stimulation in ABCA1 Knockdown Cell Lines

Real-time quantitative PCR analysis showed that after 6 hours of Pam3CSK4 stimulation, ATF1 and ATF2 transcription levels were downregulated in both RAW264.7-ABCA1-NC and RAW264.7-ABCA1-sh1 groups compared to PBS-treated groups. However, no significant differences in ATF1 and ATF2 transcription were observed between the two groups after Pam3CSK4 stimulation (P>0.05, [Figure 3: see original paper]A and 3B). After 6 hours of Pam3CSK4 stimulation, ATF4 and ATF5 transcription levels showed no significant changes compared to PBS-treated groups in either RAW264.7-ABCA1-NC or RAW264.7-ABCA1-sh1 cells [Figure 3: see original paper]C and 3D, P>0.05).

Discussion

ABCA1 can reduce the expression of MyD88-dependent pro-inflammatory cytokines such as TNF- , IL-1 , IL-6, and IL-12p40 through cholesterol transport and modulation of cell surface lipid microdomains. Macrophage-specific deletion of ABCA1 causes massive cholesterol accumulation, upregulates these cytokines, and triggers plaque inflammation during atherosclerosis progression. Correspondingly, other studies have reported that myeloid-specific ABCA1 knockout does not exacerbate inflammation in adipose tissue of high-fat diet-fed, insulin-resistant obese mice. Therefore, the direction of ABCA1' s inflammatory regulation requires further investigation.

In this study, we transfected RAW264.7 cells with shRNA vectors and selected stable resistant cell lines using puromycin. Real-time quantitative PCR and Western blotting confirmed the establishment of a stable ABCA1-knockdown RAW264.7 cell line, designated RAW264.7-ABCA1-sh1. Using this cell line and control RAW264.7-ABCA1-NC cells, we stimulated both groups with Pam3CSK4 and first observed that pro-inflammatory factors IL-1 , IL-6, and TNF- were significantly upregulated in Pam3CSK4-stimulated groups compared to unstimulated PBS controls. In Pam3CSK4-stimulated cells, RAW264.7-ABCA1-sh1 showed greater upregulation of IL-1 , IL-6, and TNF- transcription compared to RAW264.7-ABCA1-NC, indicating that ABCA1 knockdown significantly enhances Pam3CSK4-induced upregulation of inflammation-related cytokines. This is consistent with previous reports that ABCA1 knockdown upregulates inflammatory cytokine expression, validating ABCA1' s important role in inflammation suppression.

De Nardo et al. found that nascent HDL can upregulate ATF3 transcription in mouse macrophages, leading us to hypothesize that ABCA1, as a key receptor for nascent HDL cholesterol reverse cholesterol transport, might participate

in ATF3 transcriptional regulation. To test whether ABCA1 regulates ATF3 transcription, we examined ATF3 and other family members in our ABCA1 knockdown inflammatory cell model. After establishing the stable ABCA1 knockdown RAW264.7 cell line and stimulating with Pam3CSK4, we compared cytokine expression between knockdown and control groups. We found that while RAW264.7-ABCA1-NC cells showed upregulated pro-inflammatory cytokine transcription without significant ATF3 changes after Pam3CSK4 stimulation, RAW264.7-ABCA1-sh1 cells exhibited further enhanced pro-inflammatory cytokine upregulation along with significant ATF3 upregulation. Notably, other ATF family members (ATF1, ATF2, ATF4, and ATF5) showed no significant transcriptional changes in RAW264.7-ABCA1-sh1 cells under Pam3CSK4 stimulation. These results demonstrate that in Pam3CSK4-stimulated RAW264.7 macrophages, ABCA1 knockdown not only enhances pro-inflammatory cytokine expression but also upregulates the inflammatory suppressor ATF3, an effect not observed for other ATF family proteins. This suggests that ABCA1 may bidirectionally regulate Pam3CSK4-induced inflammatory responses, and its mechanism for upregulating ATF3 transcription appears to be specific and distinct from regulatory mechanisms affecting other ATF family members, warranting further investigation.

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Note: Figure translations are in progress. See original paper for figures.