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IFN-γ down-regulates ABCA1 expression by inhibiting LXRα in a JAK/STAT signaling pathway-dependent manner

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ABSTRACT

Interferon gamma (IFN-γ) is an immunomodulatory and anti-microbial cytokine, which has a variety of proatherogenic effects. It has been reported that IFN-γ can down-regulate ABCA1 expression. However, its mechanism is elusive. In the present study, we have investigated the effect of IFN-γ on ABCA1 expression and cholesterol efflux in THP-1 macrophage-derived foam cells. IFN-γ decreased ABCA1 expression at both transcriptional and translational levels in a dose-dependent manner. Cellular cholesterol content was increased while cholesterol efflux was decreased by IFN-γ treatment. Liver X receptor α (LXRα), which can regulate the expression of ABCA1, was also down-regulated by IFN-γ treatment. LXRα-specific activation by LXRα agonist almost compensated the down-regulation of ABCA1 expression by IFN-γ, while siRNA of LXRα led to down-regulation of ABCA1 expression more significantly than IFN-γ. IFN-γ induced phosphorylation of STAT1 and expression of STAT1α in the nucleus, which was inhibited by a JAK inhibitor AG-490. Treatment with STAT1 siRNA further enhanced down-regulation of LXRα mRNA by IFN-γ. Furthermore, AG-490 and STAT1 siRNA almost compensated the effect of IFN-γ on ABCA1 expression and cholesterol efflux. In conclusion, IFN-γ may first down-regulate expression of LXRα through the JAK/STAT1 signaling pathway and then decrease expression of ABCA1 and cholesterol efflux in THP-1 macrophage-derived foam cells. Therefore, our study may be useful in understanding the critical effect of IFN-γ in pathogenesis of atherosclerosis.

1. Introduction

Atherosclerosis is a chronic inflammatory condition, involving enhanced monocyte/endothelial cell interactions. The demonstration of activated T cells and macrophages within the atherosclerotic lesion provides an in situ evidence for the inflammatory components of the disease [1,2]. Activation of macrophages is central to atherosclerosis progression, ranging from the uptake of modified LDL by activated macrophages and the ensuing foam cell formation through elaboration of chemokines, cytokines, proteases and coagulation factors, and it has an important impact on the process of reverse cholesterol transport [3,4]. Central to the process of macrophage activation is the immunomodulatory cytokine IFN-γ, which has been demonstrated to have a variety of proatherogenic effects. IFN-γ has been reported to up-regulate the expression of vascular cell adhesion molecule-1 on endothelial cells [5] and class II antigens on macrophages and smooth muscle cells [6], modulate type A and B scavenger receptors [7–9], increase acyl coenzyme A:cholesterol-*O*-acyltransferase (ACAT) activity [10], decrease apoE secretion [11,12] and (as recently demonstrated) ATP-binding cassette transporter A1 (ABCA1) expression [13]. These in vitro observations are consistent with the previous report that the double knockout (KO) mice, by back-crossing apoE KO mice with IFN-γ receptor KO mice, displayed reduction in lesion size and lipid accumulation [14]. Taken together, the in vitro and in vivo data support the pathological role of IFN-γ in the progression of atherosclerotic disease, and with effects on scavenger receptors and ABCA1, at least a part of these effects could contribute towards inhibition of the reverse cholesterol transport.

Abbreviations: IFN-γ, interferon gamma; ABCA1, ATP-binding cassette transporter A1; JAK, Janus kinase; STAT, signal transducer and activator of transcription; LXRs, liver X receptors; TC, total cholesterol; FC, free cholesterol; CE, cholesterol ester.

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ABCA1 is a key player in reverse cholesterol transport (RCT) and is critical in regulating cellular cholesterol homeostasis [15–18]. A growing body of evidence indicates that ABCA1 not only plays a major role in HDL biogenesis and RCT process, but also has emerged as potential targets for therapies designed to inhibit the development of atherosclerotic vascular disease [19,20]. ABCA1 is regulated both at the transcriptional level via liver and retinoid X receptors and at the post-transcriptional level via changes in trafficking and the turnover rate of ABCA1 protein [16,18]. ABCA1 expression can also be increased by cAMP elevation and in macrophages exposed to transforming growth factor-α [21,22]. However, the down-regulation of ABCA1 expression is less well understood, with IFN-γ recently being reported to decrease cholesterol efflux and ABCA1 expression [13].

Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway is one of the most important cytokine signal transcription pathways [23]. The JAK family consists of four members in mammals, JAK1-3 and TYK2 [24]. While JAK1, JAK2, and TYK2 are expressed in all cell types [25], including human endothelial cells, the expression of JAK3 is restricted to cells of the myeloid and lymphoid lineages [26]. JAK activation is mediated by phosphorylation of specific tyrosine residues [25]. Phosphorylation of tyrosine residues 1007/1008 is a marker of JAK2 activation [27]. JAKs are activated by autophosphorylation via direct association with cell surface receptors [25], such as the interferon (IFN) receptor [28], or through interaction with tyrosine kinases, such as the Src family of kinases [29]. The major action of JAK is to promote gene transcription by activating STAT proteins [30]. To date, seven mammalian STAT proteins have been identified, referred to as STAT1-4, 5A, 5B, and 6 [31]. STAT3 activation can be detected as phosphorylation of tyrosine 705 and serine 727 [32]. Once activated, STAT proteins homo- or heterodimerize and translocate to nucleus, where they activate gene transcription through binding to specific promoter response elements [23]. Most STAT dimmers recognize and bind to members of the gamma-IFN activation sequence (GAS) [33] or the IFN stimulated response element (ISRE) [34] family of enhancers to promote gene transcription. To date, the homodimerized STAT3 has only been shown to have affinity for and bind to the GAS [35,36].

Previous studies have demonstrated that IFN-γ can down-regulate the expression of ABCA1 and decrease the efflux of cholesterol from foam cells. In an attempt to further determine the possible mechanism through which IFN-γ affects ABCA1 expression and cholesterol efflux, we treated THP-1 macrophage-derived foam cells with various items after a 24-h treatment with IFN-γ. A series of assays were conducted and it turned out that IFN-γ could down-regulate ABCA1 expression and cholesterol efflux in THP-1 macrophage-derived foam cells. At the same time, expression of LXRα was also inhibited while pSTAT and nuclear level of STAT1α were increased, which could be compensated by a JAK inhibitor AG-490. Furthermore, AG-490 blocked the effects of IFN-γ towards the expression of ABCA1 and cholesterol efflux. Taken together, our data suggested that IFN-γ might decrease the expression of ABCA1 and cholesterol efflux in THP-1 macrophage-derived foam cells by down-regulating the expression of LXRα, through which course the JAK/STAT signaling pathway was involved.

2. Materials and methods

2.1. Materials

Mouse monoclonal [AB.H10] to ABCA1 (ab18180) (Abcam plc 332 Cambridge Science Park, Cambridge, CB4 0FW, UK), rabbit polyclonal mouse STAT1α p91 antibodies and goat anti-rabbit HRP conjugated polyclonal antibody (Santa Cruz Biotechnology, CA, USA), rabbit polyclonal phospho-STAT1 (Tyr701) antibody (Cell Signaling Technology Inc., Beverly, MA, USA), 22 (*R*)-Hch and tyrphostin AG-490 (Sigma Chemical Co., St Louis, MO, USA), TRIzol Reagent (Invitrogen, 1600 Faraday Ave, Carlsbad, USA), ReverAid^{TM} First Strand cDNA Synthesis Kit (#k1622) (Fermentas, 830 Harrington Court, Burlington, Ontario, Canada), DyNAmoTM SYBR® Green qPCR Kits (Finnzymes, keilaranta 16, 02150 Espoo, Finland), recombinant human γ-interferon (R&D systems, Minneapolis, MN, USA) and immobilon-P transfer membranes (Millipore, 290 Concord Road, Massachusetts, USA) were obtained as indicated.

2.2. Cell culture

Human THP-1 cells were cultured in RPMI-1640 supplemented with 0.1% nonessential amino acids, penicillin (100 U/mL), streptomycin (100 μg/mL) and 20% fetal bovine serum (FBS) at 37 °C in 5% CO_2 at a cell density of 0.2–1.0 × 10^6/mL. After 3–4 days, cells were treated with PMA (160 nmol/L) for 24 h, and then the medium was replaced by serum-free medium containing oxLDL (50 μg/mL) for 48 h to become fully differentiated macrophages before their use in experiments.

2.3. Preparation of cell lysates

Cells were collected following stimulation, lysed in cold buffer containing 20 mM Tris–HCl, pH 8.0, 140 mM NaCl, 10% glycerol (v/v), 1% igepal (v/v), 25 μM nitrophenyl guanidinobenzoate, 10 μM sodium fluoride, 1 mM sodium orthovanadate, 25 μg/mL leupeptin and aprotinin. For pSTAT1 Western immunoblotting, the cells were solubilized in cold lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 2 mM sodium orthovanadate, 80 μM leupeptin, 1 μg/mL aprotinin, 1 mM NaF, 1 μg/mL pepstatin, 2 mM sodiumpyrophosphate, 0.25% sodiumdeoxycholate and 10 μM *N*-octyl-β-D-glucopyranoside). After incubation for 15 min on ice, lysates were centrifuged at 12,000 rpm for 5 min at 4 °C. The protein content of the supernatants was measured by the Coomassie blue method. Otherwise the lysis was performed as described above. The samples were boiled in SDS sample buffer and stored at −20 °C until used.

2.4. Preparation of nuclear extracts

Cell stimulation was terminated by the addition of ice-cold PBS and nuclear extracts were prepared according to the microscale preparation protocol. In brief, sedimented cells were resuspended in 400 μL of cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.0 mM DTT, and 0.5 mM PMSF). After 15 min on ice, 25 μL of 10% igepal (v/v) was added, and the lysate was vortexed for 10 s and centrifuged for 30 s at 12,000 *g*. The supernatant was discarded and the cell pellet was resuspended in 100 μL of cold buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF). Cells were then rocked vigorously at 4 °C for 15 min. Cellular debris was removed by centrifugation at 12,000 rpm for 5 min at 4 °C. The protein content of the supernatant was measured by the Coomassie blue method. The samples were boiled in SDS sample buffer and stored at −20 °C until used.

2.5. Western immunoblotting

Protein (20 μg of lysates or nuclear extracts) was loaded on 8% SDS-polyacrylamide electrophoresis gel and was electrophoresed for 2 h at 100 V in buffer containing 25 mM Tris base, 250 mM glycine and 0.1% SDS. After electrophoresis, the proteins were electrically transferred to the immobilon-P transfer membrane in buffer

containing 25 mM Tris, 192 mM glycine, 20% methanol, and 0.005% SDS. After transfer, the membrane was blocked in TBST (20 mM Tris base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% skimmed milk for 4 h at room temperature. The membrane was incubated with antibodies against STAT1α or pSTAT1 or ABCA1 in the blocking solution at 4 °C overnight. Thereafter the membrane was washed three times with TBST for 30 min, incubated with secondary antibody in the blocking solution for 50 min at room temperature, and washed three times with TBST for 30 min. Immunoreactivity was detected by ECL test. Protein content was calculated by densitometry using Labwords analysis software.

2.6. Transfection of siRNA

The siRNA against LXRα and STAT1α and an irrelevant 21-nucleotide siRNA duplex which was used as a control were purchased from Biology Engineering Corporation in Shanghai, China. THP-1 macrophage-derived foam cells (2×10^6 cells/well) were transfected with the siRNA of LXRα, STAT1α or control, in the absence or presence of appropriate plasmids using Lipofectamine 2000 (Invitrogen). After 4 h incubation, the medium was changed to medium A, and real-time RT-PCR was performed. In comparison to the control siRNA, the siRNA of LXRα and STAT1α suppressed the expression of these proteins by 86% and 77%, respectively, according to Western blot analysis.

2.7. RNA Isolation and real-time quantitative PCR analysis

Total RNA from cells was extracted by using TRIzol reagent in accordance with the manufacture's instructions. Real-time quantitative PCR, using SYBR Green detection chemistry, was performed on Roche light Cycler Run 5.32 real-time PCR system. Primer sequences are shown in Table 1. Melt curve analyses of all real-time PCR products were performed and shown to produce a single DNA duplex. Quantitative measurements were determined using the ΔΔ Ct method and expression of β-actin was used as the internal control.

2.8. Cellular cholesterol efflux experiments

Cells were cultured as indicated above. Then they were labeled with 0.2 μCi/mL [^{3}H]cholesterol. After 72 h, cells were subsequently washed with phosphate-buffered saline (PBS) and incubated overnight in RPMI 1640 medium containing 0.1% (w/v) bovine serum albumin (BSA) to allow equilibration of [^{3}H]cholesterol in all cellular pools. Equilibrated [^{3}H]cholesterol-labeled cells were washed with PBS and incubated in 2 mL of efflux medium containing RPMI 1640 medium and 0.1% BSA with 25 μg/mL human plasma apoA-I. A 150-μL sample of efflux medium was obtained at the times designated and passed through a 0.45-μm filter to remove any floating cells. Monolayers were washed twice in PBS, and cellular lipids were extracted with isopropanol. Medium and cell-associated [^{3}H]cholesterol was then measured by liquid scintillation counting. Percent efflux was calculated by the following equation: (total media counts/(total cellular counts + total media counts)) × 100.

2.9. Phospholipid efflux experiments

Foam cells were induced with IFN-γ for 24 h, and then incubated with 2 μCi/mL of [^{3}H]choline chloride to label the choline-containing phosphatidylcholine and sphingomyelin. 72 h later, cells were subsequently washed with PBS and incubated overnight in RPMI 1640 medium containing 0.1% (w/v) BSA, and after 6 h of incubation with medium containing 10 mg/mL apoA-I, efflux medium was collected, centrifuged to remove cell debris as above, and aliquots were taken for extraction and separated by thin-layer chromatography with the use of silica G plates developed in chloroform/methanol/ammonia (25% [w/v])/water (50:65:5:4 [v/v]). Phosphatidylcholine and sphingomyelin spots were visualized by I2 vapors and identified by comigration with standards. Relative radioactivity was measured by Phosphoscreen and quantified by PhosphorImager (Molecular Dynamics Inc.). Phospholipid efflux was expressed as percent counts in the supernatant vs total for each individual lipid.

2.10. High performance liquid chromatography assays

HPLC analysis was conducted as described previously. Briefly, cells were washed with PBS for three times. The appropriate volume (usually 1 mL) of 0.5% NaCl was added to about 50–200 μg cellular proteins per mL. Cells were sonicated using an ultrasonic processor for 2 min. The protein concentration in cell solution was measured using BCA kit. 0.1 mL aliquot cell solution (containing 5–20 μg protein) was used to measure the free cholesterol, and another aliquot for total cholesterol detection. Free cholesterol was dissolved in isopropanol (1 mg cholesterol/mL) and stored at −20 °C as stock solution. Cholesterol standard calibration solution ranging from 0 to 40 μg of cholesterol per mL was obtained by diluting the cholesterol stock solution in the same cell lysed buffer.

0.1 mL of each sample (cholesterol standard calibration solutions, or cell solutions) was supplemented with 10 μL reaction mixture including 500 mM $MgC1_2$, 500 mM Tris–HCl (pH 7.4), 10 mM dithiothreitol, and 5% NaCl. 0.4 U cholesterol oxidase in 10 μL 0.5% NaCl was added to each tube for free cholesterol determination, or 0.4 U cholesterol oxidase plus 0.4 U of cholesterol esterase for total cholesterol measurement. The total reaction solution in each tube was incubated at 37 °C for 30 min, and 100 μL methanol:ethanol (1:1) was added to stop the reaction. Each solution was kept cold for 30 min to allow protein precipitation, and then centrifuged at 1500 rpm for 10 min at 15 °C 10 μL of supernatant was applied onto a System Chromatographer (PerkinElmer Inc.) including a PerkinElmer series 200 vacuum degasser, a pump, a PerkinElmer series 600 LINK, and a PerkinElmer series 200 UV–vis detector and a Disovery C-18 HLPC column (Supelco Inc.). The column was eluted using isopropanol:*n*-heptane:acetonitrile (35:13:52) at a flow rate of 1 mL/min for 8 min. Absorbance at 216 nm was monitored. Data were analyzed with TotalChrom software from PerkinElmer.

2.11. Statistical analysis

Data are expressed as means ± S.D. Results were analyzed by one-way ANOVA and Student's *t* test, using SPSS 13.0 software.

Table 1
Primer sequences for real-time quantitative PCR

Gene	Forward primer	Reverse primer	Size
ABCA1	5′-GATTGGCTTCAGGATGTCCATGTTGGAA-3′	5′-GTATTTTTGCAAGGCTACCAGTTACATTTGACAA-3′	177 bp
LXRα	5′-AGCGTCCACTCAGAGCAAGT-3′	5′-GGGGACAGAACAGTCATTCG-3′	107 bp

Statistical significance was obtained when P values were less than 0.05.

3. Results

3.1. IFN-γ down-regulates ABCA1 expression in THP-1 macrophage-derived foam cells

We firstly examined the effect of IFN-γ on ABCA1 expression in THP-1 macrophage-derived foam cells by real-time quantitative PCR and Western immunoblotting assays. As shown (Fig. 1A and B), IFN-γ decreased ABCA1 expression at both transcriptional and translational levels in a dose-dependent manner.

ABCA1 is a key player in reverse cholesterol transport and is critical in regulating cellular cholesterol homeostasis. As ABCA1 was down-regulated by IFN-γ, we next examined the effect of IFN-γ on cholesterol content and cholesterol efflux in THP-1 macrophage-derived foam cells by high performance liquid chromatography (Table 2) and liquid scintillation counting assays (Fig. 1C). Cellular cholesterol content was increased while cholesterol efflux was decreased by IFN-γ, suggesting that ABCA1 expression can be down-regulated by IFN-γ in THP-1 macrophage-derived foam cells.

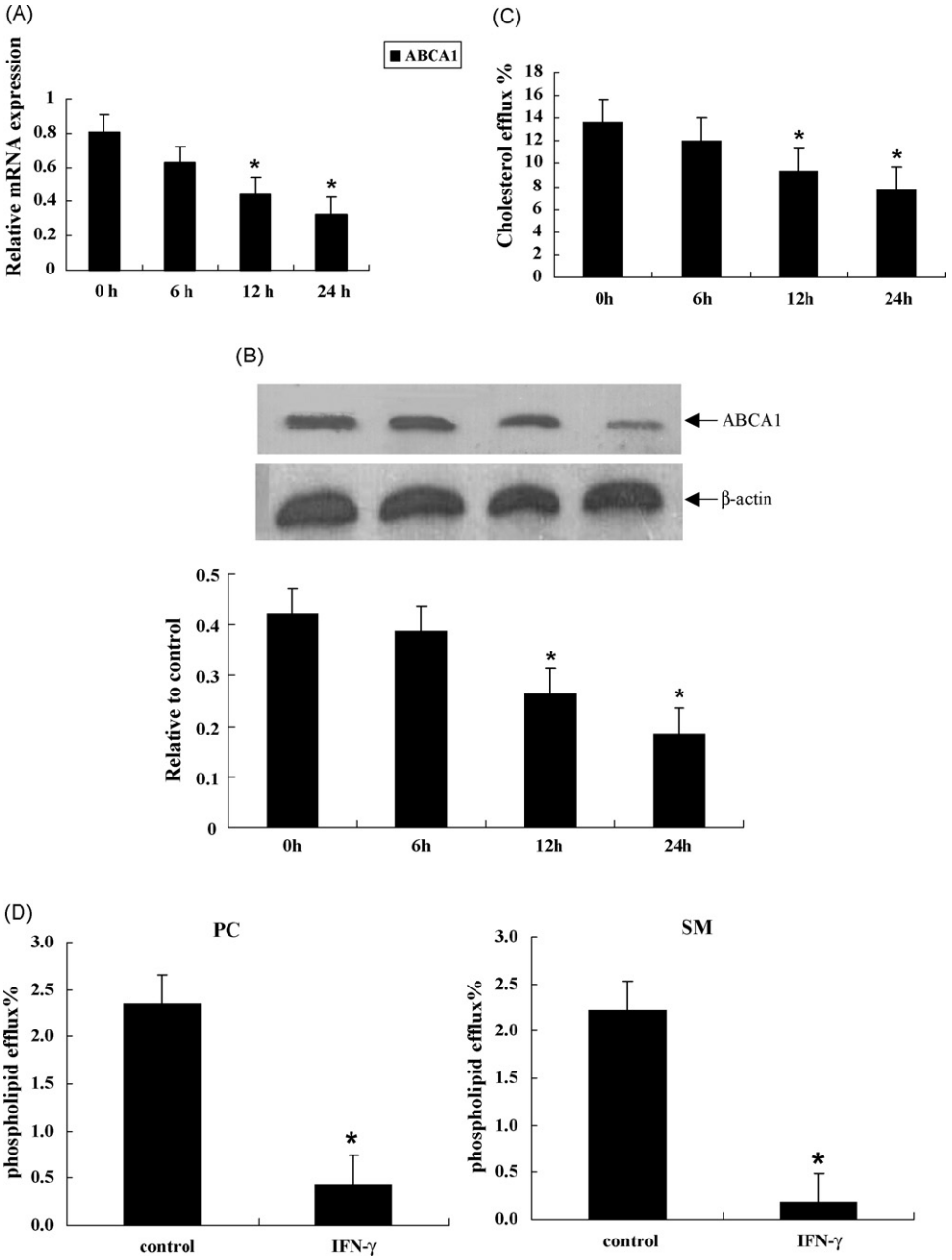

Fig. 1. IFN-γ inhibits ABCA1 expression in THP-1 macrophage-derived foam cells. Cells were treated with IFN-γ (100 μg/L) for 0 h, 6 h, 12 h and 24 h respectively. (A) and (B) ABCA1 mRNA and protein expressions were measured by real-time quantitative PCR and Western immunoblotting assays. (C) Cellular cholesterol efflux was analyzed by liquid scintillation counting assays as shown above. (D) Foam cells were treated with IFN-γ (100 μg/L) for 24 h, and apoA-I-mediated phospholipid efflux was calculated by subtracting the efflux to medium and expressed as the percentage of total cellular and medium phospholipid. All the results are expressed as mean ± S.D. from three independent experiments, each performed in triplicate. $^{*}P < 0.05$ vs vehicle.

The defect in lipidation of apoA-I has been suggested to be a major cause of the impaired ability of apoA-I to stimulate cholesterol efflux from Tangier cells [37,38]. To confirm that this process was also down-regulated by IFN-γ, foam cells were incubated with [^{3}H]choline chloride to label the choline-containing phospholipids, phosphatidylcholine and sphingomyelin. As demonstrated (Fig. 1D), IFN-γ resulted in significant reduction of apoA-I-mediated phosphatidylcholine and sphingomyelin efflux.

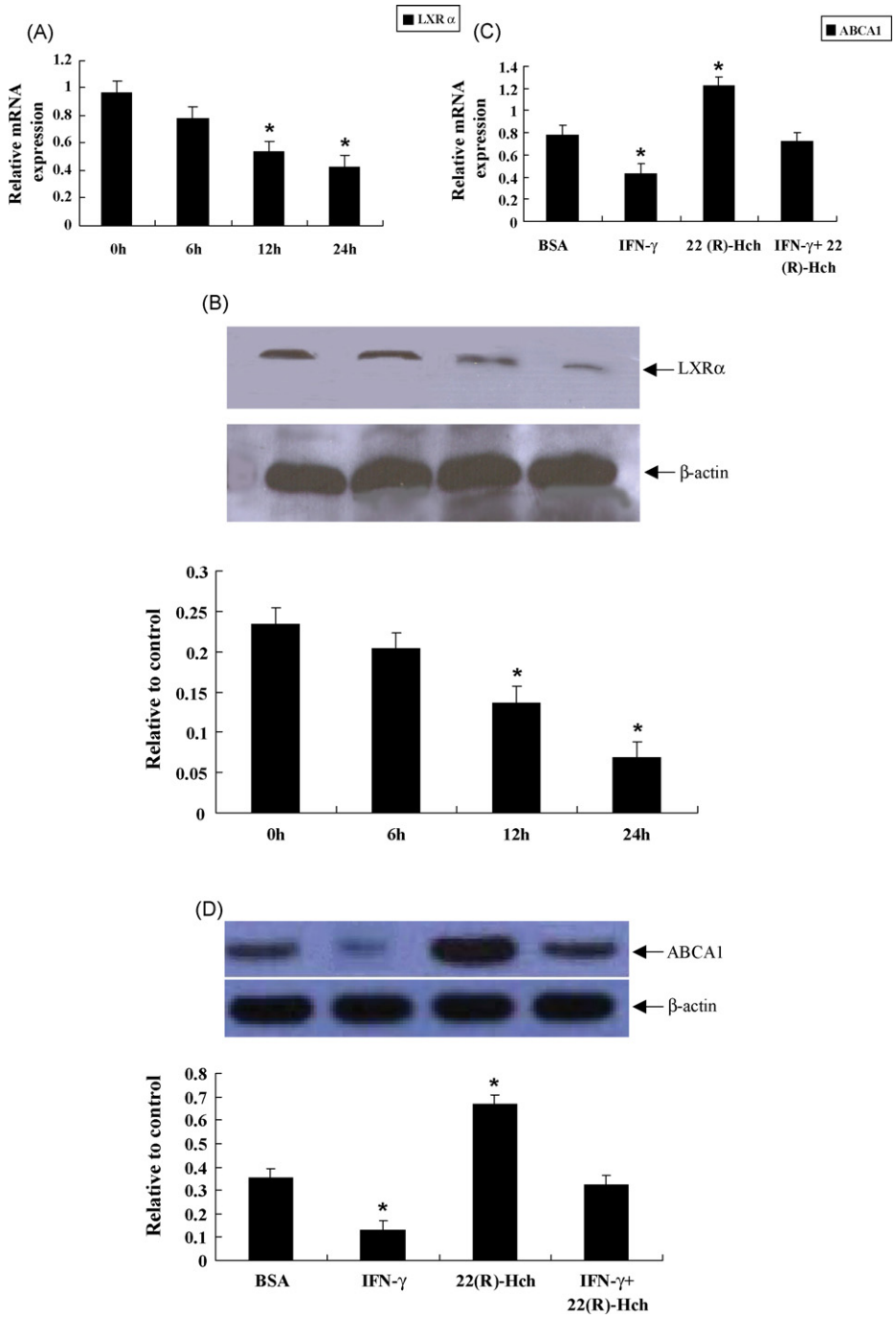

Fig. 2. LXRα is involved in the down-regulation of ABCA1 expression induced by IFN-γ. (A and B) IFN-γ decreased the expression of LXRα at both mRNA and protein levels. Cells were treated with IFN-γ (100 μg/L) for 0 h, 6 h, 12 h and 24 h, respectively. (A) Total RNA was extracted and real-time quantitative PCR was performed to determine the expression of LXRα mRNA. (B) Western immunoblotting assays using antibody against human LXRα and β-actin were conducted. Similar results were obtained in three independent experiments. Data are mean ± S.D. $^*P < 0.05$ vs baseline. (C) and (D) LXRα agonist 22 (*R*)-Hch up-regulated ABCA1 mRNA and protein expressions. THP-1 macrophage-derived foam cells were divided into four groups and treated with BSA or IFN-γ (100 μg/L) and/or 22 (*R*)-Hch for 24 h respectively. Real-time quantitative PCR and Western immunoblotting were performed as described above. Similar results were obtained in three independent experiments. Data are mean ± S.D. $^*P < 0.05$ vs baseline. (E–H) THP-1 macrophage-derived foam cells were transfected with control or LXRα siRNA, and then incubated with IFN-γ (100 μg/L) for 24 h. (E) Protein samples were immunoblotted with anti-LXRα or anti-β-actin antibodies. Data represent three experiments with different cell preparations. (F–H) ABCA1 mRNA and protein expressions were determined using real-time quantitative PCR and Western immunoblotting assays. Cellular cholesterol efflux was analyzed by liquid scintillation counting assays as shown above. Similar results were obtained in three independent experiments. Data are mean ± S.D. $^*P < 0.05$ vs baseline. $^\#P < 0.05$ vs cont and IFN-γ group.

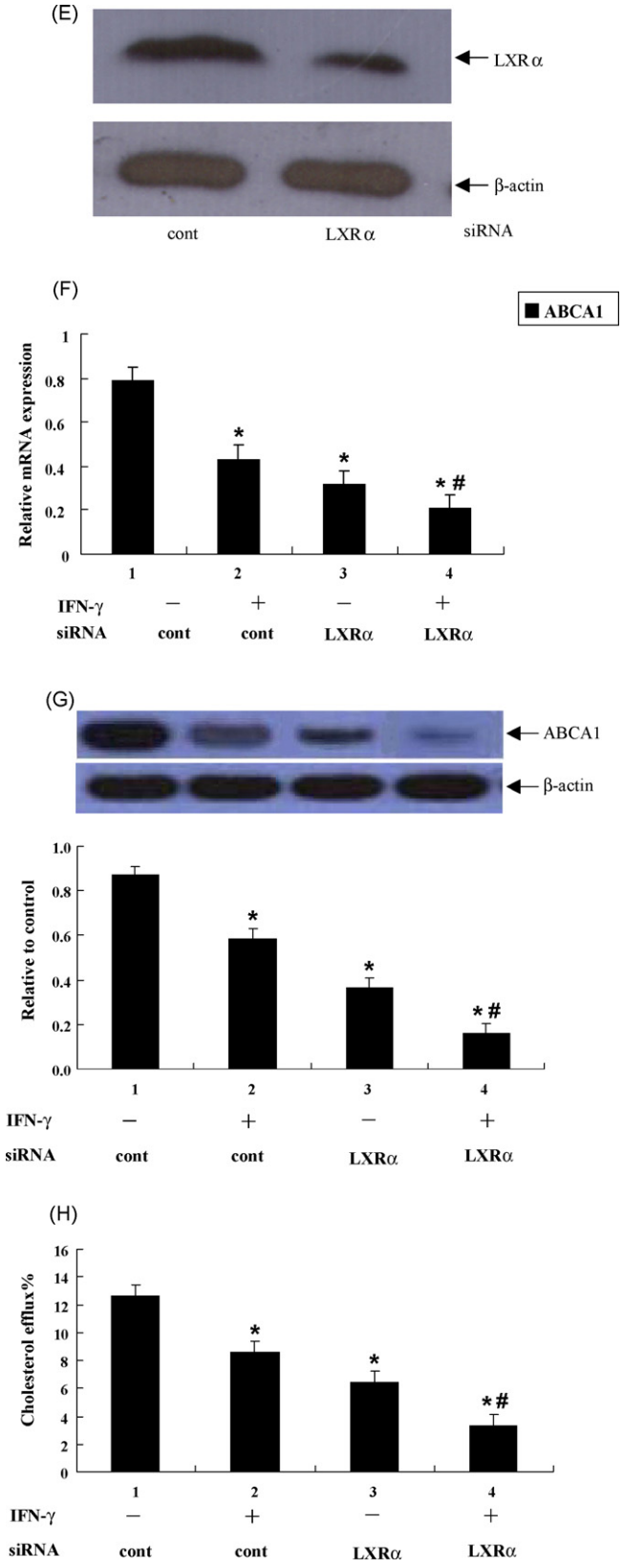

Fig. 2. (*Continued*)

Table 2
Effect of IFN-γ on cholesterol content in THP-1 macrophage-derived foam cells

IFN-γ	0 h	6 h	12 h	24 h
TC	497 ± 48	509 ± 53	667 ± 68*	795 ± 59*
FC	197 ± 21	205 ± 26	261 ± 24*	327 ± 23*
CE	301 ± 34	304 ± 31	405 ± 41*	469 ± 36*
CE/TC (%)	60.6	59.7	60.7	59.0

THP-1 macrophage-derived foam cells were divided into four groups and cultured in medium at 37 °C containing IFN-γ (100 μg/L) for 0 h, 6 h, 12 h and 24 h respectively. Cellular cholesterol and cholesterol ester were extracted as described above. And HPLC was performed to determine the cellular total cholesterol, free cholesterol and cholesterol ester. The results are expressed as mean ± S.D. from three independent experiments, each performed in triplicate.

* $P < 0.05$ vs control group.

3.2. LXRα is involved in the down-regulation of ABCA1 induced by IFN-γ in THP-1 macrophage-derived foam cells

The LXRs have been shown to regulate the expression of ABCA1, which serve as free-cholesterol and phospholipid translocators enabling cholesterol efflux from the macrophage to various acceptors, including nascent cholesterol-poor HDL, and thus have a central role in the regulation of reverse cholesterol transport [39]. To confirm whether the LXRs expression can be affected by IFN-γ, real-time quantitative PCR and Western immunoblotting analysis were performed. As shown (Fig. 2A and B), the expression of LXRα mRNA and protein was decreased when cells were treated with IFN-γ.

We then examined the effect of LXRα agonist 22 (*R*)-Hch on the down-regulation of ABCA1 induced by IFN-γ. As demonstrated (Fig. 2C), ABCA1 mRNA expression was significantly up-regulated in cells treated by 22 (*R*)-Hch compared with that in cells treated by BSA. Down-regulation of ABCA1 mRNA expression by IFN-γ was almost totally compensated by the addition of 22 (*R*)-Hch. Similar result was also found using Western immunoblotting to determine the expression of ABCA1 protein (Fig. 2D). We further investigated the effect of LXRα siRNA on the down-regulation of ABCA1 induced by IFN-γ. Treatment with siRNA for LXRα down-regulated LXRα protein expression by 88% (Fig. 2E) and made the down-regulation of IFN-γ on ABCA1 expression even more obvious (Fig. 2F and G). At the same time, cellular cholesterol efflux in cells treated by the combination of LXRα siRNA and IFN-γ was significantly decreased as compared with these treated by IFN-γ alone (Fig. 2H).

3.3. Down-regulation of LXRα expression induced by IFN-γ was mediated by JAK/STAT signaling pathway

STAT proteins are latent cytoplasmic transcription factors that are phosphorylated by JAK in response to cytokines, such as IFN-γ. Phosphorylated STAT proteins translocate to the nucleus, where they transiently turn on specific sets of cytokine-inducible genes [40]. In J774 mouse macrophages, the activation of the JAK2/STAT1 pathway by IFN-γ has been previously demonstrated [41]. Here we treated THP-1 macrophage-derived foam cells with IFN-γ (100 μg/L), tyrosine (Tyr701) phosphorylation of STAT1 was detected 15 min after addition of IFN-γ and it was further enhanced up to 60 min (Fig. 3A). Phosphorylated STATs dimerize and diffuse into the nucleus to initiate transcription [42]. Therefore we investigated the nuclear translocation of STAT1α in IFN-γ-stimulated THP-1 macrophage-derived foam cells. The presence of STAT1α in nuclear extracts was measured by Western immunoblotting. The level of STAT1α in the nucleus increased in a time-dependent manner after addition of IFN-γ into the culture. In nuclei, low levels of STAT1α were detected already after 10 min of exposure to IFN-γ and it increased up to 30 min (Fig. 3B).

JAK inhibitor AG-490 has previously been shown to prevent JAK2 phosphorylation and to decrease STAT1 phosphorylation in J774 cells [43] and to decrease activation of STAT1 pathway in B-cell chronic lymphocyticleukemia (B-CLL) cells [44]. So we tested the action of JAK inhibitor AG-490 on STAT1 activation by measuring its effect on nuclear translocation of STAT1α in IFN-γ-stimulated cells. As demonstrated (Fig. 3C), AG-490 decreased the nuclear translocation of STAT1α in a concentration-dependent manner. This result clearly demonstrates that JAK inhibitor AG-490 interferes with the activation of STAT1 signaling induced by IFN-γ, by capturing and sequestration of the STATs in the high-molecular-weight complexes.

We further investigated the effect of STAT1 siRNA on LXRα expression in IFN-γ-stimulated THP-1 macrophage-derived foam cells. Treatment with siRNA for STAT1 down-regulated STAT1 protein expression by 86% (Fig. 3D). There was no significant difference in the expression of LXRα mRNA in cells treated by IFN-γ and STAT1 siRNA as compared with control, while the expression of LXRα mRNA was significantly down-regulated in cells treated by IFN-γ alone (Fig. 3E).

3.4. Role of JAK/STAT signaling pathway in the down-regulation of IFN-γ on ABCA1 expression in THP-1 macrophage-derived foam cells

In an attempt to define the role of JAK/STAT signaling pathway in the down-regulation of IFN-γ on ABCA1 expression, we firstly treated THP-1 macrophage-derived foam cells with BSA or IFN-γ (100 μg/L) and/or AG-490 (30 μM). Over the course of 24 h, ABCA1 mRNA and protein levels were analyzed by real-time quantitative PCR assays (Fig. 4A) and Western immunoblotting (Fig. 4B), and liquid scintillation counting was performed to determine the cholesterol efflux (Fig. 4C). It was found that AG-490 blocked the effects of IFN-γ towards the expression of ABCA1 and cholesterol efflux.

Then we investigated the effect of STAT1 siRNA on ABCA1 mRNA expression and cholesterol efflux in IFN-γ-stimulated THP-1 macrophage-derived foam cells. As shown in Fig. 4D and E, there were no differences in ABCA1 mRNA expression and cholesterol efflux in cells that were treated with IFN-γ in combination of STAT1 siRNA together compared with control, while ABCA1 mRNA expression and cholesterol efflux were significantly down-regulated in cells treated by IFN-γ alone.

4. Discussion

The demonstration of the role of ABCA1 in promoting active cholesterol efflux to lipid-poor apoA-I was detected first in patients with Tangier disease, where mutations in the ABCA1 gene has been reported [45]. It serves as the gatekeeper in the reverse cholesterol transport and is critical in regulating cellular cholesterol homeostasis. Liver X receptor can up-regulate ABCA1 expression through forming heterodimers with retinoid X receptor [34,35]. IFN-γ has been demonstrated to have a negative role in cholesterol trafficking by inhibiting cellular processes associated with reverse cholesterol transport. JAK–STAT pathway is one of the most important ways in which cytokines affect their target genes.

Previous studies of our group have reported that expressions of ABCA1 as well as LXRα were elevated in many tissues of the diabetic miniature pigs fed with high-fat/high-sucrose diets. IFN-γ can decrease cholesterol efflux and the expression of ABCA1 in THP-1 macrophage-derived foam cells [46,47]. The synthetic LXR agonist T0901317 can reduce the atherosclerotic lesion area and elevates ABCA1 gene and protein expression in apoE−/− mice [48,49].

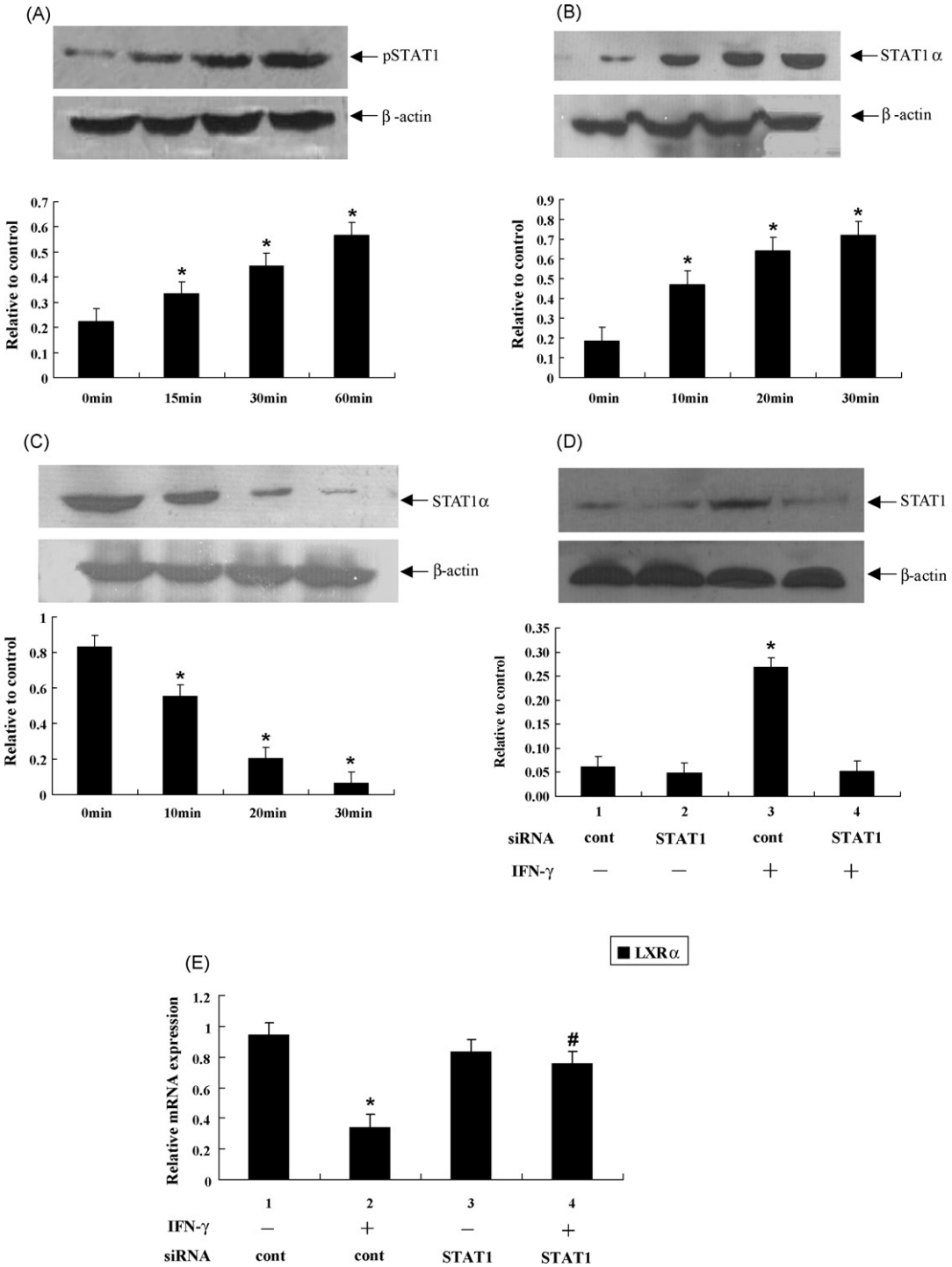

Fig. 3. Down-regulation of LXRα induced by IFN-γ is mediated through the JAK/STAT signaling pathway in THP-1 macrophage-derived foam cells. (A) Time-dependent activation (Tyr701 phosphorylation) of STAT1 by IFN-γ in THP-1 macrophage-derived foam cells. Cells were treated with IFN-γ (100 μg/L) for different time as indicated. Protein was extracted with modified RIPA-buffer, and the protein content was measured. Equal amounts of lysates (20 μg protein) were subjected to immunoblot analysis with antibodies specific for STAT1 phosphorylated at the tyrosine residue 701 and β-actin. Similar results were obtained in three independent experiments. (B) Time-dependent nuclear translocation of STAT1α in IFN-γ-stimulated THP-1 macrophage-derived foam cells. Cells were treated with IFN-γ (100 μg/L) for different time as indicated. The nuclear protein was extracted as described in materials and methods. The protein content of the samples was measured, and equal amounts of lysates (20 μg) were subjected to immunoblot analysis with antibodies against STAT1α and β-actin. Similar results were obtained in three independent experiments. Data are mean ± S.D. $^{*}P < 0.05$ vs baseline. (C) JAK inhibitor AG-490 interferes with the activation of STAT1 signaling in IFN-γ-stimulated THP-1 macrophage-derived foam cells. Cells were pretreated with IFN-γ (100 μg/L) for 30 min. Thereafter, the medium was replaced with fresh medium containing the AG-490 (30 μM). Then cells were incubated for another 30 min, and the nuclear protein was extracted as described in materials and methods. The protein content of the samples was measured and equal amounts (20 μg) were subjected to immunoblot analysis with antibody against STAT1α. Similar results were obtained in three independent experiments. Data are mean ± S.D. $^{*}P < 0.05$ vs baseline. (D) and (E) THP-1 macrophage-derived foam cells were transfected with control or STAT1 siRNA, and then incubated with IFN-γ (100 μg/L) for 24 h. (D) Protein samples were immunoblotted with anti-STAT1α or anti-β-actin antibodies. Data represent three experiments with different cell preparations. (E) LXRα mRNA expression was determined using real-time quantitative PCR assays. Similar results were obtained in three independent experiments. Data are mean ± S.D. $^{*}P < 0.05$ vs baseline. $^{\#}P < 0.05$ vs cont and IFN-γ group.

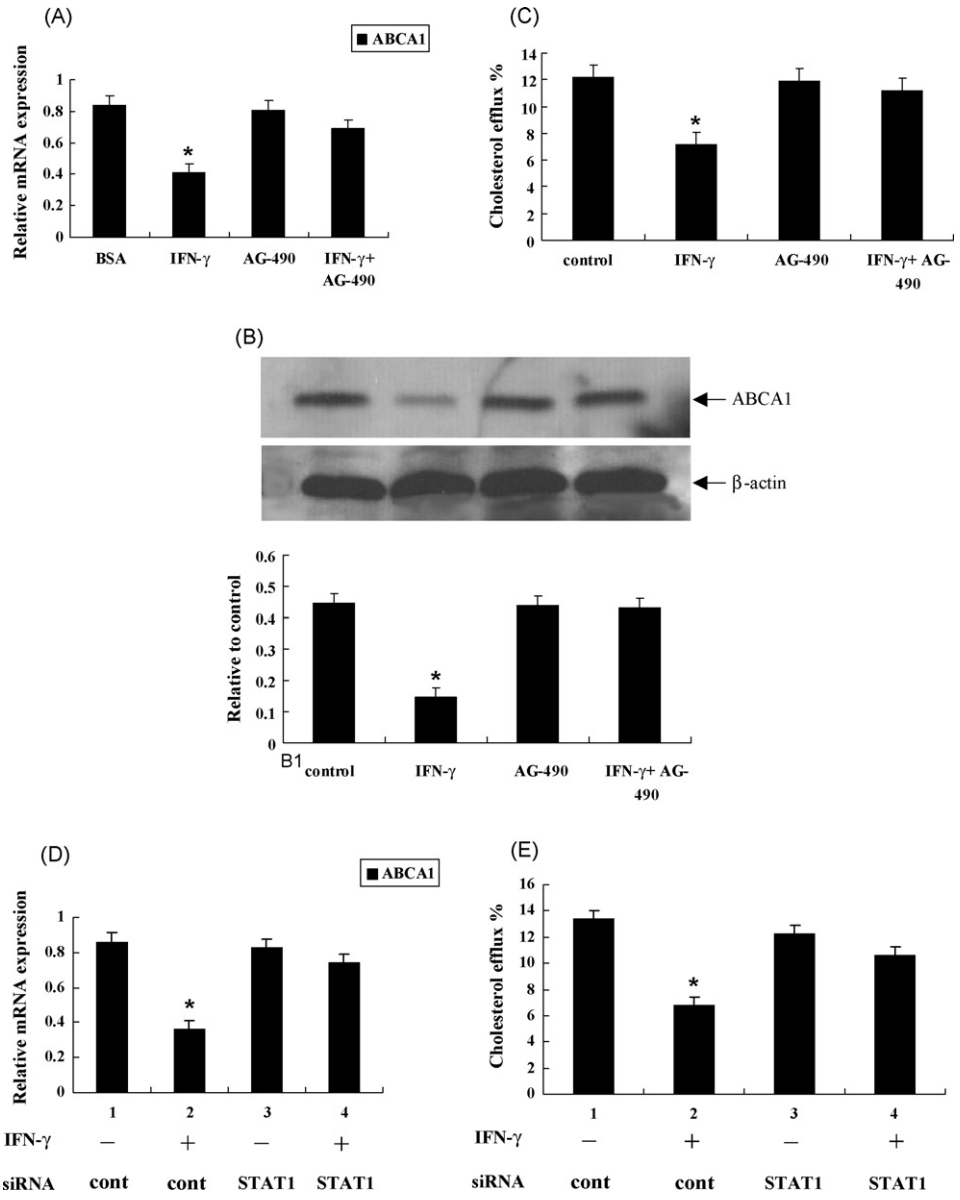

Fig. 4. Role of JAK/STAT signaling pathway in the down-regulation of IFN-γ on ABCA1 expression in THP-1 macrophage-derived foam cells. (A–C) JAK inhibitor AG-490 compensated the effects of IFN-γ on ABCA1 expression and cholesterol efflux. THP-1 macrophage-derived foam cells were divided into four groups and treated with BSA or IFN-γ (100 μg/L) and/or AG-490 (30 μM) for 24 h, respectively. (A) Total RNA was extracted and real-time quantitative PCR was performed to determine the expression of LXRα mRNA. (B) ABCA1 protein expression was determined by Western immunoblotting using antibodies against human ABCA1 and β-actin as described previously. (C) Cellular cholesterol efflux was analyzed by liquid scintillation counting assays as shown above. All the results are expressed as mean ± S.D. from three independent experiments, each performed in triplicate. $^{*}P < 0.05$ vs vehicle. (D) and (E) THP-1 macrophage-derived foam cells were transfected with control or STAT1 siRNA, and then incubated with IFN-γ (100 μg/L) for 24 h. (D) ABCA1 mRNA expression was determined using real-time quantitative PCR assays. (E) Cellular cholesterol efflux was analyzed by liquid scintillation counting assays as shown above. All the results are expressed as mean ± S.D. from three independent experiments, each performed in triplicate. $^{*}P < 0.05$ vs vehicle.

In the present study, we firstly demonstrated that IFN-γ could down-regulate the expression of ABCA1 and cholesterol efflux in THP-1 macrophage-derived foam cells, which was in accordance with results from previous studies.

LXRs have originally been described as regulators of cholesterol metabolism and RCT, which can increase the expression of ABCA1 in apoE−/− mice. And recent data suggest that these receptors may directly influence the expression of pro-inflammatory mediators in macrophages [50,51]. There are also some reports showing that LXR activation can reduce Th-1 cytokine such as IFN-γ expression in human CD4-positive lymphocytes [52]. So now the questions raise around whether the effects of IFN-γ on the expression of ABCA1 and cholesterol efflux is through some signaling pathway to directly influence ABCA1 expression, or by influencing LXRs and then affecting ABCA1.

We tested the effect of IFN-γ on the expression of LXRα to find out whether the expression of LXRα is changed during the course. It turned out that LXRα expression was also decreased by IFN-γ in THP-1 macrophage-derived foam cells. LXRα agonist 22 (*R*)-Hch and LXRα siRNA were then used to see whether LXRα is involved in the down-regulation of ABCA1 induced by IFN-γ. ABCA1 mRNA and protein expression were significantly up-regulated by 22 (*R*)-Hch. And LXRα siRNA made the down-regulation of IFN-γ on ABCA1 expression even more obvious. At the same time cellular cholesterol efflux was also significantly decreased by the combination of LXRα siRNA and IFN-γ. These results suggested that LXRα was

involved in the down-regulation of ABCA1 induced by IFN-γ in THP-1 macrophage-derived foam cells.

IFN-γ is known to activate STAT1 by tyrosine phosphorylation on Tyr701 induced by JAK [53]. Phosphorylation of STAT1 induces STAT1 dimerization, nuclear translocation, and initiation of transcription of GAS-driven genes [23]. In the present study, we examined the activation of the JAK/STAT pathway (STAT1 activation) after IFN-γ stimulation in THP-1 macrophage-derived foam cells by detecting STAT1 (Tyr701) phosphorylation and by probing nuclear lysates for STAT1α at different time points. We observed that STAT1 was activated in 15 min after IFN-γ stimulation. Similar results have been reported recently when whole cell and nuclear lysates of J774 cells were immunoblotted for phosphorylated STAT1 [54].

In our study, the phosphorylation and nuclear translocation of STAT1 induced by IFN-γ were inhibited by AG-490 in THP-1 macrophage-derived foam cells. Similarly to our results, AG-490 has previously been shown to prevent JAK2 phosphorylation and to decrease STAT1 phosphorylation in J774 cells [43] and to decrease activation of STAT1 pathway in B-cell chronic lymphocytic leukemia (B-CLL) cells [44]. Here we extend the earlier data by showing that STAT1 activation is also inhibited by AG-490 in IFN-γ-treated macrophages. We further investigated the effect of STAT1 siRNA on LXRα expression in IFN-γ-stimulated THP-1 macrophage-derived foam cells. LXRα expression in cells treated by IFN-γ and STAT1 siRNA was much more higher than in those treated by IFN-γ alone. So we concluded that the down-regulation of LXRα expression induced by IFN-γ was mediated by JAK/STAT signaling pathway.

To further understand the role of JAK/STAT signaling pathway in the down-regulation of IFN-γ on ABCA1 expression, we investigated the effect of AG-490 and STAT1 siRNA on ABCA1 expression and cholesterol efflux in IFN-γ-stimulated THP-1 macrophage-derived foam cells. We demonstrated that both AG-490 and STAT1 siRNA blocked the effects of IFN-γ towards the expression of ABCA1 and cholesterol efflux, which suggested that the JAK/STAT signaling pathway had something to do with the effect of IFN-γ on ABCA1 expression.

According to our research, IFN-γ can activate the JAK/STAT signaling pathway to regulate genes expression. LXR-α is one of IFN-γ's target genes which can be down-regulated by IFN-γ. As shown in Fig. 3E, LXR-α expression in cells treated by STAT1 siRNA and IFN-γ was much more higher than in those treated by control and IFN-γ, which made us come to the conclusion that the down-regulation of LXRα induced by IFN-γ was mediated through the JAK/STAT signaling pathway. IFN-γ may firstly induced the phosphorylation of STAT1 and the nuclear translocation of STAT1α and thus regulates the expression of LXRα.

Different regulatory mechanisms for the expression of ABCA1 have been described (see details in a recent review [55]). ABCA1 is transcriptionally regulated by LXR/RXR heterodimers via a DR4 site in the ABCA1 promoter region in macrophages [56,57]. In endothelial cells (ECs), the DR4 mutation, pABCA1(-928 DR4 mut)-Luc, greatly diminishes both LDL and cholesterol-induced ABCA1 promoter activities. LDL also activates the LXRE-driven reporter. Thus, LDL most likely regulates the ABCA1 promoter through the DR4 site [58]. Results showing that LDL further increases LXRE activities in the cotransfection of hLXRα suggest that LDL provides ligands to LXR for activation [57]. The oxysterols might be metabolized from cholesterol loaded by LDL in the cell and thus activate LXR.

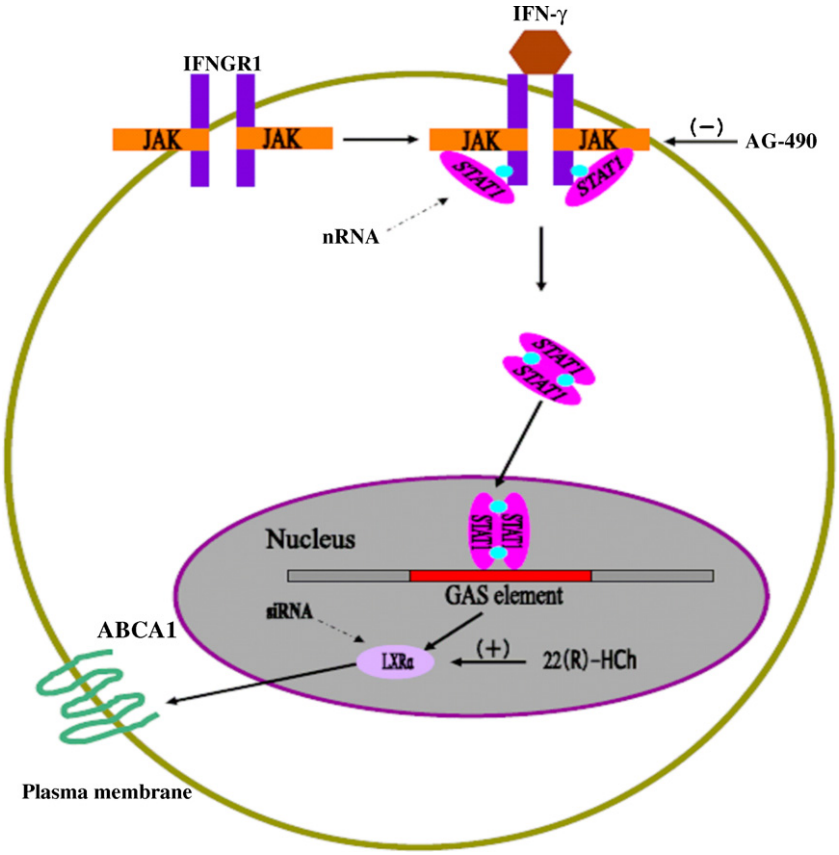

Fig. 5. Schematic representation of the effects of IFN-γ on cholesterol efflux and ABCA1 expression in THP-1 macrophage-derived foam cells. The results of the present study revealed the following scheme for the possible mechanism in which IFN-γ down-regulated of cholesterol efflux and ABCA1 expression. When THP-1 macrophage-derived foam cells are treated with IFN-γ, the JAK/STAT signaling pathway is activated and the STAT dimers bind to the GAS element in the nucleus. Then the expression of LXRα is down-regulated, which in turn decrease the expression of ABCA1 and cholesterol efflux. (+) Activation; (−) inhibition; (- · - · - ▶) interference.

Recently, 27-hydroxycholesterol was reported to be an endogenous ligand for LXR in cholesterol-loaded macrophages [59]. The zinc finger gene 202 was reported to be a transcriptional repressor of ABCA1. Overexpression of this protein in macrophages prevented the induction of ABCA1 gene expression by oxysterols [60]. LDL may activate the DR4 site by down-regulating this repressor. Here, we confirm earlier studies demonstrating ABCA1 depression by IFN-γ and regulation by LXRα in human THP-1 macrophage-derived foam cells. Whether LDL regulation of LXR and the zinc finger protein 202 was also involved in the down-regulation of ABCA1 has not yet been investigated. Further identification of potential promoter regulatory elements and ABCA1 agonists that modulate cholesterol-mediated ABCA1 gene expression will facilitate the development of new pharmacological agents for treating low HDL and atherosclerosis.

As a tyrosine kinase inhibitor, AG-490 is selective for the JAK family kinases, whereas other lymphocytic tyrosine kinases, including Lck, Lyn, Btk, Syk, and Src, are not targets [61,62]. Because no subtype-specific agonists for JAKs have been described so far, we cannot distinguish whether the effect of AG-490 is mediated via one or two of the JAK subtypes or all. Further studies are needed to establish subtype-specific inhibitors, thus allowing further elucidation of the role of JAK subtypes in the regulation of the cellular signaling pathway.

In our study, human THP-1 monocyte-macrophages were cultured and further differentiated into foam cells before used in the experiments. THP-1 cells are a leukemic cell line derived from a patient with acute monocytic leukemia. Unlike other leukemic cell lines, THP-1 cells have no prominent chromosomal abnormalities [63]. Human THP-1 monocyte-macrophages have been established as a valuable cell line for studying the lipid metabolism of human macrophages and appear to represent a more differentiated, committed macrophage cell line than previously available human monocytoid lines [64]. THP-1 cells have been particularly useful for studying the role of ABCA1 in RCT and atherosclerosis. THP-1 macrophage-derived foam cells were the only one cell line we used. In vitro derived foam cells have been shown to correlate less well to the in vivo situation than in vivo derived foam cells. So there are some limitations using in vitro derived foam cells and further studies using in vivo cells such as mice peritoneal macrophages are required.

In summary, as shown in Fig. 5, our results demonstrate that IFN-γ down-regulates ABCA1 expression and cholesterol efflux in THP-1 macrophage-derived foam cells. At the same time, expression of LXRα is also inhibited while pSTAT and nuclear level of STAT1α are increased, which can be compensated by a JAK inhibitor AG-490. All these findings suggest that IFN-γ may firstly down-regulate the expression of LXRα through the JAK/STAT1 signaling pathway and then decrease the expression of ABCA1 and cholesterol efflux in THP-1 macrophage-derived foam cells. Therefore, our study may thus be useful in understanding the critical effect of IFN-γ in pathogenesis of atherosclerosis.

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