Interferon-γ–Mediated Downregulation of Cholesterol Efflux and ABC1 Expression Is by the Stat1 Pathway

Xue-Qing Wang, Constantinos G. Panousis, M. Leticia Alfaro, Glenn F. Evans, Steven H. Zuckerman

Abstract—The pathological role of interferon-γ (IFN-γ) in atherosclerosis is mediated through effects on macrophages, foam cells, and other vascular cells. Recently, we reported that ATP-binding cassette transporter 1 (ABC1) message and protein levels were decreased 3- to 4-fold in foam cells by IFN-γ. In the present study, the pathway by which IFN-γ inhibited ABC1 expression was investigated with signal transducers and activators of transcription (Stat1) knockout mice. IFN-γ–stimulated, wild-type, macrophage-derived foam cells, as previously reported, exhibited a decrease in cholesterol efflux and ABC1 expression as well as an increase in acyl coenzyme A:cholesterol-O-acyltransferase activity. However, IFN-γ treatment of foam cells from Stat1 knockout mice failed to demonstrate reductions in efflux or ABC1 expression at the message or protein levels, nor were there any increases in acyl coenzyme A:cholesterol-O-acyltransferase activity. However, ABC1 mRNA expression in macrophages from Stat1 knockout mice could still be demonstrated to be increased by lipid loading with acetylated low density lipoprotein. Finally, Stat1–independent gene activation by IFN-γ was intact in the Stat1 KO macrophages, inasmuch as IFN-γ was shown to stimulate increases in interleukin-6 production in the Stat1 KO macrophages that were comparable to those observed in the wild-type macrophages. Therefore, Stat1 signaling is necessary and sufficient for the inhibitory effects of IFN-γ on cholesterol efflux and ABC1 expression.

Key Words: interferon-γ ■ atherosclerosis ■ knockout mice ■ inflammation

The concept of atherosclerosis as an inflammatory disease has been supported by in vitro and in vivo studies. The demonstration of activated T cells and macrophages within the atherosclerotic lesion provides in situ evidence for the inflammatory component of the disease. Macrophage activation is central to atheroma progression at the cellular and secretory levels, ranging from the uptake of modified LDL and the ensuing foam cell formation through the elaboration of chemokines, cytokines, proteases, and coagulation factors, and it has an impact on the process of reverse cholesterol transport. Central to the process of macrophage activation is the CD4 + T–cell–derived cytokine interferon-γ (IFN-γ), which has been demonstrated to have a variety of proatherogenic effects. IFN-γ has been reported to increase vascular cell adhesion molecule-1 on endothelial cells and class II antigens on macrophages and smooth muscle cells, to modulate type A and B scavenger receptors, to increase acyl coenzyme A:cholesterol-O-acyltransferase (ACAT) activity, and to decrease apoE secretion and as recently demonstrated ATP-binding cassette transporter 1 (ABC1) expression. These in vitro observations are consistent with the report that apoE knockout (KO) mice crossed with IFN-γ receptor KO mice displayed reductions in lesion size and lipid accumulation. Together, the in vitro and in vivo data support a pathological role for IFN-γ in the progression of atherosclerotic disease, and with the effects on scavenger receptors and ABC1, at least part of these effects could contribute toward inhibiting reverse cholesterol transport.

The demonstration of the role of ABC1 in promoting active cholesterol efflux to lipid-poor apoA-I and of the phenotype of patients with Tangier disease, in whom mutations in the ABC1 gene have been reported, suggests the fundamental role that ABC1 has in this process. Positive regulation of ABC1 expression has been demonstrated in macrophages exposed to lipid loading and is mediated by the heterodimeric receptor pair produced by the liver X receptor binding with the retinoid X receptor through the direct repeat response element within the ABC1 promoter. ABC1 expression can also be increased by cAMP elevation and in macrophages exposed to transforming growth factor-β. However, the downregulation of ABC1 expression is less well understood, with IFN-γ recently being reported to decrease cholesterol efflux and ABC1 expression. The present study was designed to identify the pathway by which IFN-γ regulates ABC1 expression and cholesterol efflux. We now report that the effects of IFN-γ on cholesterol efflux and ABC1 expression can be abrogated in signal transducers and activators of transcription (Stat1) KO mice.

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but not in the parental line. These results demonstrate that the IFN-γ effects on ABC1 expression are directly mediated by the Stat1 pathway.

**Methods**

**Cells and Cholesterol Efflux**

Peritoneal macrophages were collected from thioglycolate-elicited Stat1 KO mice (129S6/SvEv-Stat tm1, Taconic, Germantown, NY) or from the parental line (129S6/SvEv) by lavage and cultured in RPMI 1640 medium containing 2% FCS serum (Hyclone Laboratories). Cholesterol efflux was measured as previously described. Briefly, peritoneal macrophages were converted to foam cells by incubation with 50 μg/mL acetylated LDL (ALDL, Intracel) in RPMI 1640 medium containing 2% serum for 48 hours in the presence of 0.4 μCi/mL [4-14C]cholesterol (New England Nuclear). Subsequently, cells were washed with PBS, and 100 U/mL recombinant murine IFN-γ (Biosource International), 1 μg/mL endotoxin (Escherichia coli O55:B5, DIFCO Labs), or diluent was added for an additional 48 hours in RPMI 1640 containing 1 mg/mL fatty acid–free BSA. After treatment, the cells were washed and incubated with either BSA or 50 μg/mL apoA-I (Intracel) for 6 hours. Cholesterol efflux was expressed as the percentage of counts in the supernatant versus total [14C]cholesterol counts.

**ACAT Assay**

ACAT activity was measured by the incorporation of [14C]oleate (Amersham Life Sciences Inc) into cellular cholesteryl esters, as previously described. Briefly, foam cells were incubated with RPMI 1640 containing 9 μmol/L [14C]oleate and 3 μmol/L BSA for 2 hours at 37°C. Cells were washed and extracted with hexane-isopropanol (3:2 [vol/vol]), and lipids were dried, resuspended in chloroform-methanol (2:1), and separated by thin-layer chromatography on silica G plates (Whatman Ltd). Lipid spots were visualized by I 2 vapors, and radioactivity was measured by scintillation counting. ACAT activity was expressed as counts of incorporated [14C]oleate in cholesteryl esters per milligram of cell protein.

**Immunoblots**

Control and IFN-γ–stimulated foam cells from wild-type and Stat1 KO mice were washed with PBS and solubilized in sample buffer containing 8 mol/L urea, 2% SDS, 50 mmol/L Tris (pH 8.0), and 10% glycerol. Sample aliquots, equal to 5 × 105 cells, were separated by a 3% to 8% polyacrylamide gel (Novex) in Tris-acetate buffer, and proteins were transferred to nitrocellulose membranes (Amersham), and probed with an affinity-purified neutralizing rabbit antibody against murine ABC1 (1378Glu-1566Val). Nitrocellulose membranes were also probed with an antibody followed by enhanced chemiluminescence (ECL, peroxidase-conjugated secondary goat anti-rabbit immunoglobulin antibodies, Santa Cruz Biotechnology) as an additional control for protein loading. Immunopositive bands were visualized by using a peroxidase-conjugated secondary goat anti-rabbit immunoglobulin antibody followed by enhanced chemiluminescence (ECL, Promega).

**Real-Time Quantitative Reverse Transcription–Polymerase Chain Reaction Analysis**

Macrophages from wild-type and Stat1 KO mice were plated in 6-well plates at 106 cells per milliliter and stimulated with IFN-γ for 48 hours. Total RNA was isolated with Trizol reagent (Life Technologies), and mRNA for each treatment was quantified by real-time quantitative polymerase chain reaction with TaqMan Universal Master Mix reaction by using a sequence detection instrument (PE Applied Biosystems Prism, model 7700). The murine ABC1 primers ABC1F (gggtgagaggttataaatagttg) and ABC1R (ttccgggaagccagctg) and probe (6-FAM cgaatagcaggctccaaccctgacc TAMRA) were provided by Dr. Laura Michael (Lilly Research Laboratories). The threshold cycle (Ct), which inversely correlates with the target mRNA levels, was measured as the cycle number at which the reporter fluorescent emission increases above a threshold level. A standard curve was constructed by using varying concentrations of RNA from a control preparation, and the Ct values were obtained. All experimental samples were quantified by using sample dilutions that ensured that the Ct value was on the linear portion of the standard curve. Internal 18S rRNA was used for normalization of the mRNA amount for the different treatments, and all ABC1 data are expressed as a ratio of the amount of ABC1 message to 18S RNA based on their respective Ct values.

**IL-6 Expression**

Macrophages from wild-type or Stat1 KO mice were incubated in the presence or absence of IFN-γ for 60 hours, at which time, supernatants were assayed for interleukin-6 (IL-6) levels by ELISA (BioSource International).

**Results**

Macrophage activation by IFN-γ is associated with a decrease in cholesterol efflux to apoA-I, a decrease in ABC1 expression, and an increase in ACAT activity. In the present study, 100 U/mL IFN-γ resulted in an ∼3-fold reduction in efflux. These effects were completely blocked in foam cells derived from Stat1 KO mice (Figure 1). Thus, these data would suggest that the effects of IFN-γ in downregulating cholesterol efflux were through the Stat1 pathway. In additional studies (Figure 2), it was also observed that cholesterol efflux was significantly reduced by ∼50% in endotoxin-stimulated foam cells, and here too, this inhibitory effect on efflux was not observed in foam cells from Stat1 KO mice. To confirm that these effects on cholesterol efflux were at the level of ABC1 expression, protein and message levels were determined on foam cells from wild-type and Stat1 KO mice. As demonstrated in Figure 3, the reduction in cholesterol efflux to IFN-γ in foam cells was consistent with a reduction in ABC1 protein. This decrease in ABC1 expression was not due to either protein loading or a more general effect on metabolic activity because the levels of extracellular signal–regulated kinases detected were similar in the presence or absence of IFN-γ. In distinction and consistent with the efflux data, the level of ABC1 protein expression in the Stat1 KO foam cells remained unchanged in response to IFN-γ. Furthermore, although the effects of IFN-γ on ABC1 expression were also apparent in wild-type macrophages at
the message level, ABC1 message in the Stat1 KO macrophages remained unchanged with IFN-γ/H9253 (Figure 4A). In contrast, ABC1 regulation to other stimuli not related to the Stat1 pathway remained intact in these cells, inasmuch as lipid loading of the macrophages with ALDL led to a significant increase in ABC1 message, which was apparent in parental and Stat1 KO macrophages (Figure 4B). Collectively, these results demonstrate that the downregulation of cholesterol efflux in foam cells by IFN-γ/H9253 occurs through modulating ABC1 expression at the message and protein levels and that Stat1 signaling is necessary and sufficient for mediating these effects.

Previous studies have reported that macrophage-derived foam cells stimulated with IFN-γ also demonstrate an increase in ACAT activity that could contribute to the reduction in cholesterol efflux mediated by IFN-γ. Therefore, the Stat1 dependence for this effect was also evaluated by using the Stat1 KO macrophages. As demonstrated (Figure 5), IFN-γ-stimulated wild-type macrophages exhibited a 2-fold increase in ACAT activity, whereas there was no similar change with IFN-γ in the Stat1 KO macrophage-derived foam cells. These results suggest that many of the potential pathological effects of IFN-γ on reverse cholesterol transport are mediated through the Stat1 pathway. Finally, Stat1-independent IFN-γ signaling in the Stat1 KO macrophages remains intact, inasmuch as IFN-γ-stimulated wild-type and Stat1 KO macrophages have been shown to exhibit a comparable increase in IL-6 levels in the culture supernatants (Figure 6). Therefore, IFN-γ was able to induce IL-6 secretion in Stat1 KO macrophages through a Stat1-independent pathway while being unable to decrease ABC1 expression or increase ACAT activity, suggesting the dependence of these latter responses on Stat1 signaling.

**Discussion**

The central role of ABC1 in promoting cholesterol efflux from foam cells and its contributions to the process of reverse cholesterol transport have been delineated by identifying the mutation in patients with Tangier disease as occurring in ABC1. Although there has been recent progress in understanding the mechanism(s) by which ABC1 expression...
can be upregulated through liver X receptor and retinoid X receptor heterodimers, there is less known regarding the mechanisms of downregulation. Recent studies have demonstrated that ABC1 expression is downregulated by IFN-γ at the message and protein levels in macrophage-derived foam cells, resulting in a reduction in acetyl cholesterol efflux to apoA-I. These studies have been extended by the present investigation, which has demonstrated that the ability of IFN-γ as well as lipopolysaccharide (LPS) to downregulate cholesterol efflux is dependent on the Stat1 pathway. The effects of IFN-γ on downregulating cholesterol efflux have been previously reported to occur through the downregulation of ABC1 expression as well as the increase in ACAT activity. As demonstrated in the present study, these effects of IFN-γ on cholesterol efflux are mediated through the Stat1 pathway. Distinct from the wild-type macrophages, macrophages from Stat1 KO mice did not exhibit any reduction in either ABC1 expression or cholesterol efflux or any increase in ACAT activity when they were treated with IFN-γ. However, although ABC1 regulation by IFN-γ is lost in the Stat1 KO mice, macrophage-derived foam cells from the wild-type parental mice as well as the Stat1 KO mice still demonstrated significant increases in ABC1 mRNA levels in response to lipid loading.

Recent studies have demonstrated that IFN-γ signaling can occur through Stat1-dependent as well as Stat1-independent pathways. IFN-γ signaling through the Stat1 pathway is well defined. After ligand binding, IFN-γ type I and II receptor oligomerization occurs, and the associated Janus kinases 1 and 2 are phosphorylated, resulting in the phosphorylation of Tyr 440 toward the C-terminus of the IFN-γ type I receptor. Phosphorylation of this residue generates a docking site for Stat1, which is then phosphorylated on Tyr701. This phosphorylation results in Stat1 dimerization and translocation to the nucleus, where it can bind to γ-activated sequences (GAS elements) on the promoters of IFN-γ-inducible genes or at other sites by further interaction with other transcription factors, resulting in IFN-γ-mediated transcriptional regulation. However, further studies have reported that fibroblasts and bone marrow–derived macrophages from Stat1 KO mice are capable of responding to IFN-γ stimulation with the induction of genes seen in wild-type and Stat1 KO mice. Examples include osteopontin, c-Jun, and suppressors of cytokine signaling (SOCS 2 and 3) in fibroblasts and chemokines and the chemokine receptor CXCR4 in macrophages. These studies raised the question as to whether the effects of IFN-γ on cholesterol efflux and ABC1 expression may have been mediated in part by or were completely independent of the Stat1 pathway. The importance of this question relates to whether Stat1-dependent pathways may serve as potential targets to inhibit the negative effects of IFN-γ on reverse cholesterol transport. As the present study demonstrates, the effect of IFN-γ on reverse cholesterol transport by the macrophage is mediated entirely by the Stat1 pathway. However, Stat1 KO macrophages were IFN-γ responsive for other functions, inasmuch as IL-6 production after IFN-γ stimulation was similar between wild-type and KO macrophages. Although the mechanism by which Stat1 activation results in reduced ABC1 expression remains unclear, these results suggest the possibility of a Stat1-activated repressor that could interact with the ABC1 promoter and downregulate ABC1 message levels. The dependence of ABC1 expression on the Stat1 signaling pathway suggests appropriate pharmacological intervention sites that may prevent a downregulation of cholesterol efflux within the atherosclerotic lesion and further supports the concept of intervening in the inflammatory process as a necessary strategy for the management of atherosclerotic disease.

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**References**

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IFN-γ Inhibition of ABC1 Is Stat1 Dependent

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