TGF-β increases cholesterol efflux and ABC-1 expression in macrophage-derived foam cells: opposing the effects of IFN-γ

Constantinos G. Panousis,[1],* Glenn Evans,* and Steven H. Zuckerman[2],*

Division of Cardiovascular Research,* Lilly Research Laboratories, Lilly Corporate Center, Building 48/2, Indianapolis, IN 46285

Abstract **The regulation of ATP-binding cassette transporter 1 (ABC-1) expression by cytokines present within the microenvironment of the atheroma may play an important role in determining the impact of reverse cholesterol transport on the atherosclerotic lesion. We recently reported that the macrophage-activating cytokine interferon (IFN)-γ inhibited both cholesterol efflux and ABC-1 expression. In the present study, we investigated the effects of transforming growth factor (TGF)-β, a cytokine also apparent within the atheroma, on cholesterol efflux, ABC-1 expression, and its ability to antagonize the inhibitory effects of IFN-γ. TGF-β significantly increased cholesterol efflux in macrophage-derived foam cells from apolipoprotein E (apoE) knockout mice, with maximal effects apparent at 300 pg/ml. The increases in efflux occurred without any effect on the passive diffusion component of efflux mediated by β-cyclodextrin. Furthermore, the increase in cholesterol efflux occurred without any changes in free or esterified cholesterol pools and was consistent with an increase in both ABC-1 message and protein. Finally, TGF-β was also demonstrated to inhibit the IFN-γ-mediated down-regulation of ABC-1. These results further demonstrate the importance of cytokine cross-talk to impact the process of reverse cholesterol transport through a multitude of processes including the regulation of ABC-1.**—Panousis, C. G., G. Evans, and S. H. Zuckerman. **TGF-β increases cholesterol efflux and ABC-1 expression in macrophage-derived foam cells: opposing the effects of IFN-γ.** ***J. Lipid Res.*** **2001.** 42: **856–863.**

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Macrophage-high density lipoprotein (HDL) interactions are believed to play a critical role in modulating the process of reverse cholesterol transport (1–3). The regulation of reverse cholesterol transport within the microenvironment of the atherosclerotic lesion is likely to reflect the balance between lipoprotein acceptor species, as well as cytokine concentrations (both pro- and anti-inflammatory) to which the macrophage-derived foam cells are exposed. Previous studies from this laboratory, as well as others, have focused on the effects of the pro-inflammatory macrophage-activating factor interferon (IFN)-γ in promoting atherosclerotic lesion progression (4–6). The effects of IFN-γ are multifactorial and are believed to be due to macrophage activation. To this extent, IFN-γ-activated macrophages exhibit decreased apolipoprotein E (apoE) secretion, increased metalloproteinases and class II antigen expression, increased production of nitric oxide, thus contributing to oxidant radical injury, and reduced scavenger receptor (SR) activity (7–11). Additional changes involve cholesterol trafficking within the foam cell, including a reduction in cholesterol efflux associated with both an increase in acyl-CoA:cholesterol acyltransferase (ACAT) activity, a reduction in ABC-1 expression, and a reduction in surface binding of fluorochrome-conjugated HDL (4, 5, 11, 12).

In contrast to these effects, TGF-β is believed to have anti-inflammatory properties, as evidenced by the profound systemic inflammatory response reported for TGF-β knockout (KO) mice (13). TGF-β effects on macrophages include the induction of tissue inhibitors of metalloproteinases, the inhibition of both nitric oxide and superoxide production, and the increase in expression of the interleukin-1 receptor antagonist (14–16). Through the inhibition of metalloproteinase activity and increase in matrix deposition, it would be expected that TGF-β could contribute to plaque stability. Furthermore, the ability of TGF-β to increase apoE synthesis could also, by reverse cholesterol transport, result in plaque regression (17). However, these effects are confounded by the ability of TGF-β to inhibit both type A and B SR expression including the down-regulation of SR-BI and CD36 in macrophage-derived foam cells (12, 18). In contrast, TGF-β has been suggested to contribute to the pathology associated with arterial rest-

Abbreviations: ABC-1, ATP-binding cassette transporter 1; Ac-LDL, acetylated low density lipoprotein; apo, apolipoprotein; BSA, bovine serum albumin; ECL, enhanced chemiluminescence; HDL, high density lipoprotein; IFN, interferon; KO, knockout; PBS, phosphate-buffered saline; SR, scavenger receptor; TGF, transforming growth factor.

[1] Present address: Roche Laboratories, Basel, Switzerland.
[2] To whom correspondence should be addressed.
e-mail: Zuckerman_Steven@Lilly.com

enosis. Vascular injury following balloon angioplasty has been reported to increase TGF-β expression in smooth muscle cells with the ensuing intimal hyperplasia being reduced with neutralizing anti-TGF-β antisera (19, 20).

Human clinical studies measuring total and active TGF-β have produced conflicting results in terms of TGF-β levels and atherosclerosis. Serum concentrations of active TGF-β were reported to be reduced in patients with atherosclerosis (21, 22). In contrast, an increase in plasma concentrations of active TGF-β associated with the presence of coronary artery disease, and in situ localization of TGF-β has been reported in fibrofatty lesions, as well as in human vascular restenotic lesions (23–25).

Whether TGF-β plays a role in lipid accumulation and fatty streak formation within the intima is still not known. It has been suggested that the protective effects of tamoxifen in lipid lesion formation in apoE KO mice were mediated through the induction of increased levels of TGF-β in the aorta (26). Furthermore, in apoE KO mice treated with anti-CD40L antibody, increased levels of TGF-β were associated with lipid poor lesions, supporting a "protective cytokine" hypothesis (27).

The present study was designed to further address the role of TGF-β in modulating reverse cholesterol transport by focusing on its effects in cholesterol efflux from macrophage-derived foam cells. TGF-β-treated macrophages exhibited a significant increase in cholesterol efflux mediated either by lipid-free apoA-I or HDL but not cyclodextrin, suggesting that only the active process of efflux was up-regulated. Furthermore, the demonstration of these effects in foam cells derived from apoE KO mice clearly eliminates a confounding role for apoE in modulating efflux. The increases in A-I-mediated efflux were consistent with an increase at both the transcriptional and translational levels in the amount of ABC-1 expression in TGF-β-treated foam cells. Finally, and in accordance with the ability of TGF-β to compete with IFN-γ effects, TGF-β reversed the IFN-γ-mediated inhibition of cholesterol efflux in macrophage-derived foam cells by mitigating the inhibitory effects of IFN-γ on ABC-1 expression. These findings further support the hypothesis that TGF-β may have atheroprotective properties by increasing efflux and reducing macrophage foam cell formation.

EXPERIMENTAL PROCEDURES

Reagents and cells

Peritoneal macrophages were collected from thioglycolate-elicited apoE KO or BALB/c mice (Jackson Laboratories, Bar Harbor, ME) by lavage, and cultured in RPMI 1640 medium containing 2% fetal calf serum (Hyclone Laboratories, Logan, UT). TGF-β1 was obtained from R&D Systems, Inc. (Minneapolis, MN), and recombinant murine IFN-γ from Biosource International (Camarillo, CA). Acetylated low density lipoprotein (Ac-LDL), HDL, and lipid-free apoA-I were purchased from Intracel (Issaquah, WA). Methyl-β-cyclodextrin, fatty acid-free bovine serum albumin (BSA), and anti-rabbit immunoglobulin G (I_gG) conjugated to horseradish peroxidase were purchased from Sigma (St. Louis, MO). [4-^{14}C]cholesterol was obtained from New England Nuclear (Boston, MA); [α-^{32}P]dATP from Amersham (Arlington Heights, IL).

Cholesterol efflux

Cholesterol efflux was measured as previously described (4). Briefly, peritoneal macrophages were converted to foam cells by incubation with 50 μg/ml of Ac-LDL in RPMI 1640 medium containing 2% serum for 48 h, in the presence of 0.4 μCi/ml [4-^{14}C]cholesterol. Subsequently, cells were washed with phosphate-buffered saline (PBS), and treatments added for an additional 48 h in RPMI 1640 containing 1 mg/ml fatty acid-free BSA. After treatments, cells were washed once again, and incubated with either BSA, HDL, apoA-I, or methyl-β-cyclodextrin for 6 h. Following incubation, radioactivity in the supernatants and monolayers was measured. Cholesterol efflux was expressed as the percentage of counts in the supernatant versus total [^{14}-C] cholesterol counts.

Neutralizing ABC-1 antisera

A 189-amino acid sequence from mouse ABC-1 (1378 Glu-1566 Val) corresponding to the predicted extracellular loop between the sixth and seventh transmembrane domain was amplified by reverse transcription polymerase chain reaction (PCR) using the primers 5′-AGCCTGGAATTCCAGCCCTGGATGT-3′ and 5′-ATCTGCGGCCGCCTACTTGGTCAGCTTCA-3′, incorporating a 5′-EcoRI and a 3′-Not I restriction site, and cloned to PET-30a(+) vector (Promega, Madison, WI) downstream of a 6xHis-tag. The fusion protein was solubilized under denaturing conditions using 8 M urea, affinity purified using Ni-NTA superflow resin (Qiagen, Valencia, CA), and used for immunizing rabbits (Zymed). The animals were boosted at 6 weeks, bled at 10 weeks, and sera affinity purified against the fusion protein.

Immunoblots

Peritoneal macrophages from apoE KO mice were seeded at 2×10^6 cells/well in a 6-well plate, and converted to foam cells by incubation with 50 μg/ml Ac-LDL for 48 h. Following incubation, foam cells were incubated for an additional 48 h with TGF-β, IFN-γ, or diluent for 48 h in 0.1% BSA, RPMI 1640. Foam cells were washed once with PBS, and solubilized in sample buffer containing 8 M urea, 2% sodium dodecyl sulfate, 50 mM Tris, pH 8.0, and 10% glycerol. Sample aliquots, equal to 5×10^5 cells, were separated by a 3–8% polyacrylamide gel (Novex) in Tris-Acetate buffer. Equal loading was confirmed by replica Coomasie blue stained gels. Proteins were transferred to nitrocellulose membranes (Amersham) and probed with the affinity purified anti-ABC-1 sera. Immunopositive bands were visualized using a peroxidase conjugated secondary goat anti-rabbit I_g antibody, followed by enhanced chemiluminescence (ECL; Promega). Bands corresponding to ABC-1 were quantified using densitometry.

Northern blots

Total RNA was extracted from cells using the DNA/RNA isolation kit (Qiagen) followed by a polyA$^+$ purification with an Oligotex mRNA kit. Two micrograms of polyA$^+$ RNA were electrophoresed on a 0.7% denaturing agarose-formaldehyde gel and transferred to Nytran nylon membranes overnight by using the Turboblotter system (Schleicher & Schuell). Hybridizations were performed overnight at 42°C with [α-^{32}P]dATP PCR-amplified DNA probes labeled with random primers (Gibco). Primers for ABC-1, ACAT-1, and S-29 have previously been reported (5). Quantification was performed using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Cholesterol analysis

Lipids from [^{14}C]cholesterol-labeled foam cells, untreated or treated with TGF-β, were extracted with hexane–isopropanol 3:2 (v/v), dried under vacuum, resuspended in chloroform–methanol 2:1 (v/v) and separated by thin-layer chromatography on silica G plates (Whatman, Kent, England). The chromatogram was developed in a solvent system containing petroleum ether–ethyl ether–acetic acid 90:10:1 (v/v/v) and then air dried. Radiolabeled bands that co-migrated with cholesterol and cholesteryl oleate standards were quantitated by phosphorimaging, and expressed as relative counts of triplicates.

RESULTS

Previous studies from this lab (4, 5) have demonstrated that the macrophage-activating cytokine IFN-γ decreased cholesterol efflux from macrophage-derived foam cells through processes that involved the up-regulation of ACAT activity and levels, as well as a decrease in ABC-1 expression. In the present study, the anti-inflammatory cytokine TGF-β was investigated both in terms of its effects on cholesterol efflux and its impact on the reduction in cholesterol efflux mediated by IFN-γ. In contrast to the effects of IFN-γ, treatment of peritoneal macrophage-derived foam cells with TGF-β for 48 h resulted in a 20% increase in cholesterol efflux compared with untreated cells, using either HDL or lipid-free apoA-I as the acceptor species (**Table 1**). TGF-β, however, increases apoE (an apo known to mediate cholesterol efflux) secretion 3-fold, suggesting a possible mechanism for the TGF-β effect on macrophages (17). To exclude the up-regulation of apoE secretion as contributing to the increase in cholesterol efflux, all subsequent efflux experiments were performed using peritoneal macrophages from apoE KO mice and lipid-free apoA-I as a cholesterol acceptor. As demonstrated (**Fig. 1A**), the TGF-β effect was even more pronounced in this cell type and reached up to a 60% increase in cholesterol efflux compared with the untreated cells, suggesting that the effect of TGF-β is independent of apoE secretion. The increase in cholesterol efflux was dose dependent and reached maximum levels at 0.3 ng/ml of TGF-β. Furthermore, the TGF-β effect was apparent at all concentrations of apoA-I in which efflux was detected, and reached a plateau for both untreated and treated cells at 20 μg/ml apoA-I, suggesting that TGF-β up-regulates a rate-limited process involved with cholesterol efflux (Fig. 1B).

Cholesterol efflux can be mediated either by an active process that requires the apo part of an HDL particle, or through a passive pathway that involves the movement of cholesterol from the plasma membrane to an acceptor down a cholesterol concentration gradient (28–31). To determine the pathways TGF-β affected, the rate of cho-

TABLE 1. TGF-β increases cholesterol efflux in macrophage-derived foam cells

TGF-β[a]	Cholesterol Efflux[b]
ng/ml	%
0	18.49 (0.48)
0.01	19.68 (0.32)
0.1	20.00 (0.23)
1	20.47 (0.88)
10	22.10 (0.18)[c]

[a] Peritoneal macrophages from BALB/c mice were converted to foam cells with 50 μg/ml Ac-LDL in the presence of [^{14}C]cholesterol for 48 h. Subsequently, foam cells were treated in the presence or absence of increasing concentrations of TGF-β in RPMI 1640 medium containing 1 mg/ml fatty acid free BSA for an additional 48 h.

[b] Cholesterol efflux to 20 μg/ml lipid-free apoA-I was measured for 6 h and expressed as a percentage of [^{14}C]cholesterol efflux. Representative experiment of three, parentheses indicate the standard deviation of the mean from triplicate cultures.

[c] Significant difference with control ($P < 0.01$) by a two-tailed unpaired Students *t*-test.

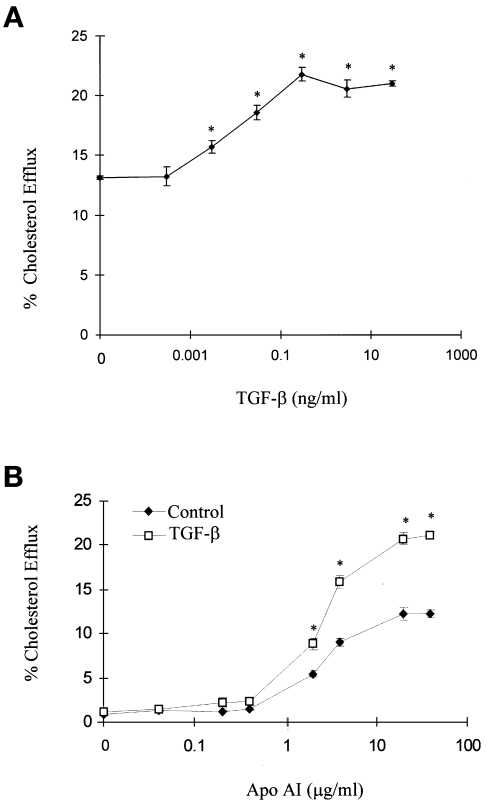

Fig. 1. TGF-β increases cholesterol efflux in macrophage-derived foam cells. Peritoneal macrophages from apoE KO mice were converted to foam cells with 50 μg/ml Ac-LDL in the presence of [^{14}C]cholesterol for 48 h. Subsequently, foam cells were treated in the presence or absence of increasing concentrations of TGF-β in RPMI 1640 medium containing 1 mg/ml fatty acid-free BSA for an additional 48 h. A: Cholesterol efflux to 20 μg/ml lipid-free apoA-I was measured for 6 h and expressed as a percentage of [^{14}C]cholesterol efflux. * Indicates a significant difference from the non-TGF-β-treated control efflux ($P < 0.01$) by a two-tailed Student's unpaired *t*-test. Brackets represent the mean ± standard deviation from triplicate cultures. B: The percentage of cholesterol efflux was evaluated with increasing concentrations of apoA-I as the acceptor species in control foam cells and those incubated with 1 ng/ml TGF-β. Representative experiment of three, each value represents the mean ± standard deviation of triplicate cultures. * Statistically significant difference from the control at comparable apoA-I concentrations that by a two-tailed Student's unpaired *t*-test, $P < 0.03$ at 2 and 40 μg/ml of apoA-I, and $P < 0.01$ at 4 and 20 μg/ml of apoA-I concentrations.

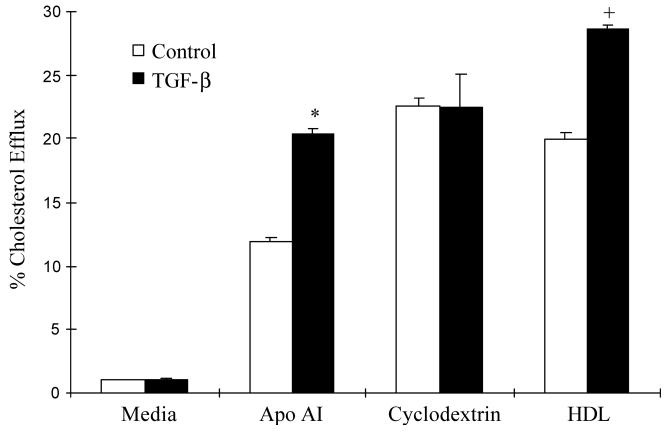

Fig. 2. TGF-β specifically up-regulates apo-mediated cholesterol efflux. Foam cells were incubated in the presence or absence of 1 ng/ml TGF-β for 48 h. Cholesterol efflux was measured in either media alone, 20 μg/ml apoA-I, 1 mM methyl-β-cyclodextrin, or 100 μg/ml HDL. Representative experiment of three, brackets indicate the standard deviation of the mean. * Significant at $P < 0.01$, $^{+}$ significance at $P < 0.00001$ by a two-tailed unpaired Student's *t*-test.

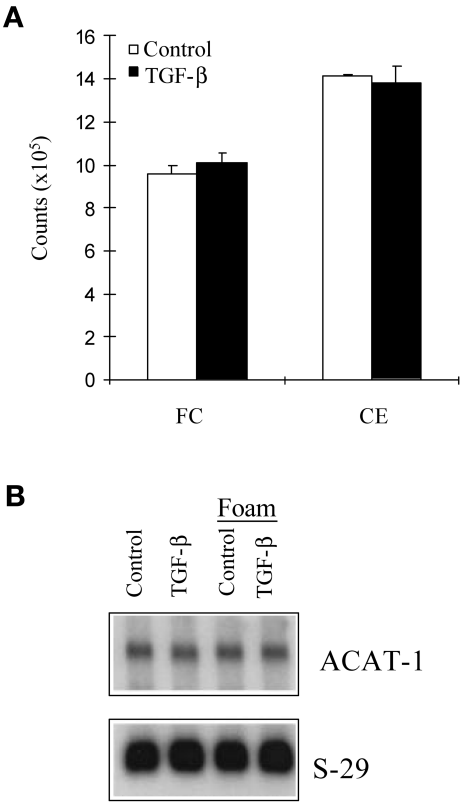

Fig. 3. Lack of effect of TGF-β on cholesterol pools or ACAT-1 message levels. A: Foam cells labeled with [^{14}C]cholesterol were incubated in the presence or absence of 5 ng/ml TGF-β for 48 h. Lipids were extracted using hexane–isopropanol (3:2), and free cholesterol (FC) and cholesteryl esters (CE) were separated by thin-layer chromatography, and quantitated by phosphorimaging. Each value represents the mean ± the standard deviation as indicated by the error bars. B: PolyA^{+} RNA isolated from control or TGF-β-incubated macrophages derived from both non-lipid-laden and foam cells were evaluated by Northern analysis using PCR-radiolabeled probes for ACAT-1 and S-29.

lesterol efflux between control and TGF-β-treated foam cells was evaluated using apoA-I, methyl-β-cyclodextrin, or HDL as the cholesterol acceptor: apoA-I is involved in the active process of cholesterol efflux, methyl-β-cyclodextrin in the passive pathway, and HDL particles can mediate efflux by both processes (29, 32). As demonstrated (**Fig. 2**), TGF-β-treated foam cells exhibited a significant increase in cholesterol efflux using 20 μg/ml apoA-I or 100 μg/ml HDL as cholesterol acceptors. In contrast, although a significant cholesterol efflux (23%) occurred within 6 h with methyl-β-cyclodextrin (1 mM), no difference was apparent with TGF-β. These results suggest that TGF-β specifically up-regulates only the active process of cholesterol efflux and has no effect on the passive diffusion of free cholesterol from the plasma membrane to cyclodextrin. Furthermore, and consistent with the lack of effect of TGF-β on passive efflux, there were no changes in the free and esterified cholesterol pools (**Fig. 3A**) or in ACAT-1 expression (Fig. 3B). These results suggest that the increase in cholesterol efflux was not due to a shift in intracellular cholesterol pools to free cholesterol or to a reduction in ACAT message.

To determine whether the TGF-β effect on cholesterol efflux was mediated through an up-regulation of ABC-1, a Northern analysis was performed in foam cells treated with TGF-β for 48 h. As shown (**Fig. 4A**), treatment with TGF-β resulted in a 34% increase in ABC-1 mRNA ($P < 0.001$) compared with untreated foam cells, suggesting a possible mechanism by which TGF-β stimulated cholesterol efflux. It has been shown that ABC-1 is a sterol sensitive gene (33). Loading macrophages with Ac-LDL induces an increase in ABC-1 expression, whereas de-loading the cholesterol from these cells by inducing cholesterol efflux results in the down-regulation of ABC-1. To investigate whether the mechanism by which TGF-β induces ABC-1 also involves a sterol component, ABC-1 message was quantitated before and after efflux in foam cells. As shown (Fig. 4B), TGF-β was not able to prevent the down-regulation induced by cholesterol efflux because in both treated and untreated cells, a 65% and 61% reduction of message, respectively, was observed. These results thus demonstrate that TGF-β-mediated increases in cholesterol efflux were consistent with an increase in ABC-1 expression.

Although TGF-β was unable to inhibit the reduction in ABC-1 levels following cholesterol efflux, the ability of IFN-γ to reduce efflux in macrophage-derived foam cells raised the possibility that TGF-β might inhibit the IFN-γ-mediated effects on efflux and ABC-1 expression. To explore this possibility, macrophage-derived foam cells were treated with increasing concentrations of IFN-γ in the presence or absence of 5 ng/ml TGF-β (**Fig. 5**). The down-regulation of cholesterol efflux by IFN-γ was observed at 0.5 U/ml and was maximal by 3 U/ml. Treatment with TGF-β resulted in a dose-dependent inhibition of the IFN-γ effect. At 1 U/ml of IFN-γ, cholesterol efflux was down to 5.4% from 13.8% in the untreated cells, and 17.2% in the IFN-γ + TGF-β-treated cells compared with

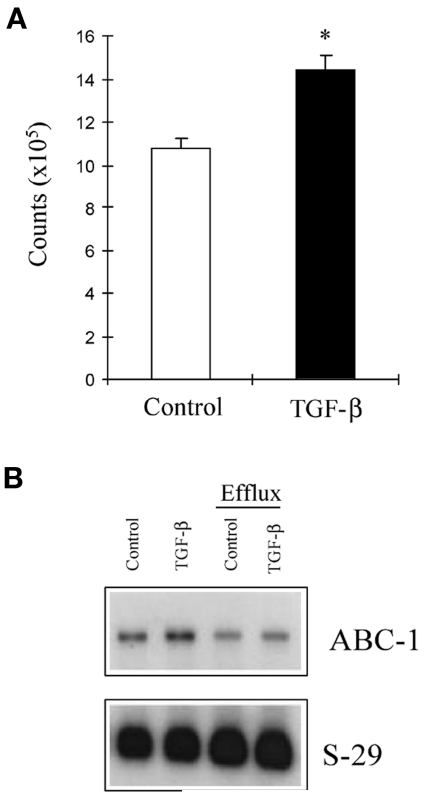

Fig. 4. Up-regulation of ABC-1 expression by TGF-β. Macrophages were converted to foam cells by incubation with Ac-LDL for 48 h, and then for an additional 48 h in the presence or absence of 5 ng/ml TGF-β. A: Following treatment, polyA$^+$ RNA was isolated and subjected to Northern blot analysis using PCR-radiolabeled probes specific for mouse ABC-1 and S-29. Radiolabeled bands from three independent experiments were quantified by a phosphorimager, and expression was adjusted to S-29. Brackets indicate the standard deviation of the mean. * Significant difference between control and TGF-β treated cells ($P < 0.001$) by a two-tailed unpaired Student's *t*-test. B: Representative Northern blot demonstrating ABC-1 expression before and after 6-h cholesterol efflux induced by 100 μg/ml HDL in control and TGF-β-treated foam cells.

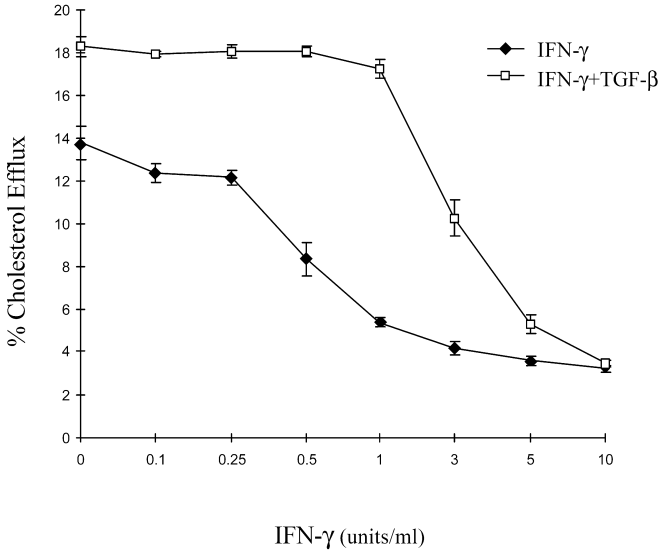

Fig. 5. Dose-dependent reversal of the IFN-γ effect on cholesterol efflux by TGF-β. Peritoneal macrophages were incubated with 50 μg/ml Ac-LDL in the presence of [^{14}C]cholesterol for 48 h. Following incubation, cells were co-incubated with increasing concentrations of IFN-γ in the presence or absence of 5 ng/ml TGF-β. Cholesterol efflux was measured after 6-h incubation with 20 μg/ml apoA-I, and expressed as the percentage of total [^{14}C]cholesterol appearing in the medium. Representative experiment of three, each value represents the mean ± SD of triplicate cultures.

18.2% in the cells treated with TGF-β alone. These results suggest an inhibition of the IFN-γ effect by TGF-β. Finally, at 10 U/ml of IFN-γ, cholesterol efflux was down to 3.2% and remained inhibited despite the presence of TGF-β, suggesting that at higher concentrations of IFN-γ, the ability of TGF-β to mitigate the inhibitory effects on cholesterol efflux was no longer apparent.

To determine whether the TGF-β-mediated antagonism of IFN-γ on cholesterol efflux involved similar changes in ABC-1 expression, protein levels of ABC-1 were tested by Western blot. For this analysis, an affinity-purified antiserum was developed against the predicted extracellular loop between the sixth and seventh transmembrane domain (1378 Glu-1566 Val) of ABC-1. As shown (**Fig. 6**), treatment with 5 ng/ml of TGF-β resulted in an approximately 2-fold increase in ABC-1 protein, whereas consistent with our previous mRNA results (5), IFN-γ significantly reduced the expression of ABC-1. When the two cytokines were added simultaneously, TGF-β was able to counteract the IFN-γ effect in a dose-dependent manner. At 3 U/ml of IFN-γ, co-incubation with TGF-β resulted in an inhibition of the IFN-γ effect as ABC-1 protein was raised back to levels observed when cells were incubated with TGF-β alone. However, at 5 U/ml of IFN-γ, and consistent with the efflux results, TGF-β was not able to block the down-regulation of ABC-1 by IFN-γ. These results suggest that TGF-β can antagonize and thus have a protective effect against the down-regulation of ABC-1 and, consequently, the reduction of cholesterol efflux mediated by IFN-γ.

Finally, in an attempt to determine directly the contribution of ABC-1 surface expression on apoA-I-mediated cholesterol efflux, foam cells were pretreated with the anti-ABC-1 sera prior to the addition of apoA-I, and efflux was measured 6 h later. As apparent (**Fig. 7**), this antisera was capable of reducing A-I-mediated efflux by 40% compared with a rabbit IgG control. The specificity of this antisera on ABC-1-mediated efflux was apparent, as it had no effect on cyclodextrin-mediated cholesterol efflux compared with the IgG control. Although the extent of efflux inhibition was not comparable with that observed with optimal concentrations of IFN-γ, it was difficult to achieve a significant molar excess of antibody over A-I acceptor and still have significant efflux through the active A-I-mediated process. Thus, these results further support a role for TGF-β in promoting the process of reverse cholesterol transport.

DISCUSSION

The mechanistic understanding of carrier-mediated efflux in reverse cholesterol transport has been aided through

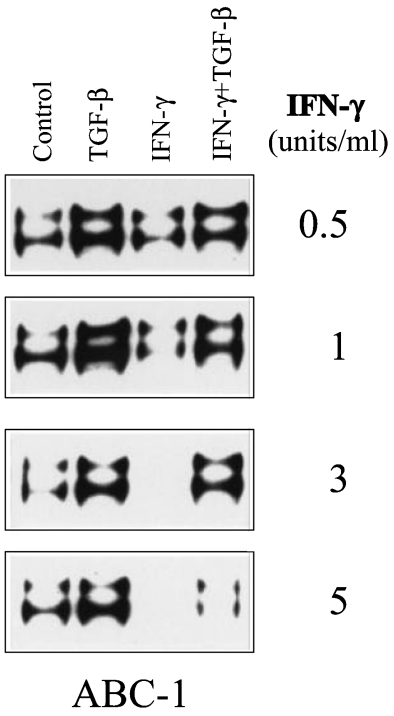

Fig. 6. TGF-β increases ABC-1 expression and inhibits its down-regulation by IFN-γ. Macrophage-derived foam cells were treated or not with 5 ng/ml TGF-β in the absence or presence of increasing concentrations of IFN-γ for 48 h. Following incubation, cell lysates corresponding to 5×10^5 cells were electrophoresed on 3–8% gradient gels and analyzed by Western blot using rabbit anti-mouse ABC-1 antisera at 1.2 μg/ml, followed by a peroxidase-conjugated secondary antibody, and visualized by ECL. A doublet (≈220 kDa) corresponding to ABC-1 was detected with this antisera that was not apparent with an appropriate I_gG control.

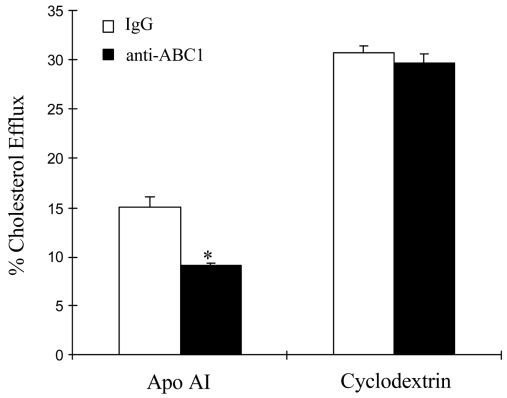

Fig. 7. Anti-ABC-1 antibody decreases cholesterol efflux to apoA-I. Peritoneal macrophages from apoE KO mice were converted to foam cells by incubation with 50 μg/ml Ac-LDL for 48 h. Following incubation, the lipoprotein-containing medium was changed to 0.1% BSA, RPMI 1640 for 24 h. Cholesterol efflux to either 4 μg/ml apoA-I or 1 mM methyl-β-cyclodextrin was measured in the presence of 40 μg/ml anti-ABC-1 antibody or 40 μg/ml rabbit I_gG control. Error bars indicate the standard deviation of the mean from triplicates. This experiment was repeated twice. * Significant difference between anti-ABC-1 and control IgG ($P < 0.05$) by a two-tailed unpaired Student's *t*-test.

the cloning and association of mutations in the ABC-1 as the basis for Tangier disease (34–36). In our previous studies, a phenotype similar to that observed in Tangier disease was apparent in IFN-γ-stimulated macrophage-derived foam cells (4, 5). IFN-γ resulted in inhibition of cholesterol efflux through a mechanism that involved both the up-regulation of ACAT-1 and the decrease in ABC-1 expression. These studies provided the first evidence for a direct link between inflammation and ABC-1 regulation. In the present study, the effects of TGF-β on control and IFN-γ-stimulated foam cells were investigated. TGF-β, in contrast to IFN-γ, increased cholesterol efflux from foam cells. The increase in cholesterol efflux was defined only in the active component of this process and was associated with an up-regulation of ABC-1 at both the transcriptional and translational levels. Furthermore, and consistent with its potential anti-inflammatory properties and in atherogenesis (37), TGF-β abrogated the IFN-γ-mediated inhibition of both cholesterol efflux and the down-regulation of ABC-1.

In Tangier cells where the ABC-1 gene is mutated, it has been shown that apo-mediated cholesterol efflux to free apoA-I or to HDL particles was defective, whereas passive efflux mediated by β-cyclodextrin remained unimpaired (38, 39). In the present study, TGF-β increased apo-mediated cholesterol efflux without affecting cyclodextrin-mediated efflux. These effects on efflux were consistent with the increased ABC-1 expression induced by TGF-β. The fact that TGF-β up-regulated only the active component of cholesterol efflux was further confirmed by the demonstration that TGF-β treatment did not result in changes in either ACAT-1 message or in free and esterified cholesterol pools. These results are distinct from those previously reported for IFN-γ where changes were detected in ACAT-1 activity, free cholesterol pools, as well as in ABC-1 levels (4, 5).

Recently, the ability of SR-BI to inhibit ABC-1-mediated cholesterol efflux has been reported (40). SR-BI-transfected RAW264.7 cells exhibited reduced cholesterol efflux to lipid-free apoA-I or HDL upon stimulation with cAMP. Furthermore, a neutralizing antibody against SR-BI caused an increase in cholesterol efflux, thus suggesting that SR-BI competes with ABC-1-mediated cholesterol efflux for the re-uptake of nascent HDL cholesterol. In addition, we recently reported that TGF-β mediates the down-regulation of HDL binding in foam cells and that the effect was consistent with the down-regulation of the type B SRs, CD36 and SR-BI (12). These observations suggest that the up-regulation of apo-mediated efflux by TGF-β may involve mechanisms beyond ABC-1 including the dynamic balance between type B SRs and members of the ABC transporter family. Clearly, the presumed decreased rate of the re-uptake of nascent HDL cholesterol due to down-regulation of the type B SRs may also play a role. However, the contribution of SR-BI in peritoneal macrophages may be limited, as message levels for CD36 are far more abundant (12). Although its efficiency is 7-fold less than SR-BI, CD36 can also facilitate the selective uptake of HDL cholesterol (31), suggesting that the down-

regulation of CD36 by TGF-β could result in reduced HDL cholesterol re-uptake, and thus contribute to the increase in cholesterol efflux.

TGF-β has potent immunomodulatory effects such as the inhibition of Th1 development and the reversal of IFN-γ-induced macrophage activation (41, 42). TGF-β inhibits IFN-γ-mediated processes including the up-regulation of nitric oxide synthase, the release of TNF-α and oxygen radicals, and the killing of intracellular microorganisms (15, 42, 43). In this study, the down-regulation of ABC-1 by IFN-γ in peritoneal macrophages was also inhibited by TGF-β. The mechanism by which TGF-β inhibits IFN-γ responses in macrophages is still not completely understood (44, 45). It has been reported that TGF-β decreases IFN-γ receptor expression by 30–35% in murine bone marrow macrophages (44); however, this reduction alone is unlikely to explain the abrogation of the IFN-γ response. The ability of IFN-γ to regulate TGF-β-stimulated processes has also been reported. IFN-γ, for example, can inhibit TGF-β signaling pathways by inducing the expression of Smad7, an antagonistic Smad that prevents the interaction of Smad3 with the TGF receptor (46). Whether the up-regulation of ABC-1 by TGF-β is mediated directly through an interaction of Smad with the ABC-1 promoter remains to be determined.

Regulation of ABC-1 expression in macrophages has been reported through cAMP-dependent processes and by changes in intracellular sterol concentrations (33, 47, 48). Recently, however, it was shown that sterol loading per se does not directly regulate ABC-1 expression but, rather, the availability of sterol-derived liver X receptor (LXR) ligands. Costet et al. (49) reported that sterol-dependent transactivation of ABC-1 is mediated through a LXR/retinoid X receptor (RXR) heterodimer, and identified a DR4 element in the human ABC-1 promoter that is required for the activation of the gene. These results were further confirmed using specific ligands for the LXR and RXR receptors, and suggests the importance of these nuclear receptors in regulating ABC-1 expression (50).

The regulation of ABC-1 expression and the ensuing impact on apoA-I-mediated cholesterol efflux can therefore be mediated by sterols, cyclic nucleotides, and, as further characterized in the present study, cytokines modulating macrophage activation. Clearly, the role of inflammation in mediating the progression of atherosclerosis is established. The mechanisms by which this occurs and the relationship between pro- and anti-inflammatory cytokines within the atherosclerotic lesion in modulating the process of reverse cholesterol transport remain an area requiring further understanding.

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REFERENCES

1. Barter, P. J., and K-A. Rye. 1996. Molecular mechanisms of reverse cholesterol transport. *Curr. Opin. Lipidol.* **7:** 82–87.
2. Phillips, M. C., K. L. Gillotte, M. P. Haynes, W. J. Johnson, S. Lund-Katz, and G. H. Rothblat. 1998. Mechanisms of high density lipoprotein-mediated efflux of cholesterol from cell plasma membranes. *Atherosclerosis.* **137:** S13–S17.
3. Tall, A. R. 1998. An overview of reverse cholesterol transport. *Eur. Heart J.* **19:** A31–A35.
4. Panousis, C. G., and S. H. Zuckerman. 2000. Regulation of cholesterol distribution in macrophage-derived foam cells by gamma interferon (IFN-γ). *J. Lipid Res.* **41:** 75–83.
5. Panousis, C. G., and S. H. Zuckerman. 2000. Reduction of cholesterol efflux to free apolipoprotein AI by interferon gamma is associated with the down regulation of the expression of the Tangier's disease gene ABC1. *Arterioscler. Thromb. Vasc. Biol.* **20:** 1565–1571.
6. Gupta, S., A. M. Pablo, X. C. Jiang, N. Wang, A. R. Tall, and C. Schindler. 1997. IFN-gamma potentiates atherosclerosis in ApoE knock-out mice. *J. Clin. Invest.* **99:** 2752–2761.
7. Oropeza, R., R. Schreiber, and Z. Werb. 1985. Regulation of apolipoprotein E expression in macrophages by γ-interferon. *In* Cellular and Molecular Biology of Lymphokines. C. Sorg and S. Shimpl, editors. Academic Press, New York. 303–307.
8. Jonasson, G., J. Holm, O. Skalli, G. Gabiani, and G. K. Hansson. 1985. Expression of class II transplantation antigen on vascular smooth muscle cells in human atherosclerosis. *J. Clin. Invest.* **76:** 125–131.
9. Nathan, C. F., H. W. Murray, M. E. Wiebe, and B. Y. Rubin. 1983. Identification of interferon-γ as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J. Exp. Med.* **158:** 670–689.
10. Geng, Y. J., and G. K. Hansson. 1992. Interferon-γ inhibits scavenger receptor expression and foam cell formation in human monocyte-derived macrophages. *J. Clin. Invest.* **89:** 1322–1330.
11. Zuckerman, S. H., C. G. Panousis, J. Mizrahi, and G. F. Evans. 2000. The effect of γ-interferon to inhibit macrophage-high density lipoprotein interactions is reversed by 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2. *Lipids.* **35:** 1239–1247.
12. Zuckerman, S. H., C. G. Panousis, and G. F. Evans. 2001. Down regulation of macrophage CD36 expression and HDL binding by TGF-β. *Atherosclerosis.* **155:** 79–85.
13. Shull, M. M., I. Ormsby, A. B. Kier, S. Pawlowski, R. L. Diebold, M. Yin, R. Allen, C. Sidman, G. Proetzel, D. Calvin, N. Annunziata, and T. Doetschman. 1992. Targeted disruption of the mouse transforming growth factor-β1 gene results in multifocal inflammatory disease. *Nature.* **359:** 693–699.
14. Edwards, D. R., G. Murphy, J. J. Reynolds, S. E. Whitman, A. J. Docherty, P. Angel, and J. K. Heath. 1987. Tranforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. *EMBO J.* **6:** 1899–1904.
15. Ding, A., C. F. Nathan, J. Graycar, R. Derynk, D. J. Stuehr, and S. Srimal. 1990. Macrophage deactivating factor and transforming factor-β1, -β2, and -β3 inhibit induction of macrophage nitric oxide synthesis by IFN-γ. *J. Immunol.* **145:** 940–944.
16. Di Febbo, C., G. Baccante, M. Reale, M. L. Castellani, A. Angelini, F. Cuccurullo, and E. Porreca. 1998. Transforming growth factor β1 induces IL-1 receptor antagonist production and gene expression in rat vascular smooth muscle cells. *Atherosclerosis.* **136:** 377–382.
17. Zuckerman, S. H., G. F. Evans, and L. O'Neal. 1992. Cytokine regulation of macrophage apoE secretion: opposing effects of GM-CSF and TGF-β. *Atherosclerosis.* **96:** 203–214.
18. Bottalico, L. A., R. E.Wager, L. B. Agellon, R. K. Assoian, and I. Tabas. 1991. Transforming growth factor-beta 1 inhibits scavenger receptor activity in THP-1 human macrophages. *J. Biol. Chem.* **266:** 22866–22871.
19. Majesky, M. W., V. Lindner, D. R. Twardzik, S. M. Schwartz, and M. A. Reidy. 1991. Production of transforming growth factor β1 during repair of arterial injury. *J. Clin. Invest.* **88:** 904–910.
20. Wolf, Y. G., L. M. Rasmussen, and E. Rusolahti. 1994. Antibodies against transforming growth factor β1 suppress intimal hyperplasia in a rat model. *J. Clin. Invest.* **93:** 1172–1178.
21. Grainger, D. J., P. R. Kemp, J. C. Metcalfe, A. C. Liu, R. M. Lawn, N. R. Williams, A. A. Grace, P. M. Schofield, and A. Chauan. 1995. The serum concentration of active transforming growth factor-β is severely depressed in advanced atherosclerosis. *Nat. Med.* **1:** 74–70.
22. Erren, M., H. Reinecke, R. Junker, M. Fobker, H. Schulte, J. O. Schurek, J. Kropf, S. Kerber, G. Brethardt, G. Assman, and P. Cullen. 1999. Systemic inflammatory parameters in patients with

atherosclerosis of the coronary and peripheral arteries. *Arterioscler. Thromb. Vasc. Biol.* **19:** 2355–2363.
23. Bobik, A., A. Agrotis, P. Kanellakis, R. Dilley, A. Krushinsky, V. Smirnov, E. Tararak, M. Condron, and G. Kostolias. 1999. Distinct patterns of transforming growth factor-β isoform and receptor expression in human atherosclerotic lesions. Colocalization implicates TGF-β in fibrofatty lesion development. *Circulation.* **99:** 2883–2891.
24. Wang, X. L., S. X. Liu, and D. E. L. Wilcken. 1997. Circulating transforming growth factor β1 and coronary artery disease. *Cardiovasc. Res.* **34:** 404–410.
25. Nikol, S., J. M. Isner, J. G. Pickering, M. Kearny, G. Leclerc, and L. Weir. 1991. Expression of transforming growth factor-β1 is increased in human vascular restenosis lesions. *J. Clin. Invest.* **88:** 904–910.
26. Reckless, J., J. Metcalf, and D. J. Grainger. 1997. Tamoxifen decreases cholesterol sevenfold and abolishes lipid lesion development in apolipoprotein E knockout mice. *Circulation.* **95:** 1542–1548.
27. Lutgens, E., K. B. Cleutjens, S. Heeneman, V. E. Koteliansky, L. C. Burkly, and M. J. Daemen. 2000. Both early and delayed anti-CD40L antibody treatment induces a stable plaque phenotype. *Proc. Natl. Acad. Sci. USA.* **97:** 7464–7469.
28. Oram, J. F., and S. Yokoyama. 1996. Apolipoprotein-mediated removal of cellular cholesterol and phospholipids. *J. Lipid Res.* **37:** 2473–2491.
29. Mendez, A. J. 1997. Cholesterol efflux mediated by apolipoproteins is an active process distinct from efflux mediated by passive diffusion. *J. Lipid Res.* **38:** 1807–1821.
30. Rothblat, G. H., F. H. Mahlberg, W. J. Johnson, and M. C. Phillips. 1992. Apolipoproteins, membrane cholesterol domains, and the regulation of cholesterol efflux. *J. Lipid Res.* **33:** 1091–1097.
31. de la Llera-Moya, M., G. H. Rothblat, M. A. Connely, G. Kellner-Weibel, S. W. Sakr, M. C. Phillips, and D. L. Williams. 1999. Scavenger receptor BI (SR-BI) mediates free cholesterol flux independently of HDL tethering to the cell surface. *J. Lipid Res.* **40:** 575–580.
32. Atger, V., M. de la Llera-Moya, G. Stoudt, W. Rodriguez, M. Phillips, and G. Rothblat. 1997. Cyclodextrins as catalysts for the removal of cholesterol from macrophage foam cells. *J. Clin. Invest.* **99:** 773–780.
33. Langmann, T., J. Klucken, M. Reil, G. Liebisch, M. F. Luciani, G. Chimini, W. E. Kaminski, and G. Smitz. 1999. Molecular cloning of the human ATP-binding cassette transporter 1 (hABC1): evidence for sterol-dependent regulation in macrophages. *Biochem. Biophys. Res. Commun.* **275:** 29–33.
34. Bodzioch, M., E. Orso, J. Klucken, T. Langman, A. Bottcher, W. Diederich, W. Drobnik, S. Barlage, C. Buchler, M. Porsch-Ozcurumez, W. E. Kaminski, H. W. Hahmann, K. Oette, G. Rothe, C. Aslanidis, K. J. Lackner, and G. Schimtz. 1999. The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nat. Genet.* **22:** 347–351.
35. Rust, S., M. Rosier, H. Funke, J. Real, Z. Amoura, J. C. Piette, J. F. Deleuze, H. B. Brewer, N. Duvenger, P. Denefle, and G. Assman. 1999. Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. *Nat. Genet.* **22:** 352–355.
36. Brooks-Wilson, A., M. Marcil, S. M. Clee, L. H. Zhang, K. Roomp, M. van Dam, L. Yu, C. Brewer, J. A. Collins, H. O. F. Molhuizen, O. Loubser, B. F. Ouelette, K. Fichter, K. Ashbourne-Excoffon, C. W. Sensen, S. Scherer, S. Mott, M. Denis, D. Martindale, J. Frohlich, K. Morgan, B. Koop, S. Pimstone, J. Kastelein, J. Genest, Jr., and M. R. Hayden. 1999. Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat. Genet.* **22:** 336–345.
37. Grainger, D. J., and J. C. Metcalfe. 1995. A pivotal role for TGF-β in atherogenesis? *Biol. Rev.* **70:** 571–596.
38. Lawn, R. M., D. P. Wade, M. R. Garvin, X. Wang, K. Schwartz, J. G. Porter, J. J. Seilhamer, A. M. Vaughan, and J. F. Oram. 1999. The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. *J. Clin. Invest.* **104:** R25–R31.
39. Remaley, A. T., U. K. Schumacher, J. A. Stonik, B. D. Farsi, H. Nazih, and H. B. Brewer. 1997. Decreased reverse cholesterol transport from Tangier disease fibroblasts. Acceptor specificity and effect of brefeldin on lipid efflux. *Arterioscler. Thromb. Vasc. Biol.* **17:** 1813–1821.
40. Chen, W., D. L. Silver, D. S. Jonathan, and A. R. Tall. 2000. Scavenger receptor-BI (SR-BI) inhibits ATP binding cassette transporter 1 (ABC1)-mediated cholesterol efflux in macrophages. *J. Biol. Chem.* **275:** 30794–30800.
41. Fox, F. E., R. J. Capocasale, H. C. Ford, R. J. Lamb, J. S. Moore, and P. C. Nowell. 1992. Transforming growth factor-β inhibits human T cell proliferation through multiple targets. *Lymph. Cytokine Res.* **11:** 299.
42. Tsunawaki, S., M. Sporn, A. Ding, and C. Nathan. 1988. Deactivation of macrophages by transforming growth factor-β. *Nature.* **334:** 260–262.
43. Silva, J. S., D. R. Twardzik, and S. G. Reed. 1991. Regulation of *Trypanosoma cruzi* infections in vivo by transforming growth factor β (TGF-β). *J. Exp. Med.* **174:** 539–545.
44. Pinson, D. M., R. D. LeClaire, R. B. Lorsbach, M. J. Parmely, and S. W. Russel. 1992. Regulation by transforming factor-β1 of expression and function of the receptor for IFN-γ on mouse macrophages. *J. Immunol.* **149:** 2028–2034.
45. Panek, B. R., Y. J. Lee, and E. N. Benveniste. 1995. TGF-β suppression of IFN-γ-induced class II MHC gene expression does not involve inhibition of phosphorylation of JAK1, JAK2, or signal transducers and activators of transcription, or modification of IFN-γ enhanced factor X expression. *J. Immunol.* **154:** 610–619.
46. Ulloa, L., J. Doody, and J. Massague. 1999. Inhibition of transforming growth factor-β/SMAD signaling by the interferon-γ/STAT pathway. *Nature.* **397:** 710–713.
47. Hokland, B. M., J. P. Slotte, and T. F. Johnson. 1993. Cyclic AMP stimulates efflux of intracellular sterol from cholesterol-loaded cells. *J. Biol. Chem.* **268:** 25343–25349.
48. Lawn, R. M., D. P. Wade, M. R. Garvin, X. Wang, K. Schwartz, J. G. Porter, J. J. Seilhamer, A. M. Vaughan, and J. F. Oram. 1999. The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. *J. Clin. Invest.* **104:** R25–R31.
49. Costet, P., Y. Luo, N. Wang, and A. R. Tall. 2000. Sterol dependent transactivation of the ABC1 promoter by the liver X receptor/retinoid X receptor. *J. Biol. Chem.* **275:** 28240–28245.
50. Repa, J. J., S. D. Turley, J. A. Lobaccaro, J. Medina, L. Li, K. Lustig, B. Shan, R. A. Heyman, J. M. Dietschy, and D. J. Mangelsdorf. 2000. Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science.* **289:** 1524–1529.