Regulation of cholesterol distribution in macrophage-derived foam cells by interferon-γ

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Abstract The Th1-derived cytokine gamma interferon, IFN-γ, is present within the microenvironment of an atheromatous lesion and likely contributes to lesion progression through macrophage activation. While the inflammatory effects of IFN-γ are well known, the role of this cytokine in cholesterol metabolism in macrophage derived foam cells is unclear. In the present study, the incubation of foam cells with IFN-γ resulted in the reduction of HDL_3-mediated cholesterol efflux. The decrease in cholesterol efflux was not observed with other macrophage-activating factors as colony-stimulating factors failed to demonstrate a similar effect. The reduction in cholesterol efflux was independent of apoE synthesis or SR-BI expression and was associated with a redistribution of intracellular cholesterol with an increase in cholesteryl ester accumulation. The increase in the esterified pool, primarily in cholesterol eicosapentadenoate, docosapentaenoate, arachidonate, and linoleate was associated with a 2-fold increase in acyl-CoA:cholesterol-O-acyltransferase, ACAT, activity and message without any change in neutral cholesteryl ester hydrolase activity. While CD36 message was reduced in IFN-γ-treated foam cells, the ability to reverse the decrease in efflux by the ACAT inhibitor A58035 in a dose-dependent manner suggests that the IFN-γ effect on efflux is primarily through the modulation of ACAT expression. Therefore, in addition to its inflammatory effects, IFN-γ can contribute to the progression of an atherosclerotic lesion by altering the pathway of intracellular cholesterol trafficking in macrophage derived foam cells.—Panousis, C. G., and S. H. Zuckerman. **Regulation of cholesterol distribution in macrophage-derived foam cells by interferon-γ. *J. Lipid Res.* 2000.** 41: **75–83.**

Supplementary key words cholesterol efflux • interferon-γ • ACAT • high density lipoprotein • cholesterol ester • foam cells

Atherosclerosis as a chronic inflammatory disease is characterized by the presence of lipid-laden lesions within the arterial wall (1–4). Infiltration of T-cells and monocyte-derived macrophages into the intimal cell wall is believed to result in the progression of the atherosclerotic lesion, from the early fatty streak, which is an accumulation of lipid-laden macrophages (foam cells), to the advanced fibroproliferative atherosclerotic lesion (1, 2). Macrophages play a key role in the development of these early lesions by the uptake and metabolism of modified low density lipoprotein (LDL) particles, as well as in lesion progression by secreting a range of chemokines, cytokines, proteases and coagulation factors (5). The regulation of macrophage effector functions through autocrine and paracrine mechanisms involving cytokine elaboration suggests a pathologic role in lesion progression for cytokines associated with macrophage activation (1, 2).

Activated $CD4^+$ T cells present in the atherosclerotic lesion can secrete interferon-gamma (IFN-γ), as has been demonstrated in human atherosclerotic plaques by immunofluorescence and by polymerase chain reaction (6, 7). The proatherogenic effects of IFN-γ include the induction of VCAM-1 on endothelial cells (8), MHC-II on macrophages and smooth muscle cells (9), lipoprotein receptors on smooth muscle cells (10) and decreased secretion of apolipoprotein (apo)E and expression of lipoprotein receptors on macrophages (11–13). Consistent with these in vitro observations is the recent report that apoE knockout (KO) mice crossed with IFN-γ receptor KO mice display reduced lesion size, lipid accumulation, and cellularity (14). However, IFN-γ may also have a protective role in atherosclerosis as in vitro treatment reduces LDL oxidation by monocytes (15) and the expression of scavenger receptor (SR) (16) suggesting a decrease in cholesterol accumulation. To what extent IFN-γ regulates cholesterol trafficking within macrophage-derived foam cells has not been reported and yet is important in understanding the foam cell response to IFN-γ (1–4).

In the present study, IFN-γ was demonstrated to reduce cholesterol efflux to high density lipoprotein (HDL_3) from macrophage-derived foam cells. This effect was independent of apo E secretion by macrophages or SR-BI expression, and was associated with an increase in intracellular cholesteryl ester accumulation. The increase in esterified cholesterol was associated with the induction of acyl-CoA:cholesterel acyltransferase (ACAT) without any

Abbreviations: INF-γ, interferon-γ; apo, apolipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; ACAT, acyl-CoA:cholesterol acyltransferase; SR, scavenger receptor; PMϕs, peritoneal macrophages; NCEH, neutral cholesteryl ester hydrolase.

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effect on neutral cholesteryl ester hydrolase. The inhibition of cholesterol efflux as well as the increase in cholesteryl ester accumulation in IFN-γ-treated foam cells is consistent with its proatherogenic role within an atheroma through processes associated with cholesterol trafficking.

MATERIALS AND METHODS

Macrophage cultures and cholesterol efflux

Peritoneal macrophages, PMϕs, were obtained from thioglycolate-elicited Balb/C or apoE KO mice and maintained in culture in RPMI 1640 supplemented with 2% fetal calf sera (Hyclone Laboratories, Logan, UT). PMϕs were seeded in 24-well plates at 3×10^5 cells/ml and converted into foam cells by incubation with 50 μg/ml of Ac-LDL or Ox-LDL (Intracel Corp., Rockville, MD) in the presence of 0.4 μCi/ml [^{14}C]cholesterol (NEN, Boston, MA) for 48 h. Cells were washed thrice with PBS and incubated for an additional 48 h in RPMI 1640 with 1 mg/ml fatty acid-free BSA (Sigma, St. Louis, MO) in the presence or absence of 300 units/ml recombinant murine IFNγ (Biosource Intl., Camarillo, CA) or 10 ng/ml M-CSF, GM-CSF, JE/MCP, MIP (R&D Systems, Minneapolis, MN). Cells were then washed once with PBS and incubated with 100 μg/ml HDL_3 in RPMI 1640 for 0–24 h. Radiolabeled cholesterol was quantitated in the supernatants at designated intervals after centrifugation, and cell monolayers were lysed with 250 μl of 0.1 N NaOH to obtain the total counts. Efflux was expressed as a percent of counts in the supernatant versus total [^{14}C]cholesterol counts.

HDL_3 isolation

HDL_3 was isolated from fresh plasma of healthy volunteers by sequential ultracentrifugation, as the fraction with density of 1.125–1.215 gm/ml in KBr. The resulting density gradient band was dialyzed against PBS containing 1 mm EDTA (17) and further resolved from apoB- and apoE-containing particles by heparin-agarose chromatography (Pharmacia, Piscataway, NJ) as described (18). The purified HDL_3 was filtered through a 0.22-μm filter, protein concentrations were determined by the BCA method (Pierce, Rockford, IL) and aliquots were evaluated on agarose gels.

Acyl-CoA:cholesterol-O-acyltransferase assay

ACAT activity was measured by the incorporation of [^{14}C]oleate (Amersham Life Science Inc., Arlington Heights, IL) into cellular cholesteryl esters as previously described (19). Briefly, control or foam cells were washed with PBS, and incubated for 2 h with RPMI 1640 containing 9 μm [^{14}C]oleate and 3 μm BSA at 37°C. Cells were washed with ice-cold PBS containing 1 mg/ml BSA, and lipids were extracted with hexane–isopropanol 3:2 (v/v). Lipids were then dried under vacuum, resuspended in chloroform–methanol 2:1 and separated by thin-layer chromatography (20) on silica G plates (Whatman Ltd, Kent, England). The chromatogram first was developed in chloroform–methanol–water 65:30:5 for 4 cm, dried, and then developed with a second solvent system composed of petroleum ether–ethyl ether–acetic acid 90:10:1. Lipid spots were visualized by I_2 vapors, and radioactivity was measured by scintillation counting. ACAT activity was expressed as counts incorporated [^{14}C]oleate in cholesteryl esters per mg of cell protein and was compared with the incorporation of [^{14}C]oleate into triglycerides.

Neutral cholesteryl ester hydrolase assay

The activity of neutral cholesteryl ester hydrolase, NCEH, was determined by the release of [^{14}C]cholesterol from [^{14}C]cholesteryl oleate (NEN, Boston, MA) (21). Cells from a 75 cm^2 flask were washed and detached with a rubber policeman in 1 ml of 10 mm Tris pH 7.4, 250 mm sucrose, 0.1 mm EDTA and sonicated for 20 seconds on ice using a Microson sonicator at 30% power output. A 100-μl aliquot of the sonicate was incubated with 100 μl of substrate containing 85 mm phosphate buffer, 12.5 mm Na-taurocholate, 6 μm [^{14}C]cholesteryl oleate (150 mCi/mmol), 25 μm phosphatidylcholine, 25 μm phosphatidylethanolamine, and 0.4 mg/ml albumin for 2 h at 37°C. Neutral lipids were extracted thrice with 200 μl of chloroform–methanol 1:1. The chloroform phase was collected, dried under vacuum, and lipids were separated by TLC. NCEH activity was expressed as pmole of free cholesterol released per mg of protein per hour.

High performance liquid chromatography

After incubation with Ac-LDL macrophage-derived foam cells were homogenized by sonication and 50 μg of cholesteryl heptadecanoate was added as an internal standard to 1 mg of cell lysate. Lipids, extracted as described for the NCEH assay, were resuspended in 40 μl of chloroform–methanol 2:1, and fractionated by HPLC using a 10-μl loop injector. HPLC was performed in a Waters 625 LC System with a Spherisorb S3 ODS2 250 × 4 mm column (Waters, Milford, MA), heated with a Systec column heater (Systec Inc., New Brighton, MN) at 40°C. Cholesterol and cholesteryl esters were eluted isocratically using isopropanol–n-heptane–acetonitrile 35:12:52 as an eluent and detected by UV absorption at 206 nm (22).

Northern blot analysis

Macrophage-derived foam cells were incubated in the absence or presence of 300 unit/ml of IFN-γ for 48 h. Total RNA and subsequently poly A+ RNA was isolated using RNA isolation kits (Qiagen, Santa Clarita, CA). Poly A+ RNA was denatured for 5 min at 65°C, separated on 0.7% formaldehyde agarose gels, and transferred to Nytran nylon membranes overnight using the turboblotter system (Schliecher and Schuell Inc., Keene, NH). Membranes were prehybridized in hybridization buffer (23) and hybridized with [α-^{32}P]dATP PCR-amplified DNA probes labeled with random primers (GIBCO, Grand Island, NY) in fresh hybridization buffer. The following sets of primers were used to amplify: SRBI; sense (5′-TCCTGAGCCCCGAGAGCCCCTTCGGC-3′) antisense (5′-CTGGCTGCGCAGTTGGCAGATGATGGC-3′), CD36; sense (5′-CAGCCCAATGGAGCCATC-3′) antisense (5′-CAGCGT AGATAGACCTGC-3′), ACAT; sense (5′-GGACACATACAGAAA TGGTCAC AT-3) antisense (5′-GCACAAAACCTAGAACTCCAA GTT-3′), S29; sense (5′-TCTGAAGGCAAGATGGGTCACCA-3′) antisense (5′-TTTGTGTACAAAGACTAGCATGAT-3′). Membranes were washed in 2× SSC 1% SDS and exposed to Kodak Biomax-MS film or to phosphoscreen. Quantitation was performed using a phosphoimager (Molecular Dynamics Inc., Sunnyvale, CA).

Statistics

Statistical analysis was performed by unpaired (two-tail) *t*-test. Values are reported as means ± SD. The 95% confidence limit was taken as significant ($P < 0.05$).

RESULTS

Macrophage activation is associated with an increase in effector functions contributing to inflammation in general and the progression of atherosclerotic disease in particular. In the current study, cytokines associated with macrophage activation were evaluated for their effect on

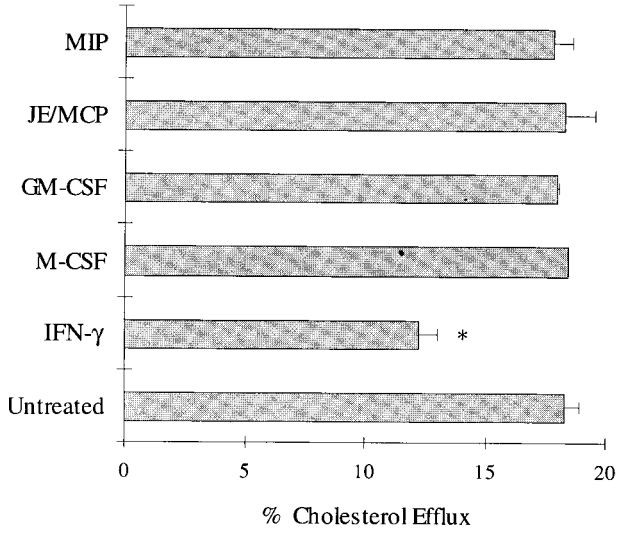

Fig. 1. Effects of macrophage-activating cytokines on cholesterol efflux. Macrophages were labeled with [^{14}C]cholesterol plus 50 μg/ml Ac-LDL for 48 h prior to treatment with 300 units/ml IFN-γ, 10 ng/ml M-CSF, GM-CSF, JE/MCP, or MIP for 48 h in 1 mg/ml BSA, RPMI 1640 medium. Efflux of [^{14}C]cholesterol was measured after 4 h incubation at 37°C with 100 μg/ml HDL_3, and is expressed as the percent of total [^{14}C]cholesterol appearing in the medium. Each value represents the mean ± SD of triplicates. * Significant difference between Control and IFN-γ ($P < 0.05$).

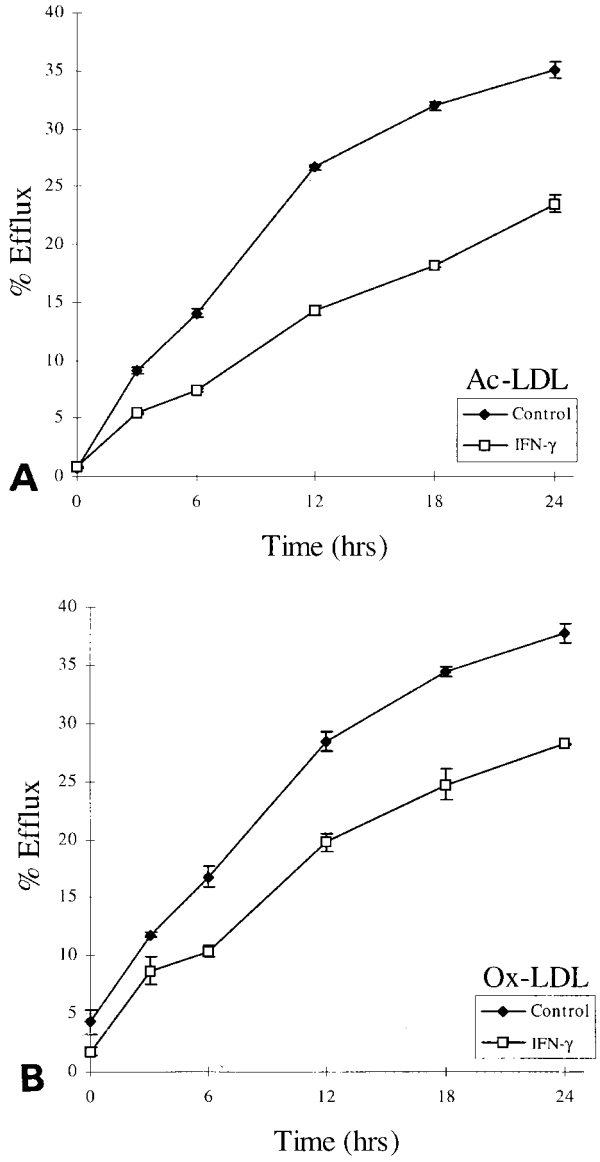

Fig. 2. Kinetics of HDL_3-mediated cholesterol efflux from macrophages. Macrophages were converted into foam cells with (A) 50 μg/ml Ac-LDL or (B) 50 μg/ml Ox-LDL and labeled concurrently with [^{14}C]cholesterol for 48 h. After the 48-h incubation, lipoprotein-containing media were changed to RPMI 1640 with 0.1% BSA medium alone (□) or medium containing 300 units/ml IFN-γ (◆) for 48 h. Efflux to HDL_3 was measured at the indicated time points and expressed as percent of total [^{14}C]cholesterol. Each value represents the mean ± SD of triplicates. Statistically different ($P < 0.01$) values were obtained for IFN-γ for all time points after "0" compared with those of the Control; representative experiment of three.

cholesterol efflux from macrophage-derived foam cells. As demonstrated in **Fig. 1**, IFN-γ in distinction to the other cytokines evaluated, inhibited efflux by 34% using HDL_3 as the acceptor species. This effect was observed even with 24 h pretreatment, but it was maximal by 48 h. A dose of 300 units/ml for IFN-γ and 10 ng/ml for the other cytokines was used in order to achieve maximal effects, even though reduction in cholesterol efflux can also be achieved with 1 unit/ml of IFN-γ. The inhibition of cholesterol efflux was specific to IFN-γ and was not related to differences in cholesterol loading as total radioactivity was equal between control and treated cells. These results suggest that the effect of IFN-γ on reducing cholesterol efflux in foam cells was not attributable to macrophage activation as none of the other cytokines exhibited a similar effect.

Inhibition of cholesterol efflux by IFN-γ was observed when either Ac-LDL or Ox-LDL (50 μg/ml) were used for conversion of macrophages to foam cells (**Fig. 2**). Furthermore, the effects of IFN-γ on efflux were apparent at all timepoints evaluated after addition of HDL_3 as the acceptor species. The effect of IFN-γ was more prominent in the Ac-LDL-treated cells where the reduction relative to the control cultures was 48% and 47% at the 6 and 12 h time point, respectively, compared to 39% and 31% for the Ox-LDL-treated cells. The kinetics observed for efflux would suggest that IFN-γ does not completely inhibit but rather reduces the rate of cholesterol efflux.

In an attempt to define the mechanism by which IFN-γ reduced cholesterol efflux, the role of apoE secretion was evaluated. As apoE secretion has been implicated in cholesterol efflux (24, 25) and IFN-γ has been reported to inhibit secretion of apoE by macrophages (11, 12), it was necessary to determine whether the reduction in efflux was mediated through decreased apoE secretion. Therefore, the effects of IFN-γ on cholesterol efflux were determined in macrophage-derived foam cells from apoE KO mice to eliminate the contribution of macrophage synthesized apoE in the efflux process. As demonstrated (**Fig. 3**), peritoneal macrophages from apoE KO or from wild-type mice loaded with Ac-LDL when exposed to IFN-γ exhib-

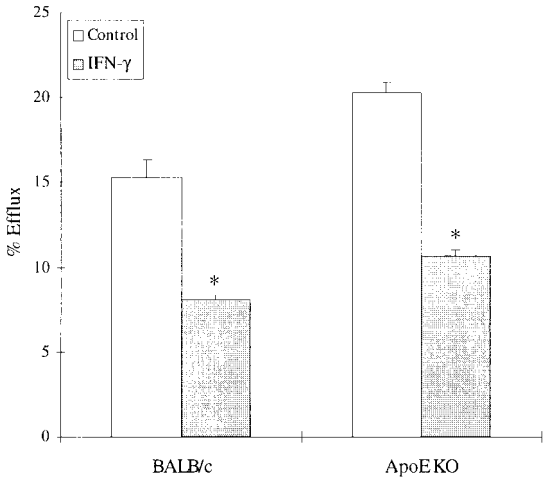

Fig. 3. IFN-γ effects on cholesterol efflux are independent of apoE synthesis. Peritoneal macrophages from BALB/c or apoE KO mice were incubated with 50 μg/ml Ac-LDL in the presence of [^{14}C]cholesterol for 48 h and then for an additional 48 h in RPMI medium containing 1 mg/ml BSA with 300 units/ml IFN-γ. Efflux was measured after incubation with 100 μg/ml HDL_3 for 6 h at 37°C. In the absence of HDL_3 as acceptor, efflux to BSA was less than 3%. * Significant difference between Control and IFN-γ ($P < 0.01$).

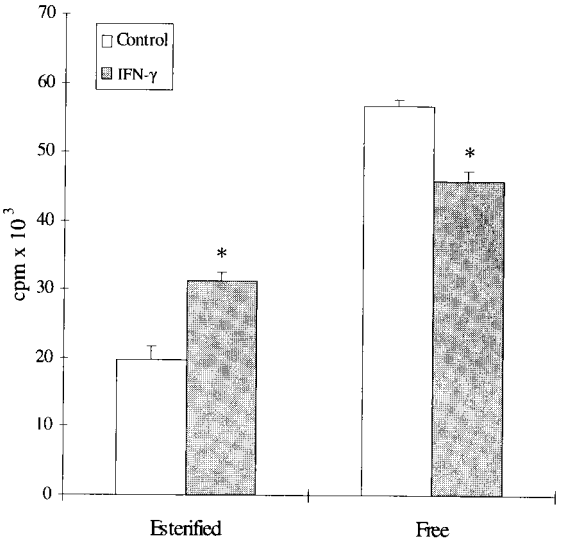

Fig. 5. IFN-γ-induced redistribution of radiolabeled cholesterol in foam cells. Peritoneal macrophages were loaded with cholesterol by incubation with 50 μg/ml Ac-LDL and labeled with [^{14}C]cholesterol for 48 h. Cells were untreated or treated with 300 units/ml IFN-γ prior to extraction of lipids using hexane–isopropanol 3:2. Cholesterol and cholesteryl esters were separated by TLC, visualized by I_2 vapors, and quantified by scintillation counting. Each value represents the mean ± SD as indicated by the error bars. * Significant difference between IFN-γ and Control ($P < 0.05$); representative experiment of five.

ited a comparable reduction in cholesterol efflux suggesting that IFN-γ exerts its effects by a mechanism independent of apoE.

As cholesterol efflux is dependent on HDL binding and only the class B scavenger receptors CD36 and SR-BI have been clearly identified as mediating HDL binding and efflux (26–28), the effects of IFN-γ on SR-BI and CD36 message expression were evaluated. As demonstrated (**Fig. 4A, B**), IFN-γ had no significant effect on SR-BI message levels whether macrophages were loaded with Ac-LDL or Ox-LDL. However, consistent with observations reported in human macrophages (29), a reduction in CD36

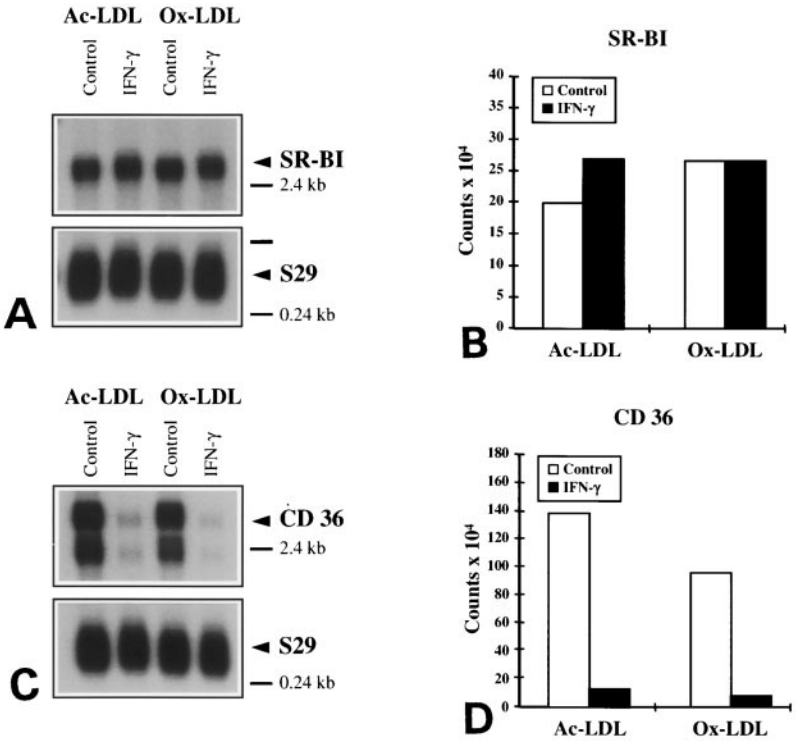

Fig. 4. Effect of IFN-γ on macrophage HDL scavenger receptors. Macrophage-derived foam cells obtained after incubation with 50 μg/ml of Ac-LDL or Ox-LDL were incubated for an additional 48 h in the presence or absence of 300 units/ml IFN-γ. Poly A^+-enriched RNA was isolated and analyzed by Northern blot analysis. Filters were hybridized with PCR-labeled probes for SR-BI (A) or CD36 (C) with S29 as an internal control. Hybridization intensity was quantitated by phosphoimager and expression was normalized for differences in loading using S29 as an internal control (B, D).

message was observed after treatment with IFN-γ (Fig. 4C, D). These results would suggest that the reduction in cholesterol efflux could occur via a receptor-related mechanism. Furthermore, and consistent with previous results (S. H. Zuckerman, C. Panousis, and G. F. Evans, unpublished results), the relative mRNA levels would suggest that CD36 represents the more abundant HDL scavenger receptor on murine peritoneal macrophage-derived foam cells.

In addition to the effect of IFN-γ on CD36, it was possible that a similar reduction in cholesterol efflux would occur if IFN-γ mediated a shift in the cellular pool of free cholesterol. In an attempt to explore this possibility further, the levels of free and esterified cholesterol were quantitated in peritoneal macrophages labeled with [^{14}C]cholesterol plus Ac-LDL for 48 h in the presence or absence of IFN-γ. As demonstrated (**Fig. 5**), a pronounced shift towards esterified cholesterol was observed upon treatment with IFN-γ. There was a 54% increase in cholesteryl esters and a 20% reduction in free cholesterol, or a shift in the free/esterified ratio from 2.8 in the control to 1.5 in the IFN-γ-treated cells. This represents 14% of the total radioactivity that moved from the free to the esterified cholesterol pool.

Equilibration of radiolabeled cholesterol between free and the esterified pool is achieved after prolonged incubations (30), which means that the specific activities of free and esterified cholesterol may be different. Therefore, changes in both free cholesterol and in the amounts of the various cholesteryl esters were quantitated by HPLC (22). As evident (**Fig. 6**), the effect of IFN-γ on increasing cholesteryl ester was significant for cholesteryl ester species including, eicosapentaenoate, docosapentaenoate, arachidonate, and linoleate. The percent change for each of the esters was calculated based on areas under the curve and averages from three chomatograms are presented (**Table 1**). While the trends in other cholesterol species were increased with IFN-γ, these changes did not achieve statistical significance. The increase by more than 50% in the total cholesteryl ester by IFN-γ was confirmed by HPLC, the reduction in the free cholesterol was only 5.5%, indicating that the specific activity of free cholesterol in IFN-γ-treated cells is 85% of that in control cells. These combined results would suggest that IFN-γ did induce a redistribution of cellular cholesterol with a decrease in radiolabeled free cholesterol associated with an increase in the esterified pool.

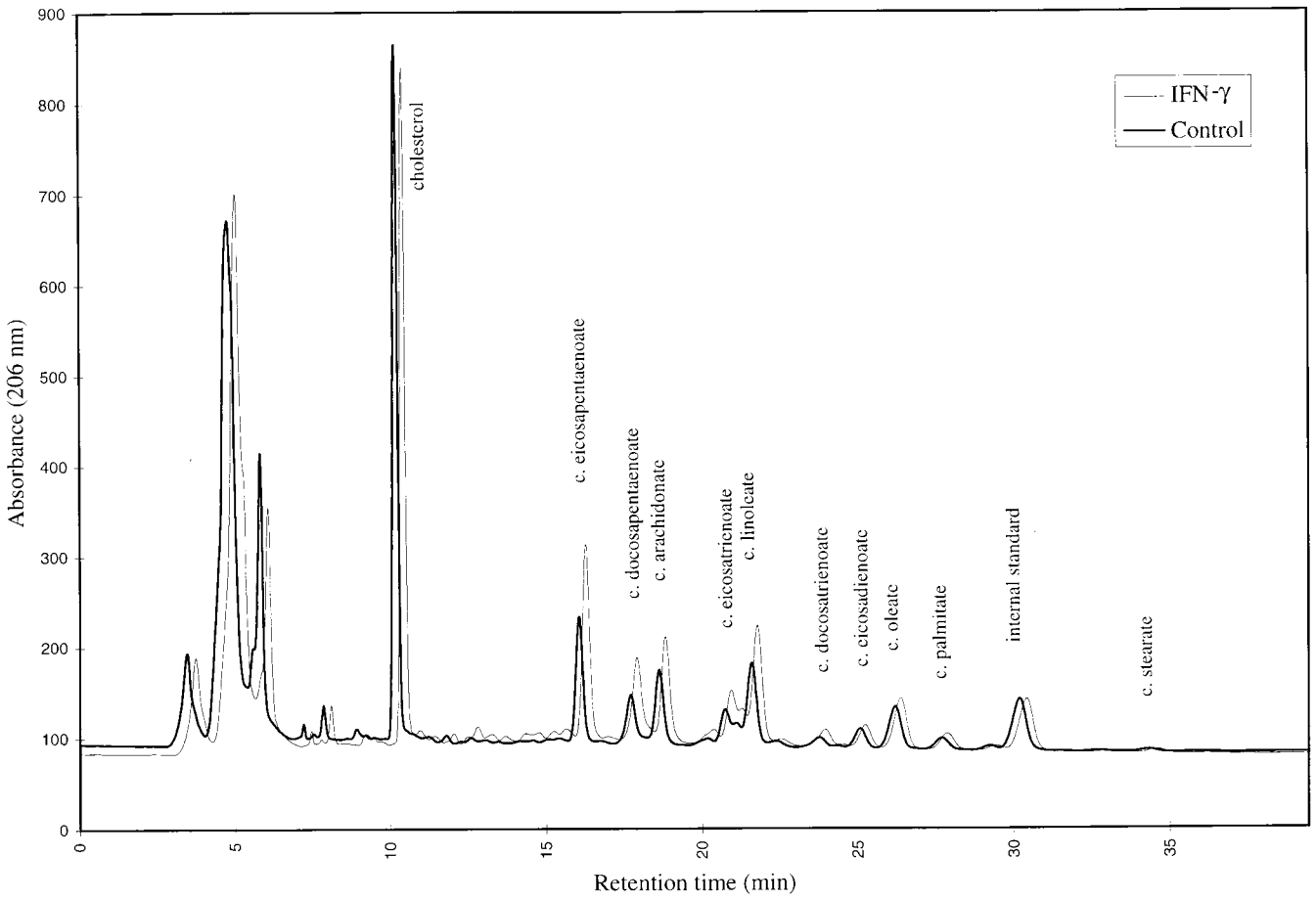

Fig. 6. IFN-γ-induced changes in cholesteryl ester content in foam cells. Macrophages converted to foam cells by incubation with Ac-LDL for 48 h were incubated for an additional 48 h in the presence or absence of 300 units/ml IFN-γ. Lipid extracts were analyzed by HPLC and the peaks for the cholesteryl esters arachidonate, linoleate, oleate, palmitate, and stearate were identified after comparison with elution times of commercially available standards. The remainder of the ester peaks were identified based on their relative elution times when compared to published values. Cholesterol heptadecanoate was used as internal standard as described in the materials and methods. The above chromatogram is representative of four. The HPLC profile for the IFN-γ extract was intentionally staggered to facilitate peak comparisons.

TABLE 1. IFN-γ-induced increase in cholesteryl esters

Cholesteryl esters	Change
	%
Eicosapentaenoate	71.5%
Docosapentaenoate	98.9%
Arachidonate	49.1%
Eicosatrienoate	57.2%
Linoleate	67.5%
Docosatrienoate	29.3%
Eicosadienoate	−6.2%
Oleate	11.5%
Palmitate	−20.0%
Stearate	58.2%

Average percent change of surface areas for the corresponding cholesterol and cholesteryl ester peaks from three chromatograms, adjusted by the internal control. SD of all CE was less than 20% except for the docosatrienoate, eicosadienoate, palmitate, and stearate that we were unable to measure accurately due to the size of the peaks.

The demonstration by both HPLC and TLC that IFN-γ-treated macrophages exhibited an increased accumulation of esterified cholesterol suggested a role for NCEH and ACAT. Modulation of either enzyme activity will result in a shift in the intracellular distribution between free and esterified cholesterol (31). As described (**Fig. 7A**), IFN-γ-treated cells did exhibit an increase in ACAT and this was independent of whether the cells were control or lipid-loaded with either Ac-LDL or Ox-LDL. IFN-γ-treated foam cells had a 2- to 3-fold increase in ACAT activity when compared to the non-IFN-γ-treated cells. This increase could not be explained by differences in the uptake of [^{14}C]oleate as both treated and untreated cells accumulate the same amount of total radioactivity in cholesteryl esters, triglycerides, and phospholipids combined (data not shown). In contrast with ACAT, no differences were observed with IFN-γ in NCEH activity whether or not the cells had been converted to foam cells with Ac-LDL (Fig. 7B). These results would suggest that the increase in cholesteryl ester accumulation mediated by IFN-γ was due to an increase in ACAT activity and not to a decrease in the catabolism of cholesterol esters by NCEH.

Northern blot analysis was then performed to determine whether the increase in ACAT activity was due to post-translational modifications of ACAT or whether it reflected changes in ACAT message levels. As demonstrated (**Fig. 8**), IFN-γ treatment resulted in a 65–75% increase in the expression of ACAT by macrophage-derived foam cells. These results were consistent with the higher basal ACAT activity observed in Ac-LDL-treated cells and the higher fold increase in ACAT activity by IFN-γ in Ox-LDL-derived foam cells. Therefore, the Northern data would suggest that the increase in ACAT activity induced by IFN-γ was due to changes in ACAT mRNA levels.

Finally, cholesterol efflux experiments in IFN-γ-treated foam cells were performed in the presence of the ACAT inhibitor A58035, to determine whether the reduction in cholesterol efflux by IFN-γ was due to changes in ACAT activity or CD36 expression. Accordingly, [^{14}C]cholesterol-labeled foam cells were incubated in the absence or presence of A58035 and efflux was evaluated 6 h after the addition of HDL_3. As demonstrated (**Fig. 9**), A58035 inhibited the IFN-γ-mediated reduction in cholesterol efflux in a dose-dependent manner. At an ACAT inhibitor concentration of 16 μg/ml, the difference in cholesterol efflux between the control and IFN-γ-treated macrophages was no longer statistically significant ($P < 0.2$). These results suggest then that the effects of IFN-γ on cholesterol efflux are mediated primarily through the up-regulation of ACAT with the reduction in CD36 expression playing a less significant role.

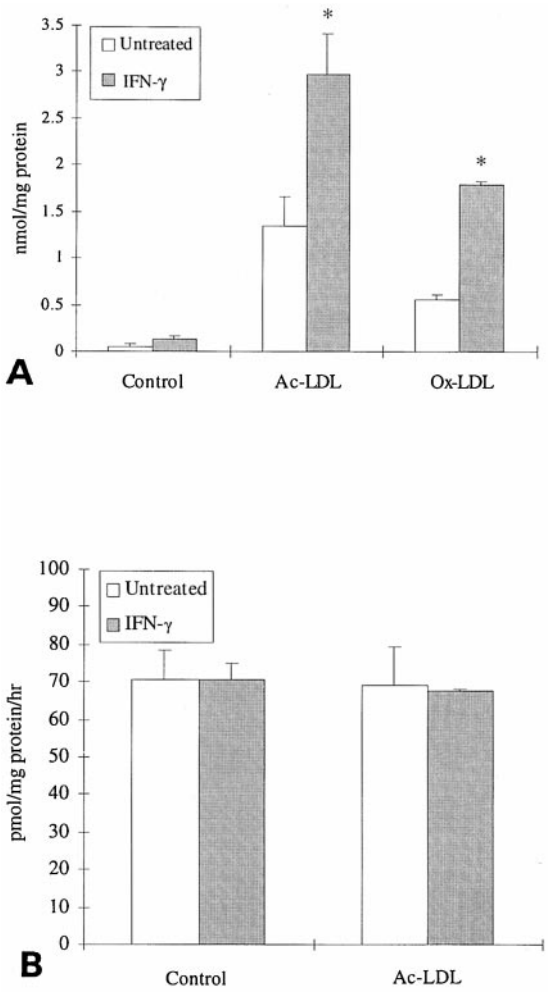

Fig. 7. Effect of IFN-γ on cholesteryl ester metabolism. (A) ACAT activity was measured in foam cells (control) which were untreated (open bars) or stimulated with 300 units/ml IFN-γ (solid bars) for 48 h. During the final 2 h, cells were pulsed labeled with [^{14}C]oleate–albumin complex, and assayed for ACAT activity by quantitating cholesteryl [^{14}C]oleate content; representative experiment of three. (B) NCEH activity was measured as described in Materials and Methods in parallel cultures. Hydrolytic activity was expressed as pmol of free [^{14}C]cholesterol released per mg of protein in 1 hour; representative experiment of two. * Significant difference between untreated and IFN-γ-treated foam cells ($P < 0.05$).

DISCUSSION

The macrophage-activating factor, IFN-γ, has been detected within the atherosclerotic lesion and its effects on

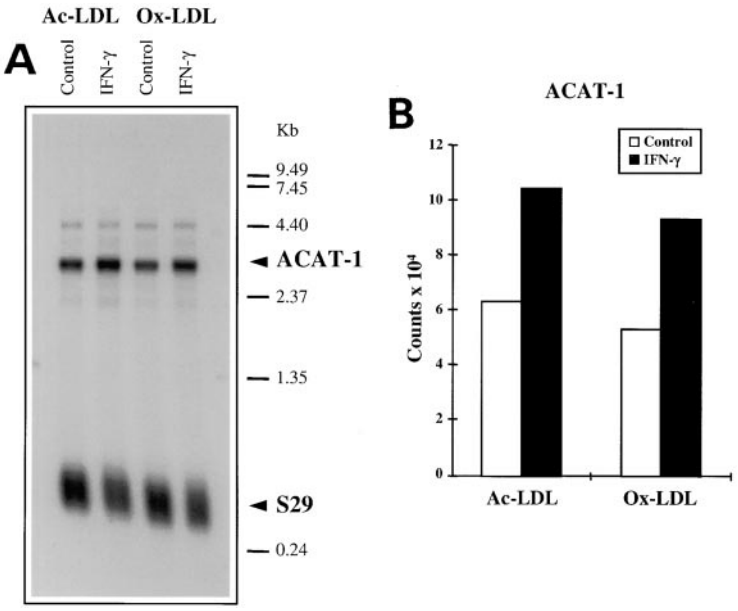

Fig. 8. Up-regulation of ACAT expression by IFN-γ. (A) Macrophages were incubated for 48 h with Ac-LDL or Ox-LDL and then for an additional 48 h in the presence or absence of 300 units/ml IFN. Foam cells were then lysed, poly A^+ mRNA was isolated and subjected to Northern blot analysis and hybridized with PCR-labeled probes specific for ACAT-1 and S29; representative experiment of three. (B) Quantitation of radiolabeled bands was performed by phosphorimager, and expression was adjusted to differences in loading by using S29 as an internal standard.

macrophage activation suggest an association with lesion development and progression (6, 7). Specifically, IFN-γ has been reported to induce iNOS, cytokine and protease secretion, expression of MHC II, and to decrease apoE secretion by macrophages (32, 33). However, while likely to contribute to lesion progression, the effect of IFN-γ on macrophage-derived foam cells remains unclear. Using murine and human macrophages for example, it has been demonstrated that IFN-γ down-regulates the expression of the scavenger receptor A (16), and the secretion of lipoprotein lipase (34), resulting in in vitro inhibition of lipid accumulation and foam cell conversion. Even though this suggests that IFN-γ will have a protective role in vivo on macrophage foam cell formation and cholesterol accumulation, Gupta et al. (14) reported that apoE KO mice crossed with IFNγR KO mice displayed a 60% reduction in lesion lipid accumulation (14). The results of the present study are consistent with this in vivo effect as IFN-γ increased the esterified cholesterol pool and decreased cholesterol efflux in macrophage-derived foam cells. The increase in cholesteryl ester accumulation was associated with an increase in both ACAT activity and mRNA levels.

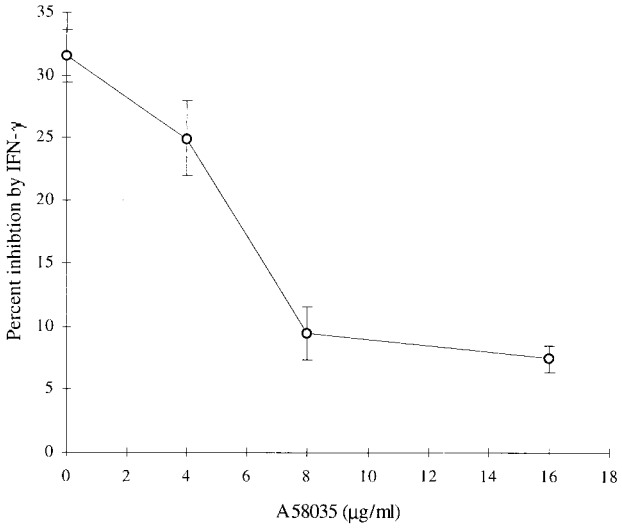

Fig. 9. IFN-γ-mediated effects on cholesterol efflux are reversed by an ACAT inhibitor. Macrophages were loaded with cholesterol by incubation with 50 μg/ml Ac-LDL, and labeled with [^{14}C]cholesterol for 48 h. Radiolabeled foam cells were incubated in the absence or presence of 300 units/ml IFN-γ concomitant with increasing concentrations of A58035. Efflux to HDL_3 from untreated and IFN-γ-treated cells was measured at 5 h, and the percent inhibition of efflux by IFN-γ was expressed relative to the A58035 concentration. Brackets indicate standard deviations.

The level of free cholesterol in mammalian cells is tightly regulated, as changes in membrane cholesterol have major effects on an array of biological functions (35) and high levels of free cholesterol are toxic for cells (36). Regulation of membrane cholesterol can be achieved through extracellular processes that involve uptake or efflux of free cholesterol between the plasma membrane and lipoproteins or can be achieved intracellularly through the actions of ACAT and NCEH (35). Changes in the redistribution of intracellular cholesterol between the free and the esterified pool have a major impact on cholesterol efflux. Increased cholesterol efflux for example, can be observed in murine macrophages upon stimulation of NCEH activity with cAMP (37) or by reducing ACAT activity with ACAT inhibitors (38). In contrast, reduction in cholesterol efflux by sequestration of free cholesterol efflux from the plasma membrane can be observed in human skin fibroblasts with monensin and brefeldin (39) or in smooth muscle cells by increased ACAT activity and a shift towards accumulation of cholesteryl esters (21). In the present study, inhibition of cholesterol efflux by IFN-γ coincided with an increased accumulation of cholesteryl esters. That the increase in cholesterol esters during incubation with IFN-γ occurred in a medium with no lipoproteins present and consequently in the absence of efflux sug-

gests that the effects of IFN-γ on cholesterol redistribution are intracellular and involve ACAT and NCEH activity.

Cholesteryl esters stored as cytoplasmic droplets are not inert, but exist in a dynamic state undergoing continuous hydrolysis by NCEH, and re-esterification by ACAT (31). In the present study, IFN-γ had no effect on NCEH activity, but increased ACAT activity 2- to 3-fold. Increased ACAT activity can be due to an increase in mRNA expression, as has been shown during the differentiation of monocytes to macrophages (40) or after dexamethasone treatment of murine macrophages (41). Alternatively, increased ACAT activity can be independent of transcriptional regulation as has been shown with cholesterol loading and with protein synthesis inhibitors (42). However, as ACAT activity in the present study was quantitated on intact cells, the observed increase with IFN-γ can reflect enhanced substrate availability instead of an actual increase in ACAT activity. This is unlikely to be the case as ACAT activity increased more than 2-fold after 24 h of incubation with IFN-γ, where almost no differences in lipid accumulation were observed (data not shown). Moreover, a 65% and 75% increase in ACAT expression with IFN-γ in Ac-LDL and Ox-LDL-loaded cells, respectively, was observed, suggesting that the increase in ACAT activity was related to increased enzyme content. The importance of the increase in ACAT activity in decreasing cholesterol efflux was demonstrated by reversing this effect with the ACAT inhibitor A58035. This reversal also suggests that the down-regulation of CD36 contributes only minimally to the observed reduction of cholesterol efflux, and that the effect is mediated mainly through the up-regulation of ACAT. The inhibition of cholesterol efflux was also independent of SR-BI, as no differences in its expression were observed upon IFN-γ treatment. These results would then suggest that the major pathway by which IFN-γ reduces cholesterol efflux is through increasing ACAT activity which leads to increased amounts of cholesteryl esters and sequestration of free cholesterol from the plasma membrane.

The isoform of ACAT that is expressed in macrophages is ACAT-1 (42, 43), while ACAT-2 is expressed primarily in the intestine and liver and is not detected in macrophages (44, 45). Peritoneal macrophages from ACAT-1 KO mice had reduced cholesteryl ester accumulation (46), and rabbits fed an atherogenic diet had a 2- to 3-fold increase in aortic ACAT-1 mRNA expression (47). Furthermore, in human atherosclerotic lesions, ACAT-1 expression was detected in macrophages, with greater expression in atheromatous plaques than in fatty streaks or in diffuse intimal thickening (48). These results suggest that ACAT-1 plays an important role in foam cell formation and the development of atherosclerosis.

In the present study, the observation that IFN-γ up-regulates ACAT-1 and can impact foam cell formation by inhibiting cholesterol efflux further supports its proatherogenic role. Additionally, the fact that a macrophage-activating cytokine can have a negative impact on cholesterol efflux provides further evidence that atherosclerosis is an inflammatory disease. Future studies will address the question of the mechanism that underlies the increase in ACAT-1 message by IFN-γ, and also the impact that IFN-γ may have on de novo synthesis of cholesterol, as an increase in total cholesterol mass by IFN-γ was observed.

In summary, the present study demonstrates that IFN-γ up-regulates ACAT-1 and thus mediates part of its proatherogenic effects by increasing cholesteryl ester accumulation and reducing cholesterol efflux. Elucidation of the pathway that leads to this up-regulation and concomitant change in intracellular cholesterol trafficking could contribute to the development of novel therapeutics that target atherosclerosis at the level of the vascular wall.

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