Interferon-γ Inhibits Macrophage Apolipoprotein E Production by Posttranslational Mechanisms

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Abstract

Macrophage-derived apolipoprotein (apo) E and multimers of a synthetic apo E-peptide display monokine-like functions by inhibiting mitogen- or antigen-driven lymphocyte proliferation. This study demonstrated how the target lymphocyte itself can modulate macrophage apo E production. The lymphokine interferon-γ (IFN) dramatically inhibited the accumulation of apo E in the supernatant of human monocytic THP-1 cells when present during phorbol myristate acetate–induced differentiation. A similar effect was observed when IFN was added to differentiated THP-1 cells. Treatment with IFN did not change the steady-state levels of apo E mRNA. Furthermore, in the presence of IFN no increased degradation or increased uptake of extracellular apo E was detected. Pulse-chase experiments indicated that IFN reduced the accumulation of extracellular apo E and increased the degradation of intracellular apo E. The inhibitory effect of IFN on apo E production also was observed in human monocyte-derived macrophages. Thus, our data demonstrated that IFN inhibited macrophage apo E production by posttranslational mechanisms. This represents a previously uncharacterized immunoregulatory interaction and lends further support to a relationship between lipid metabolism and the immune system. (*J. Clin. Invest.* 1993. 91:2031–2039.) Key words: differentiation • intracellular degradation • lymphokines • phorbol ester • THP-1 cells

Introduction

Apolipoprotein (apo) E is a major constituent of several subclasses of plasma lipoproteins and displays an important function in lipid metabolism as a targeting protein in receptor-mediated uptake of lipoproteins (1). However, unlike most apolipoproteins, which are produced mainly in the liver and the small intestine, apo E is synthesized also by numerous peripheral tissues including macrophages (2–4).

The regulation of apo E expression in macrophages is complex, and is dependent on the differentiation state of the cell (5). Cultured human monocytic THP-1 cells increase apo E expression during differentiation into macrophages, and this expression is regulated by both transcriptional and posttranscriptional mechanisms (6, 7). In mouse peritoneal macrophages, a variety of activating agents including bacterial endotoxin modulate apo E expression (8, 9). We have shown that human monocyte-derived macrophages cultured in the presence of activated platelets or the releasates of activated platelets manifest a dramatic increase in their secretion of apo E (10). In mouse macrophages and differentiated THP-1 cells, cholesterol loading increases apo E gene transcription resulting in increased levels of both apo E mRNA and apo E protein (11–13). Furthermore, a recent report indicates that HDL-induced apo E production in the mouse macrophage cell line, J774, is regulated by posttranslational mechanisms (14).

It has been suggested that apo E produced by macrophages and other extrahepatic tissues plays a role in the movement of cholesterol from peripheral tissues to the liver and may serve as a targeting molecule for local redistribution of cholesterol within tissues undergoing remodeling (1). Furthermore, our laboratory has demonstrated that apo E isolated from human monocyte-derived macrophage culture supernatants or synovial fluids also has immunoregulatory monokine-like functions (15). We have shown that macrophage-derived apo E is a potent inhibitor of mitogen- or antigen-driven lymphocyte proliferation (16) and apo E isolated from synovial fluids inhibits neutrophil functions (17). Apo E is the biologically active component of the plasma immunoregulatory lipoprotein, LDL-In (18). In addition, we have shown recently that multimers of a synthetic peptide representing amino acid residues 141–155 of the human apo E molecule can mimic its inhibitory effect on lymphocyte proliferation (19).

The present study was designed to identify mechanisms by which the target lymphocyte itself could modulate the expression of macrophage apo E. IFN is a potent lymphokine that is secreted mainly by activated T lymphocytes and has numerous effects on cells of monocytic lineage (20, 21). A preliminary report noted that IFN reduces apo E production in mouse macrophages (22). For a detailed analysis of the effects of IFN on apo E expression, we have used human THP-1 monocytic cells (23), a widely used model for studying monocyte/macrophage function (24, 25). We and others have shown that THP-1 cells take on macrophage-like characteristics when stimulated with phorbol ester (25, 26). In the present study we demonstrated that IFN dramatically inhibited the production of apo E in THP-1 macrophage-like cells by posttranslational mechanisms. In addition, we identified a similar inhibitory effect of IFN on apo E production in human monocyte-derived macrophages.

Methods

Cell culture. THP-1 cells (American Type Culture Collection, Rockville, MD) were maintained in suspension at a density of 2.0×10^5 to 1.0×10^6/ml in RPMI-1640 (low endotoxin) containing 7% fetal calf serum, 10 mM Hepes, 1 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 5×10^{-5} M β-mercaptoethanol. The experiments were performed under serum-free con-

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ditions as described earlier (26) by washing the cells and replacing the 7% fetal calf serum with 1% Nutridoma-HU (Boehringer Mannheim Biochemicals, Indianapolis, IN). Unless otherwise stated, cells were plated at a density of 5×10^5 cells in 1 ml per well in 24-well tissue culture dishes. PBMC were isolated using Ficoll-Hypague as described earlier (26). Monocytes were isolated from PBMC using Sepracell-MN (Sepratech Corp., Oklahoma City, OK) and by an additional adherence step on fibronectin-coated tissue culture plates (Collaborative Research Inc., Bedford, MA). The purity of the monocyte preparation was > 95% as determined by nonspecific esterase staining (10). The monocytes were cultured under low-endotoxin conditions at a density of 1×10^6 cells in 1 ml per well in 24-well tissue culture dishes as described (26, 27). PMA (Sigma Chemical Co., St. Louis, MO) was used at a concentration of 10^{-7} M for THP-1 cells and 10^{-8} M for isolated monocytes throughout the study. Recombinant human interferon-γ (2.5×10^7 U/mg) was obtained from Genzyme Corp., Cambridge, MA. Viability of cells was monitored by trypan blue exclusion, and cellular DNA and protein were determined as described previously (26).

Competitive apo E immunoassay. Apo E in the supernatant was measured with a solid-phase radioimmunoassay performed as described previously (10) using ^{125}I-labeled apo E purified from human plasma VLDL and a human apo E–specific monoclonal antibody, 1E.

PAGE and Western blot analysis. Electrophoresis was performed with 3–20% gradient polyacrylamide slab gels (0.1% SDS) and carried out at 12 mA for 20 h at room temperature as previously described (10). For protein staining, the gel was stained with Coomassie Blue and destained with methanol-acetic acid. The proteins were transferred to a nitrocellulose membrane by electrophoresis as described earlier (10). After transfer, the nitrocellulose membranes were incubated with ascites fluid containing an apo E–specific monoclonal antibody, 1E, washed, and incubated with polyclonal goat anti–mouse IgG antibody. Antibody binding to apo E was visualized on X-ray film using the Immune-Lite chemiluminescent assay kit (Bio Rad Laboratories, Richmond, CA).

RNA isolation, Northern blotting, and hybridization. Cells were seeded at a density of $2–3 \times 10^6$ cells in 4 ml per well in six-well culture dishes. Total cellular RNA was purified by the acid guanidinium thiocyanate-phenol-chloroform extraction (28). Northern blotting and hybridization were performed as described earlier (24). Briefly, RNA was subjected to denaturing electrophoresis in 1.2% agarose-formaldehyde gels (29) and was transferred to GeneScreen (Du Pont-New England Nuclear, Boston, MA). An apo E cDNA fragment was obtained from American Type Culture Collection (30) and used to generate a 291-bp cDNA probe. This apo E cDNA probe was radiolabeled with [α-^{32}P]dATP (7,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) by the random primer method (Boehringer-Mannheim Biochemicals, Indianapolis, IN) to give a specific activity of $\geq 10^8$ cpm/μg. Prehybridization, hybridization (1×10^6 cpm/ml), and posthybridization washes were performed using 5% SDS exactly as described (31). Blots were analyzed by autoradiography and apo E mRNA levels were quantitated by scanning densitometry using an Ultroscan XL laser densitometer (LKB Produkter, Bromma, Sweden). The level of the "housekeeping" CHO-B mRNA was used as a control to normalize the amounts of RNA loaded (24).

[^{35}S]Methionine labeling of cells. Cells were incubated in the presence of PMA±IFN for different time intervals. The cells were washed between media changes with methionine-free RPMI-1640 before the pulse period and with regular RPMI-1640 before the chase period. For labeling, cells were incubated with 0.25 ml of methionine-free RPMI-1640 medium containing 100 μCi/ml (THP-1 cells) or 200 μCi/ml (monocyte-derived macrophages) of L-[^{35}S]methionine (1100 Ci/mmol, Du Pont-New England Nuclear, Boston, MA) and 1 μM of unlabeled L-methionine. When appropriate, the pulse was followed by 0.25 ml of chase medium (regular RPMI-1640, containing 500 μM of unlabeled methionine) for the time periods indicated. Both the pulse and the chase medium contained 1% Nutridoma, 10 mM Hepes, 1 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 5×10^{-5} M β-mercaptoethanol.

At the end of the incubation period, 100 kallikrein-inactivating units aprotinin and 0.1 mM PMSF were added to the wells to give a total volume of 0.275 ml and the medium was removed to prechilled microcentrifuge tubes. The samples were spun for 5 min at 10,000 *g* in a microcentrifuge to remove any floating cells. The supernatants were then precleared by adding 0.05 ml of a 10% suspension of protein A Sepharose CL-4B (Pharmacia-LKB, Piscataway, NJ) and rotating the tubes at 4°C for 45 min. The samples were spun at 10,000 *g* for 2 min to remove the protein A Sepharose beads and frozen at −20°C.

The cells were lysed with 0.275 ml of lysis buffer containing 0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 100 kallikrein-inactivating units aprotinin, and 0.1 mM PMSF. After vigorous scraping, the cell lysate was pipetted into prechilled microcentrifuge tubes and spun for 10 min at 10,000 *g* to remove any cell debris and also precleared with 0.05 ml of protein A Sepharose CL-4B beads as described above.

Immunoprecipitation of Apo E. Immunoprecipitation was performed as described earlier (4, 14) with several modifications. To reduce nonspecific binding, protein A Sepharose CL-4B (10% suspension in immunoprecipitation buffer, see below, 0.2% BSA) was pretreated with unlabeled cell extract from dividing THP-1 cells immediately before the experiment (rotator, 4°C, 45 min). ^{35}S-incorporation into protein of cell lysate and culture supernatant was determined by TCA precipitation (10%) of 0.01 ml of sample. Equal amounts of TCA-precipitable counts of cell lysate or supernatant were incubated on a rotator at 4°C for 12 h with 5 μg of apo E–specific monoclonal antibody, 0.5 ml of immunoprecipitation buffer (0.5% Nonidet P-40, 0.5% deoxycholic acid, 150 mM NaCl, 5 mM EDTA, 50 mM Tris [pH 7.4], 0.1% SDS, 0.02% sodium azide), and 0.03 ml of protein A Sepharose CL-4B beads. Nonspecific background was reduced as described (32) by layering the entire reaction mixture over a 1 M sucrose cushion, solubilizing the samples in 1% SDS and repeating the immunoprecipitation reaction.

After immunoprecipitation, the beads were washed five times and analyzed by gel electrophoresis and scintillation counting. For gel analysis, pellets were resuspended in Laemmli's sample buffer (1×: 10% glycerol, 5% β-mercaptoethanol, 3% SDS, 62.5 mM Tris [pH 6.8], 0.002% bromophenol blue), boiled for 5 min, and spun for 2 min at 10,000 *g*. The samples were analyzed by SDS-PAGE (3% stacking gel, 10% resolving gel). After staining with Coomassie Blue, gels were washed three times with distilled water and prepared for fluorography by soaking the gels in 1.0 M sodium salicylate for 30 min. After drying, gels were exposed to X-Omat AR x-ray film (Eastman Kodak Co., Rochester, NY) with an intensifier screen for up to 8 d. Human VLDL detected by Coomassie Blue or ^{14}C-labeled protein molecular weight markers (Amersham Corp.) were used as standards. The autoradiographs were quantitated by scanning densitometry as described above. Essentially the same results were obtained when we cut the bands from the gel identified by fluorography and analyzed them by scintillation counting. For this purpose, the gel pieces were treated with tissue homogenizer TS-1 (Research Products International, Mount Prospect, IL) for 2 h at 50°C before scintillation counting.

Miscellaneous. Tumor necrosis factor α was measured as described (33). Statistical significance was calculated using the two-tailed Student's *t* test. Data in text is given as mean±standard deviation.

Results

Apo E accumulation in the supernatant in the absence or presence of IFN. Our laboratory and others have shown that human monocytic THP-1 cells can be differentiated into macrophage-like cells by incubation with PMA (25, 26). During PMA-induced differentiation, a dramatic increase in both the level of apo E mRNA and apo E protein can be observed in

these cells (7, 26). Therefore, in initial experiments, THP-1 cells were incubated for 6 d with 10^{-7} M PMA in the absence or presence of 100 U/ml of IFN and the accumulation of apo E in the supernatant was quantitated by immunoassay. This dose of IFN is used widely to study the effects of this lymphokine on monocytic cells including THP-1 cells (34, 35). Treatment with IFN dramatically decreased the concentration of apo E in the supernatant at day 6 from 268±21 to 65±10 ng/μg DNA ($P < 0.001$, $n = 3$) (Miya, T., and L. K. Curtiss, unpublished observations). In the supernatant of dividing THP-1 cells (i.e., cells without PMA), < 3 ng of apo E per μg DNA was detected and this was not modulated by the addition of IFN (data not shown).

Fig. 1 illustrates this inhibitory effect of IFN on apo E accumulation in the supernatant. THP-1 cells were incubated up to 4 d (Fig. 1 *A*) with PMA±IFN and supernatants were analyzed for the presence of apo E by Western blot analysis. On days 2, 3, and 4, PMA treatment induced a continuous increase of apo E in the supernatant that was significantly inhibited by the simultaneous exposure to IFN. Fig. 1 *B* shows a more dramatic inhibitory effect of IFN in a separate experiment in which the accumulation of apo E was analyzed after treatment for 6 d. To detect only monocyte/macrophage-derived apo E and to exclude any effects that might be derived from serum-associated lipoproteins, we used a serum-free culture system (1% Nutridoma). As demonstrated earlier (26), THP-1 cells require ~ 1–2 d to adjust to serum-free 1% Nutridoma conditions and this can explain the delayed detection of apo E.

To document specificity of the apo E response to IFN, cells were examined for secretion of TNF. It has been established that IFN enhances the secretion of TNF in macrophages (20, 27). Cells were incubated with PMA±100 U/ml of IFN for 4 d and the production of TNF was analyzed. In untreated cells, the concentration of TNF in the supernatant was < 0.09 ng/10^6 cells. After 4 d of incubation with PMA, the TNF concentration had increased to 0.16±0.02 ng/10^6 cells which was further enhanced to 1.18±0.3 ng/10^6 cells by IFN ($n = 3$). Further support for specificity was obtained from our observation that, in contrast to the results obtained with IFN, incubation with macrophage colony-stimulating factor (M-CSF) or GM-CSF, two other potent cytokines known to modulate monocytic cell function (36), had no significant effect on apo E production as determined by immunoassay or Western blot analysis either in PMA-treated or in dividing THP-1 cells (Miya, T., and L. K. Curtiss, unpublished observations).

Effect of IFN on the steady-state levels of apo E mRNA. To examine if treatment with IFN had an effect on PMA-induced levels of apo E mRNA, cells were incubated for up to 6 d±IFN. Treatment with PMA induced a dramatic and sustained increase in the steady-state levels of apo E mRNA over a period of 6 d (Fig. 2). The presence of IFN did not change the apo E mRNA levels during the 6 d of incubation. This was in contrast to the observed inhibition of apo E accumulation in the supernatant by IFN (Fig. 1). No apo E mRNA was detected in these studies in dividing THP-1 cells or in cells treated with IFN in the absence of PMA over a period of 6 d (data not shown).

Effect of IFN dose on the accumulation of intracellular and extracellular apo E. The decreased accumulation of apo E in the supernatant in the presence of IFN without a concomitant change in the levels of apo E mRNA suggested control by translational and/or posttranslational regulatory mechanisms. To further investigate the level of regulation, we studied the effect of IFN on intracellular as well as extracellular apo E accumulation (Fig. 3). THP-1 cells were incubated with PMA±IFN (10 or 100 U/ml) for 4 d. At this time the cells were labeled with [^{35}S]methionine for 6 h, before cell lysate and supernatant samples were prepared for analysis of apo E levels by immunoprecipitation. Intracellular apo E accumulation was not significantly changed by 10 U/ml of IFN, but was slightly decreased at 100 U/ml (19±2% inhibition, $n = 3$). In contrast, extracel-

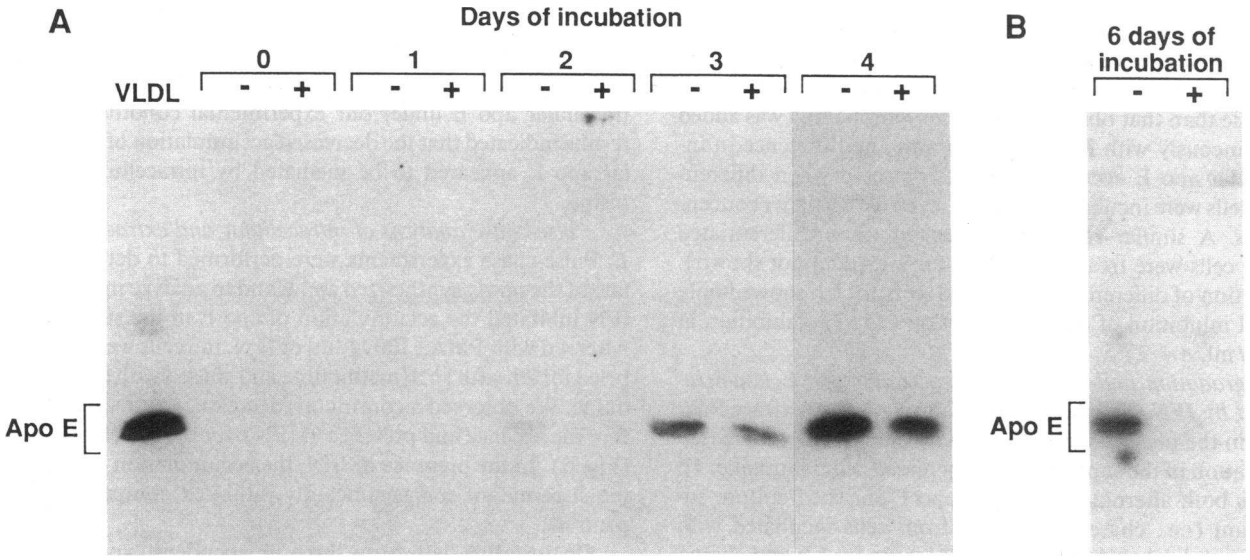

Figure 1. Accumulation of supernatant apo E induced by PMA in the absence or presence of IFN. (*A*) Cells at a density of 8×10^5/ml were incubated with 10^{-7} M PMA up to 4 d in the absence (−) and presence (+) of 100 U/ml of IFN and the supernatants were harvested daily. (*B*) Cells at a density of 5×10^5/ml were incubated with 10^{-7} M PMA±100 U/ml IFN for 6 d. The supernatants of *A* and *B* were analyzed by Western blotting with an apo E–specific monoclonal antibody. Human VLDL was used as a standard.

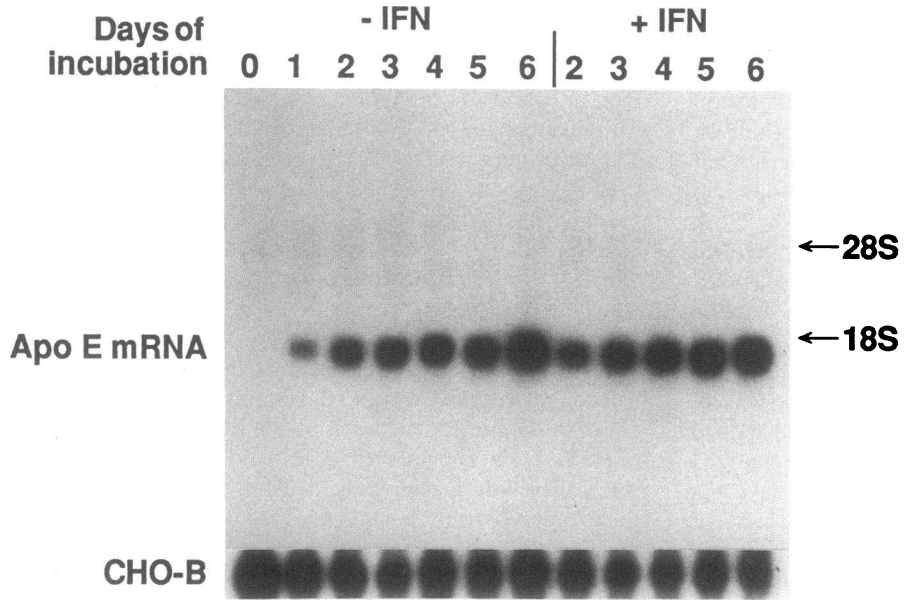

Figure 2. Time course of apo E mRNA accumulation induced by PMA in the absence and presence of IFN. Cells were incubated with PMA±100 U/ml of IFN for up to 6 d. Total RNA was prepared and the apo E mRNA content determined using a ^{32}P-labeled apo E cDNA probe. To account for variability in sample loading, the blot was rehybridized with a CHO-B cDNA probe, which detects a single mRNA species of 1.1 kb. The positions of the 18 S and 28 S ribosomal bands are indicated. Data shown are from a representative experiment which was repeated three times.

lular apo E accumulation was inhibited by 58±6% at 10 U/ml of IFN and more dramatically inhibited by 89±8% at 100 U/ml (n = 3). Several molecular weight species of apo E were observed in these blots consistent with previous studies (4, 37). The different sizes were apparently due to O-linked oligosaccharide chains containing differing amounts of sialic acid residues (4, 37). Thus, the expected shift towards higher molecular weight glycosylated isoforms in the secreted apo E compared to the intracellular apo E was observed.

Addition of IFN to differentiated THP-1 cells. To examine if IFN could exert its inhibitory effect on apo E production in already differentiated cells, THP-1 cells were incubated with PMA for 4 d. At this time, fresh medium (without PMA) was added that contained 0, 10, 100 or 500 U/ml of IFN and the cells were incubated for an additional 2 d. Treatment of differentiated cells with IFN produced a concentration-dependent inhibition of extracellular apo E accumulation (Fig. 4). The effect of IFN added after differentiation appeared to be less dramatic than that observed when this lymphokine was added simultaneously with PMA. Furthermore, no difference in intracellular apo E accumulation was detected when differentiated cells were incubated with IFN, even at the higher concentrations. A similar result was observed when differentiated THP-1 cells were treated with IFN for 1 d (data not shown). Incubation of differentiated cells with IFN for 6 h showed only a small inhibition of apo E production (15±2% inhibition at 100 U/ml, n = 2).

Degradation and uptake of extracellular apo E is not increased by IFN. The decreased accumulation of extracellular apo E in the presence of IFN could be due to either increased degradation in the supernatant or increased cellular uptake. To address both alternatives, labeled apo E-enriched culture supernatant (i.e., chase-supernatant from cells incubated with PMA for 4 d, labeled with [^{35}S]methionine for 6 h and chased for 2 h) was added to nonlabeled cells that had been treated with PMA±100 U/ml of IFN for 4 d. The supernatant and cell lysate were harvested over 4 h. We detected a similar decrease of apo E in the chase-supernatant whether it was added to non-treated or IFN-treated cells (Fig. 5). In addition, we detected a very low level of extracellular apo E uptake which increased to 3±1% in the absence and 4±1% in the presence of IFN after 4 h (n = 3). Similar results were obtained when we used labeled apo E–enriched culture supernatant from IFN-treated cells (data not shown).

Uptake of secreted apo E also was addressed by an independent experimental approach. Macrophages have high affinity sites that can bind and internalize apo E–containing lipoproteins (38, 39). This high-affinity uptake in macrophages is absolutely dependent on calcium ions. Therefore, cells were chased with medium containing EGTA at a concentration sufficient to chelate total calcium and magnesium ions in the medium. Furthermore, cells were chased in the presence of calcium- and magnesium-free Hanks' balanced salt solution. IFN inhibited the extracellular accumulation of apo E in the presence and absence of calcium by ~ 70% (Table I). Our data demonstrated that IFN did not increase degradation or uptake of extracellular apo E under our experimental conditions. These results indicated that the decreased accumulation of extracellular apo E appeared to be mediated by intracellular mechanisms.

Pulse-chase analysis of intracellular and extracellular apo E. Pulse-chase experiments were performed to determine the fate of the newly synthesized apo E and to analyze in detail how IFN inhibited the accumulation of apo E in the supernatant. After 4 d with PMA±100 U/ml of IFN, the cells were pulse-labeled for 2 h with [^{35}S]methionine and chased with cold methionine. We observed a continuous decrease in intracellular apo E in the absence and presence of IFN over the 5-h chase period (Fig. 6). In the presence of IFN, the accumulation of apo E in the supernatant was significantly inhibited compared to the control.

Quantitative data from three independent experiments is shown in Table II. In the absence of IFN, the majority of apo E lost from the intracellular pool during the chase period was recovered in the supernatant. In contrast, in the presence of IFN, the recovery of apo E in the supernatant was significantly

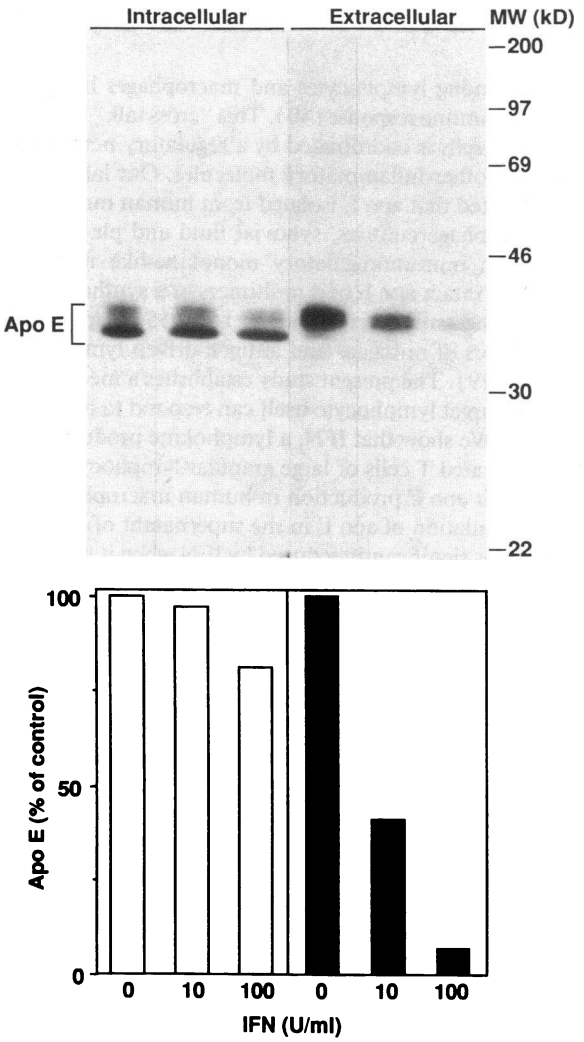

Figure 3. Effect of IFN on PMA-induced accumulation of intracellular and extracellular apo E. Cells were incubated with PMA for 4 d in the absence and presence of IFN (10 and 100 U/ml). At day 4, cells were labeled with 100 μCi/ml [^{35}S]methionine for 6 h, and cell lysate and supernatant were harvested. Apo E was analyzed by immunoprecipitation followed by SDS-PAGE. Human VLDL identified by Coomassie Blue (not shown) and ^{14}C-labeled molecular weight marker proteins were used as standards. The autoradiographs of electrophoretic analyses were quantitated by scanning densitometry. The amount of labeled apo E produced in the absence of IFN was defined as 100% for both intracellular or extracellular apo E. The experiment shown is representative of three separate studies.

reduced despite an accelerated decrease in intracellular levels of apo E. Therefore, our results indicated that IFN increased intracellular apo E degradation. This effect was even more significant because of the apparent reduced secretion efficiency, and appeared to account for the dramatically increased loss of total apo E during the chase period (Table II).

In the same experiments as described in Table II, we compared the fate of intracellular methionine-labeled total protein with that of intracellular apo E during the chase period in IFN-treated cells. The recovery of intracellular apo E was significantly lower than the recovery of total intracellular protein during the chase period (Table III). At the same time, no significant difference in the recovery of extracellular apo E or total protein was observed. This indicated that IFN specifically increased the intracellular degradation of apo E.

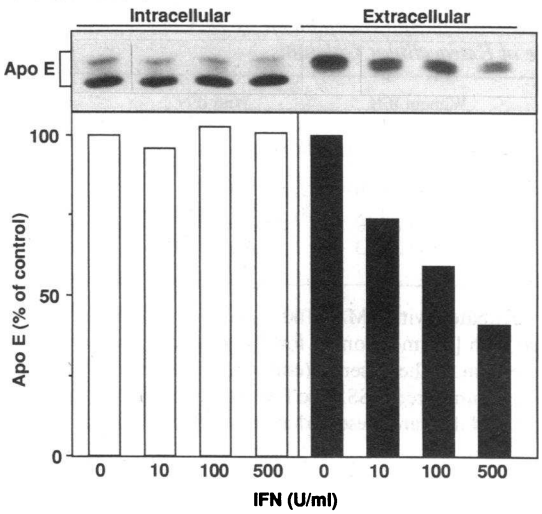

Figure 4. Effect of IFN on differentiated THP-1 cells. Cells were incubated with PMA for 4 d. At day 4, fresh medium (without PMA) was added that contained no IFN or increasing amounts of IFN (10–500 U/ml) and the cells were incubated for an additional 2 d. At day 6, cultures were labeled for 6 h with [^{35}S]methionine and cell lysate and supernatant were harvested and analyzed by immunoprecipitation as described for Fig. 3. One representative experiment of three separate experiments is shown.

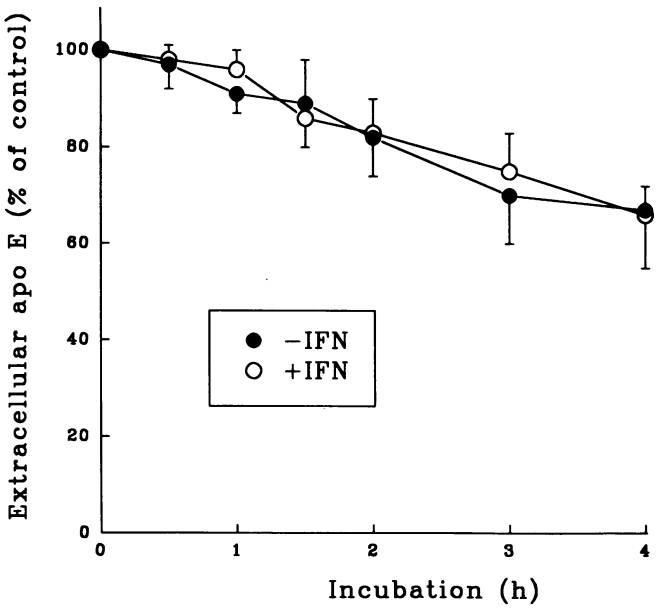

Figure 5. Extracellular degradation of apo E in the absence or presence of IFN. Labeled apo E–enriched culture supernatant (i.e., chase supernatant from cells incubated with PMA for 4 d, labeled with [^{35}S]methionine for 6 h and chased for 2 h) was added to nonlabeled cells treated with PMA±100 U/ml IFN for 4 d. The supernatants were harvested over 4 h and analyzed by immunoprecipitation. The amount of extracellular labeled apo E present at 0 h was defined as 100%. Data are shown as mean±SD (n = 3).

Table I. Inhibition of Apo E Productin by IFN in the Absence or Presence of Extracellular Calcium

	Without IFN	With IFN	Inhibition
	cpm/5 × 10⁵ cells		%
Control	23,397±1,670	6,980±231	70
EGTA	24,756±3,135	6,782±726	73
HBSS	25,636±2,123	7,431±1,001	71

Cells were incubated with PMA±100 U/ml for 4 d. At day 4, cells were labeled with [^{35}S]methionine for 2 h and chased for 2 h in complete medium in the absence (control) or presence of 1.5 mM EGTA or in calcium-free HBSS. Apo E was determined by immunoprecipitation, and data are presented as mean±SD (n = 3).

Effect of IFN on monocyte-derived macrophages. Finally, we investigated if the inhibitory effect of IFN on apo E production also could be observed in human monocyte-derived macrophages. The experiments were performed under similar conditions used for THP-1 cells. Adherent peripheral blood monocytes were incubated with 10^{-8} M PMA±IFN (10 or 100 U/ml) for 2 d (Fig. 7). At this time, the cells were labeled with [^{35}S]methionine for 6 h and apo E was analyzed in cell lysate and supernatant samples. Incubation with IFN did not change the accumulation of intracellular apo E. However, similar to the results obtained using THP-1 cells, the extracellular apo E accumulation was inhibited by IFN (70±8% by 100 U/ml, n = 3). These data suggested that a similar posttranslational effect of IFN on apo E production also existed in cultured human monocyte-derived macrophages.

Discussion

Interactions among lymphocytes and macrophages lie at the center of the immune response (40). This "cross talk" between hematopoietic cells is coordinated by a regulatory network of cytokines and other inflammatory molecules. Our laboratory has demonstrated that apo E isolated from human monocyte-derived macrophage cultures, synovial fluid and plasma can function as an immunoregulatory monokine-like molecule (15–18). Both intact apo E and multimers of a synthetic peptide representing amino acid residues 141–155 of apo E are potent inhibitors of mitogen- and antigen-driven lymphocyte proliferation (19). The present study establishes a mechanism by which the target lymphocyte itself can respond to this "apo E challenge." We show that IFN, a lymphokine produced primarily by activated T cells or large granular lymphocytes (20, 21), can inhibit apo E production in human macrophages.

The accumulation of apo E in the supernatant of cultured THP-1 cells was significantly reduced by IFN when it was present during differentiation or when added to differentiated cells. Treatment with this lymphokine did not change the steady-state levels of apo E mRNA. Furthermore, in the presence of IFN no increased degradation or increased uptake of secreted apo E was observed. Pulse-chase experiments performed in the absence of IFN indicated that the loss of intracellular apo E during the chase period was primarily due to secretion. This recovery of apo E in the supernatant was significantly inhibited by IFN. In addition, the decrease in intracellular apo E during the chase period was accelerated in IFN-treated cells versus nontreated cells. These data suggested two alternative hypotheses. Either IFN specifically inhibited the secretion of apo E that favored intracellular degradation, or IFN increased the intra-

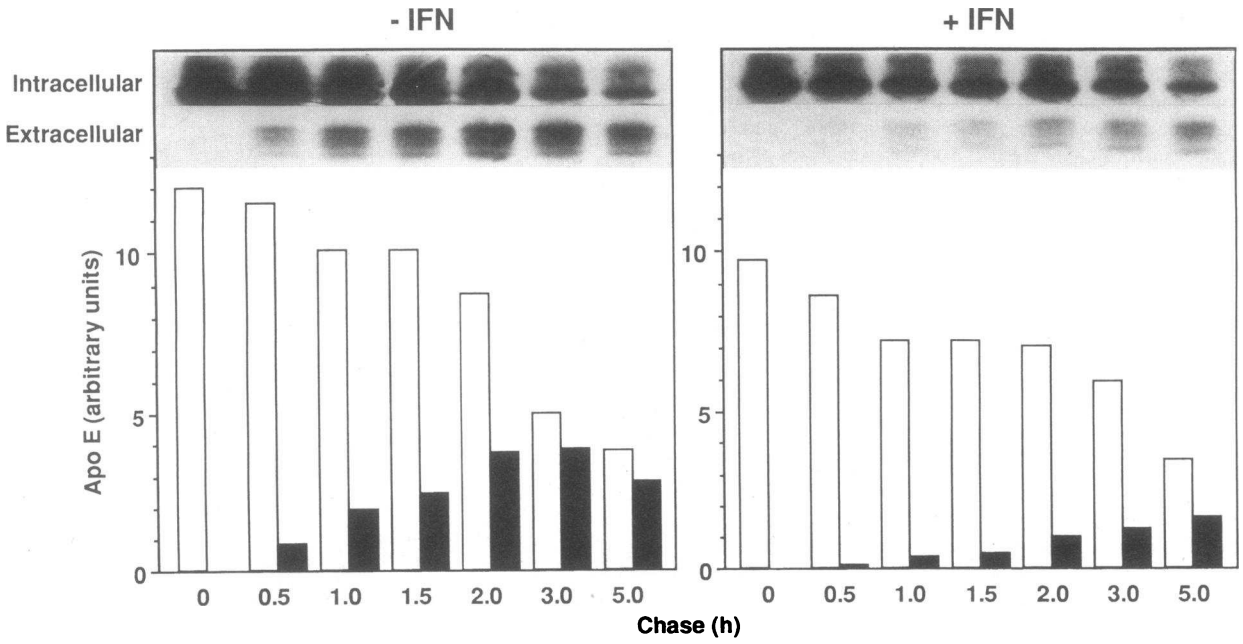

Figure 6. Pulse-chase analysis of intracellular and extracellular apo E accumulation. Cells were incubated with PMA±IFN (100 U/ml) for 4 d. At day 4, cells were labeled with [^{35}S]methionine for 2 h, followed by a chase period of 5 h. Cell lysate (*open bars*) and supernatant (*filled bars*) were harvested at the times indicated and analyzed by immunoprecipitation. A representative experiment of three experiments is depicted.

Table II. Effect of IFN on the Recovery of Intracellular and Extracellular apo E

	Recovery					
	Intracellular		Extracellular		Loss (total)	
Chase	−IFN	+IFN	−IFN	+IFN	−IFN	+IFN
h	%				%	
0	100	100	0	0	0	0
0.5	97±3*	89±3*	6±1*	1±0.3*	0	10
1.0	84±5*	74±2*	15±1*	3±1*	1	23
1.5	78±6	67±8	19±3*	5±1*	3	28

The experiment from Fig. 6 and from two additional experiments were analyzed. The amount of intracellular labeled apo E as determined by scintillation counting present at the beginning of the chase period (0 h) was defined as 100% and the data were calculated as a percentage of this value. Data for recovery were expressed as mean±SD (n = 3). The loss of total apo E was calculated by adding the averaged values for intracellular and extracellular apo E and determining the difference to 100%. * The difference between −IFN and +IFN was significant at $P < 0.05$ for intracellular values and $P < 0.005$ for extracellular values.

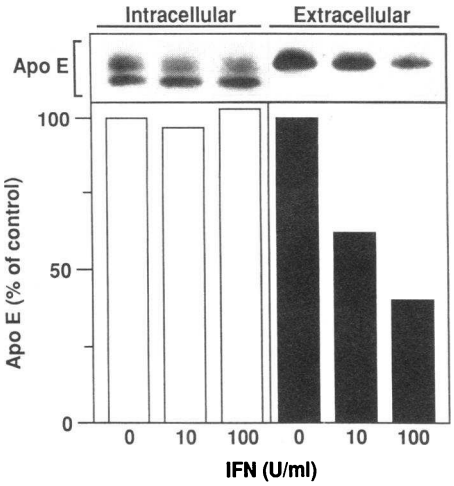

Figure 7. Effect of IFN on apo E production in monocyte-derived macrophages. Adherent human peripheral blood monocytes were incubated with 10^{-8} M PMA±IFN (10 or 100 U/ml) for 2 d. The cells were labeled with 200 μCi/ml of [^{35}S]methionine for 6 h, and cell lysates and supernatants were harvested and analyzed by immunoprecipitation. One representative experiment of three separate experiments is shown.

cellular degradation that resulted in lowered secretion. In either case we have demonstrated that the inhibition of apo E production by IFN in human macrophages was mediated by post-translational mechanisms.

IFN could facilitate intracellular degradation of apo E by several mechanisms. For example, IFN could affect protein trafficking and actively divert apo E from a secretory pathway to an intracellular compartment that favored degradation. Recently a protein, homologous to IFN-inducible antiviral Mx proteins, was shown to play an important role in protein sorting (41). It was suggested that this molecule is involved in modulating the secretory pathway. A related protein might be involved in mediating the effects of IFN on intracellular transport and degradation of apo E. Intracellular degradation in the presence of IFN could account for the observed slight inhibition of intracellular accumulation of apo E when this lymphokine was present during differentiation (Fig. 3). However, at this time we cannot exclude an additional minor translational effect of IFN on apo E synthesis. Further studies are necessary to identify the exact intracellular mechanisms by which IFN exerts its inhibitory effect on apo E production. Intracellular degradation in hepatic cells has been demonstrated to be an important regulatory mechanism controlling the level of secretion of apo B (42). Insulin and LDL inhibit secretion of apo B and increase intracellular degradation (43, 44). Oleate, on the other hand, stimulates the secretion of apo B–containing lipoproteins by inhibiting early intracellular degradation (45). A similar mechanism has been suggested for the HDL-induced apo E production in J774 cells (14). Intracellular degradation also appears to play a regulatory role in the secretion of apo E by Hep G2 cells (46).

Table III. Recovery of Total Labeled Protein and Apo E in the Presence of IFN

	Recovery			
	Intracellular		Extracellular	
Chase	Total	Apo E	Total	Apo E
h	%			
0	100	100	0	0
0.5	97±5	89±3	3±2	1±0.3
1.0	93±7*	74±2*	4±1	3±1
1.5	90±6*	67±8*	5±2	5±1

The data for apo E are derived from Table II. Total labeled protein was determined in the same experiments by TCA precipitation. The amount of total intracellular labeled protein or apo E present at the beginning of the chase (0 h) was defined as 100%. Data for recovery were expressed as mean±SD (n = 3). * The differnce in recovery between total labeled protein and apo E was significant at $P < 0.02$.

Recent reports have described the inhibitory effects of IFN on the production of macrophage lipoprotein lipase (LPL)[1] and scavenger receptor (ScR) (47, 48), which, like apo E, have been identified in atheroma (49–51). Activated T cells, the main producers of IFN, are a prominent cell type in atherosclerotic lesions (52). At particular stages of plaque development, IFN expression may predominate and reduce the expression of apo E, LPL, and ScR. This could protect the lymphocyte from the immunosuppressive effects of macrophage apo E and limit foam cell formation (47, 48). Inhibition of the production of apo E, LPL, and ScR by the same agonist, IFN, suggests a common theme and may be part of in vivo mechanisms that prevent plaque progression.

Defective production of IFN is associated with immunodeficiency conditions (53) and chronic disease states (21, 54). In-

1. *Abbreviations used in this paper:* LPL, liproprotein lipase; ScR, scavenger receptor.

terestingly, we identified a high level of immunosuppressive apo E in fetal cord blood (55), which could derive at least in part from the physiologic defect in IFN production that has been identified in human neonates (56). Therefore, we speculate that the expression of macrophage apo E in vivo can be modulated by immunoregulatory substances like IFN and possibly in acute inflammatory situations also by inflammatory molecules such as bacterial lipopolysaccharide (9). A loss of this control under conditions described above, could lead to an increased production of "lymphocyte-suppressive" apo E which could contribute to an immunodeficient condition.

Our study has demonstrated that the lymphokine IFN inhibited apo E production in human macrophages by posttranslational mechanisms, which included increased intracellular degradation and/or inhibition of secretion. Inhibition of macrophage-apo E production by IFN represents a previously uncharacterized interaction between T lymphocytes and macrophages and may serve as an example of the growing amount of evidence to support a relationship between lipid metabolism and immune cell function.

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References

1. Mahley, R. W. 1988. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science (Wash. DC)*. 240:622–630.

2. Basu, S. K., M. S. Brown, Y. K. Ho, R. J. Havel, and J. L. Goldstein. 1981. Mouse macrophages synthesize and secrete a protein resembling apolipoprotein E. *Proc. Natl. Acad. Sci. USA*. 78:7545–7549.

3. Basu, S. K., J. L. Goldstein, and M. S. Brown. 1983. Independent pathways for secretion of cholesterol and apolipoprotein E by macrophages. *Science (Wash. DC)*. 219:871–873.

4. Zannis, V. I., J. vanderSpek, and D. Silverman. 1986. Intracellular modifications of human apolipoprotein E. *J. Biol. Chem.* 261:13415–13421.

5. Werb, Z., and J. R. Chin. 1983. Onset of apoprotein E secretion during differentiation of mouse bone marrow-derived mononuclear phagocytes. *J. Cell Biol.* 97:1113–1118.

6. Auwerx, J. H., S. Deeb, J. D. Brunzell, R. Peng, and A. Chait. 1988. Transcriptional activation of the lipoprotein lipase and apolipoprotein E genes accompanies differentiation in some human macrophage-like cell lines. *Biochemistry*. 27:2651–2655.

7. Basheeruddin, K., C. Rechtoris, and T. Mazzone. 1992. Transcriptional and post-transcriptional control of apolipoprotein E gene expression in differentiating human monocytes. *J. Biol. Chem.* 267:1219–1224.

8. Werb, Z., and J. R. Chin. 1983. Apoprotein E is synthesized and secreted by resident and thioglycollate-elicited macrophages but not by pyran copolymer- or bacillus calmette-guerin-activated macrophages. *J. Exp. Med.* 158:1272–1293.

9. Werb, Z., and J. R. Chin. 1983. Endotoxin suppresses expression of apoprotein E by mouse macrophages in vivo and in culture. *J. Biol. Chem.* 258:10642–10648.

10. Takagi, Y., C. A. Dyer, and L. K. Curtiss. 1988. Platelet-enhanced apolipoprotein E production by human macrophages: a possible role in atherosclerosis. *J. Lipid Res.* 29:859–867.

11. Mazzone, T., H. Gump, P. Diller, and G. S. Getz. 1987. Macrophage free cholesterol content regulates apolipoprotein E synthesis. *J. Biol. Chem.* 262:11657–11662.

12. Mazzone, T., K. Basheeruddin, and C. Poulos. 1989. Regulation of macrophage apolipoprotein E gene expression by cholesterol. *J. Lipid Res.* 30:1055–1064.

13. Mazzone, T., and K. Basheeruddin. 1991. Dissociated regulation of macrophage LDL receptor and apolipoprotein E gene expression by sterol. *J. Lipid Res.* 32:507–514.

14. Mazzone, T., L. Pustelnikas, and C. A. Reardon. 1992. Post-translational regulation of macrophage apoprotein E production. *J. Biol. Chem.* 267:1081–1087.

15. Curtiss, L. K. 1992. Cholesterol, apolipoprotein E and immune cell function. *In* Nutrient Modulation of the Immune Response. S. Cunningham-Rundles, editor. Marcel Dekker, Inc., New York. 169–181.

16. Curtiss, L. K. 1993. Immunoregulatory plasma apolipoprotein E-structure and function. *In* Immunoregulatory Properties of Plasma Lipoproteins. M. Esfahani, and R. Bigler, editors. The Telford Press, New York. In press.

17. Terkeltaub, R. A., C. A. Dyer, J. Martin, and L. K. Curtiss. 1991. Apolipoprotein (apo) E inhibits the capacity of monosodium urate crystals to stimulate neutrophils: characterization of intraarticular apo E and demonstration of apo E binding to urate crystals in vivo. *J. Clin. Invest.* 87:20–26.

18. Pepe, M. G., and L. K. Curtiss. 1986. Apolipoprotein E is a biologically active constituent of the normal immunoregulatory lipoprotein, LDL-In. *J. Immunol.* 136:3716–3723.

19. Dyer, C. A., R. S. Smith, and L. K. Curtiss. 1991. Only multimers of a synthetic peptide of human apolipoprotein E are biologically active. *J. Biol. Chem.* 266:15009–15015.

20. Lewis, D. B., and C. B. Wilson. 1990. Gamma-interferon: an immunoregulatory lymphokine with immunotherapeutic potential. *Pediatr. Infect. Dis. J.* 9:642–651.

21. Young, H. A., and K. J. Hardy. 1990. Interferon-γ: producer cells, activation stimuli, and molecular genetic regulation. *Pharmacol. Ther.* 45:137–151.

22. Oropeza, R. L., 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. Schimpl, editors. Academic Press, Inc., New York. 303–307.

23. Tsuchiya, S., M. Yamabe, Y. Yamaguchi, Y. Kobayashi, T. Konno, and K. Tada. 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int. J. Cancer*. 26:171–176.

24. Brand, K., B. J. Fowler, T. S. Edgington, and N. Mackman. 1991. Tissue factor mRNA in THP-1 monocytic cells is regulated at both transcriptional and posttranscriptional levels in response to lipopolysaccharide. *Mol. Cell. Biol.* 11:4732–4738.

25. Auwerx, J. 1991. The human leukemia cell line, THP-1: a multifacetted model for the study of monocyte-macrophage differentiation. *Experientia*. 47:22–31.

26. Banka, C. L., A. S. Black, C. A. Dyer, and L. K. Curtiss. 1991. THP-1 cells form foam cells in response to coculture with lipoproteins but not platelets. *J. Lipid Res.* 32:35–43.

27. Sariban, E., K. Imamura, R. Luebbers, and D. Kufe. 1988. Transcriptional and posttranscriptional regulation of tumor necrosis factor gene expression in human monocytes. *J. Clin. Invest.* 81:1506–1510.

28. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

29. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

30. Breslow, J. L., J. McPherson, A. L. Nussbaum, H. W. Williams, F. Lofquist-Kahl, S. K. Karathanasis, and V. I. Zannis. 1982. Identification and DNA sequence of a human apolipoprotein E cDNA clone. *J. Biol. Chem.* 257:14639–14641.

31. Virca, G. D., W. Northemann, B. R. Shiels, G. Widera, and S. Broome. 1990. Simplified Northern blot hybridization using 5% sodium dodecyl sulfate. *BioTechniques*. 8:370–371.

32. John, N. J., and G. L. Firestone. 1986. A sensitive two-step immunoadsorption procedure to analyze low abundance polypeptides by lowering nonspecific background. *BioTechniques*. 4:404–406.

33. Heidenreich, S., J.-H. Gong, A. Schmidt, M. Nain, and D. Gemsa. 1989. Macrophage activation by granulocyte/macrophage colony-stimulating factor: priming for enhanced release of tumor necrosis factor-α and prostaglandin E_2. *J. Immunol.* 143:1198–1205.

34. Faltynek, C. R., L. R. Finch, P. Miller, and W. R. Overton. 1989. Treatment with recombinant IFN-γ decreases cell surface CD4 levels on peripheral blood monocytes and on myelomonocytic cell lines. *J. Immunol.* 142:500–508.

35. Watanabe, Y., and C. O. Jacob. 1991. Regulation of MHC class II antigen expression: opposing effects of tumor necrosis factor-α on IFN-γ-induced HLA-DR and Ia expression depends on the maturation and differentiation stage of the cell. *J. Immunol.* 146:899–905.

36. Clark, S. C., and R. Kamen. 1987. The hematopoietic colony-stimulating factors. *Science (Wash. DC)*. 236:1229–1237.

37. Menju, M., S. Tajima, and A. Yamamoto. 1989. Expression of the apolipoprotein E gene in a human macrophage-like cell line, THP-1. *J. Biochem.* 106:505–510.

38. Van Lenten, B. J., A. M. Fogelman, M. M. Hokom, L. Benson, M. E. Haberland, and P. A. Edwards. 1983. Regulation of the uptake and degradation of β-very low density lipoprotein in human monocyte macrophages. *J. Biol. Chem.* 258:5151–5157.

39. Koo, C., M. E. Wernette-Hammond, and T. L. Innerarity. 1986. Uptake of canine β-very low density lipoproteins by mouse peritoneal macrophages is mediated by a low density lipoprotein receptor. *J. Biol. Chem.* 261:11194–11201.

40. Arai, K., F. Lee, A. Miyajima, S. Miyatake, N. Arai, and T. Yokota. 1990. Cytokines: coordinators of immune and inflammatory responses. *Annu. Rev. Biochem.* 59:783–836.

41. Rothman, J. H., C. K. Raymond, T. Gilbert, P. J. O'Hara, and T. H. Stevens. 1990. A putative GTP binding protein homologous to interferon-inducible mx proteins perform an essential function in yeast protein sorting. *Cell.* 61:1063–1074.

42. Borchardt, R. A., and R. A. Davis. 1987. Intrahepatic assembly of very low density lipoproteins. Rate of transport out of the endoplasmic reticulum determines rate of secretion. *J. Biol. Chem.* 262:16394–16402.

43. Sparks, J. D., and C. E. Sparks. 1990. Insulin modulation of hepatic synthesis and secretion of apolipoprotein B by rat hepatocytes. *J. Biol. Chem.* 265:8854–8862.

44. Sato, R., T. Imanaka, A. Takatsuki, and T. Takano. 1990. Degradation of newly synthesized apolipoprotein B-100 in a pre-golgi compartment. *J. Biol. Chem.* 265:11880–11884.

45. Dixon, J. L., S. Furukawa, and H. N. Ginsberg. 1991. Oleate stimulates secretion of apolipoprotein B-containing lipoproteins from Hep G2 cells by inhibiting early intracellular degradation of apolipoprotein B. *J. Biol. Chem.* 266:5080–5086.

46. Ye, S. Q., L. M. Olson, C. A. Reardon, and G. S. Getz. 1992. Human plasma lipoproteins regulate apolipoprotein E secretion from a post-golgi compartment. *J. Biol. Chem.* 267:21961–21966.

47. Jonasson, L., G. K. Hansson, G. Bondjers, L. Noe, and J. Etienne. 1990. Interferon-gamma inhibits lipoprotein lipase in human monocyte-derived macrophages. *Biochim. Biophys. Acta.* 1053:43–48.

48. Geng, Y., 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.

49. Salomon, R. N., R. Underwood, M. V. Doyle, A. Wang, and P. Libby. 1992. Increased apolipoprotein E and c-*fms* gene expression without elevated interleukin 1 or 6 mRNA levels indicates selective activation of macrophage functions in advanced human atheroma. *Proc. Natl. Acad. Sci. USA.* 89:2814–2818.

50. O'Brien, K. D., D. Gordon, S. Deeb, M. Ferguson, and A. Chait. 1992. Lipoprotein lipase is synthesized by macrophage-derived foam cells in human coronary atherosclerotic plaques. *J. Clin. Invest.* 89:1544–1550.

51. Matsumoto, A., M. Naito, H. Itakura, S. Ikemoto, H. Asaoka, I. Hayakawa, H. Kanamori, H. Aburatani, F. Takaku, H. Suzuki, et al. 1990. Human macrophage scavenger receptors: primary structure, expression, and localization in atherosclerotic lesions. *Proc. Natl. Acad. Sci. USA.* 87:9133–9137.

52. Hansson, G. K., J. Holm, and L. Jonasson. 1989. Detection of activated T lymphocytes in the human atherosclerotic plaque. *Am. J. Pathol.* 135:169–175.

53. Murray, H. W., B. Y. Rubin, H. Masur, and R. B. Roberts. 1984. Impaired production of lymphkines and immune (gamma) interferon in the acquired immunodeficiency syndrome. *N. Engl. J. Med.* 130:883–889.

54. Sechler, J. M. G., H. L. Malech, C. J. White, and J. I. Gallin. 1988. Recombinant human interferon-gamma reconstitutes defective phagocytic function in patients with chronic granulomatous disease of children. *Proc. Natl. Acad. Sci. USA.* 85:4874–4877.

55. Curtiss, L. K., T. M. Forte, and P. A. Davis. 1984. Cord blood plasma lipoproteins inhibit mitogen-stimulated lymphocyte proliferation. *J. Immunol.* 133:1379–1384.

56. Wakasuki, N., and J.-L. Virelizier. 1985. Defective IFN-gamma production in the human neonate. I. Dysregulation rather than intrinsic abnormality. *J. Immunol.* 134:167–171.