

# Tumor Necrosis Factor- $\alpha$ Modulates Monocyte/Macrophage Apoprotein E Gene Expression

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## Abstract

apo E has been shown to modulate cholesterol balance in arterial wall cells. Production of apo E by macrophages in atherosclerotic plaques could thereby influence the development of the plaque lesion. Cytokines, including TNF $\alpha$ , have been identified in human lesions, therefore, we undertook a series of studies to evaluate the effect of TNF $\alpha$  on monocyte/macrophage apo E production. The addition of TNF $\alpha$  to freshly isolated human monocytes led to a four- to fivefold increase of apo E mRNA abundance. The addition of TNF $\alpha$  to fully differentiated macrophages either had no effect or modestly inhibited apo E mRNA expression. THP1 human monocytic cells also responded to TNF $\alpha$  in a phenotype-specific manner. Treatment of these cells with TNF $\alpha$  produced a dose- and time-dependent increase in apo E mRNA. This increase was reflected in apo E synthesis and was associated with inhibition of DNA synthesis, and with induction of *c-fos* and ICAM-1 gene expression. Cell-permanent analogues of ceramide did not reproduce TNF $\alpha$  effect on apo E, but antagonists of protein kinase C did inhibit its effect. TNF $\alpha$  induction of apo E mRNA abundance was associated with stimulation of apo E promoter-dependent gene transcription. In summary, TNF $\alpha$  stimulates apo E gene transcription, mRNA abundance, and protein synthesis in the monocyte/macrophage in a phenotype-specific manner. Such regulation could significantly modify the amount of apo E present in vessel wall lesions. (*J. Clin. Invest.* 1995; 96:915-922.) Key words: cytokines • atherosclerosis • macrophage activation • protein kinase C • ceramide

## Introduction

apo E is an important structural and functional constituent of plasma lipoproteins, including VLDL, HDL, and chylomicron remnants (1). Although apo E is primarily synthesized by the liver, significant amounts are also produced by a variety of peripheral tissues and cells (2), including macrophages, where its synthesis is regulated by the cholesterol content of the cells (3, 4). Histochemical studies have indicated that macrophages

are the major source of locally derived apo E in developing vessel wall atheromatous lesions (5). Local production of apo E in the vessel wall could impact on proximate cellular cholesterol flux and modify the local availability of bioactive molecules such as growth factors (1). Furthermore, endogenous production of apo E has been shown to influence macrophage cholesterol flux by regulating the rate of cellular cholesterol clearance to HDL<sub>3</sub> (6). Thus, modulation of macrophage apo E secretion in the vessel wall could significantly impact atheroma progression or regression.

Regulation of macrophage apo E expression is complex: it is regulated not only by cholesterol but also by activators of protein kinase C and by endotoxin (7-9). TGF $\beta$  has been demonstrated to induce macrophage apo E expression (10). On the other hand, the monocyte colony stimulating factor (M-CSF)<sup>1</sup> has been shown, in separate reports, both to inhibit and augment macrophage apo E expression (10, 11). A potential regulatory role for other cytokines, for example, TNF $\alpha$ , has been even less clear (10, 12).

Local production of TNF $\alpha$  is an important component of the vessel wall response to injury. TNF $\alpha$  positivity has been found in the cytoplasm of macrophages, and in the cytoplasm and attached to cell membranes of smooth muscle cells and endothelial cells of the human atheroma (13, 14). These observations suggest a potential for TNF $\alpha$  to modulate the evolution of the atherosclerotic plaque. Further, such modulation would be likely to occur via effects on monocyte/macrophage cells because these are primary targets of TNF $\alpha$  action. In the present study, we investigated the effect of TNF $\alpha$  on apo E expression in the human monocyte/macrophage. We examined phenotype-dependent effects of TNF $\alpha$  on apo E gene expression and compared these with its effects on the expression of other monocyte/macrophage genes (*c-fos* and intercellular adhesion molecule [ICAM]-1) and cell cycle traverse. We also investigated potential roles for ceramide second messengers and protein kinase C (pkC) activation for transducing the observed effects of TNF $\alpha$  on apo E.

## Methods

**Materials.** [<sup>35</sup>S]Methionine (10 Ci/mmol), [<sup>32</sup>P]dCTP (800 Ci/mmol), and [<sup>3</sup>H]thymidine (5 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). Human recombinant TNF $\alpha$  was purchased from Genzyme, Corp. (Boston, MA). C<sub>2</sub>-ceramide and H7 were purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). Endotoxin-free BSA came from Calbiochem Corp. (La

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1. Abbreviations used in this paper: ICAM, intercellular adhesion molecule; M-CSF, monocyte colony stimulating factor; NF $\kappa$ B, nuclear factor- $\kappa$ B; pkC, protein kinase C.

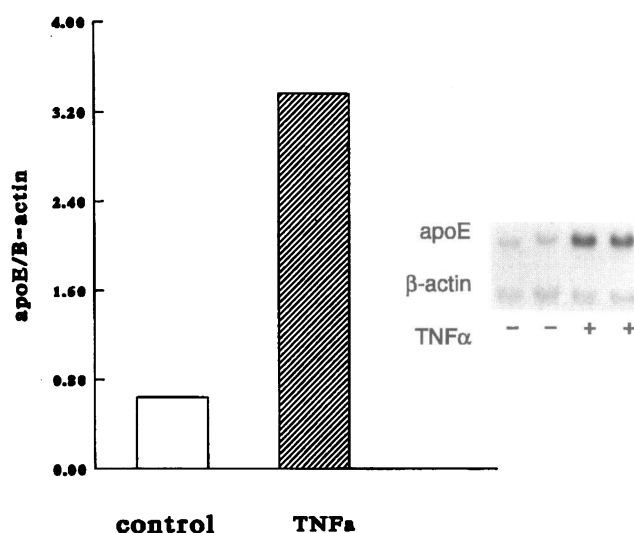
Jolla, CA). All other materials were obtained from previously identified sources (6, 9, 15, 16).

**Cell culture.** Freshly isolated human monocytes were purified by elutriation. The cell population used for experiments was > 95% monocytic, as determined by differential counts of Wright stained smears. These cells were incubated using conditions described in the figure legends. For experiments using fully differentiated human monocyte-derived macrophages, cells were allowed to differentiate in serum-containing medium for 7 d before the start of the incubation with TNF $\alpha$ . The THP1 human monocytic cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD), and cells were grown in culture flasks (Falcon Plastics, Cockeysville, MD) in RPMI-1640 medium containing 10% heat-inactivated FBS as described (15). For experiments, all cells were washed twice with serum-free RPMI-1640 and were placed in RPMI-1640 medium supplemented with 0.2% endotoxin-free BSA unless otherwise indicated.

**Immunoprecipitation of apo E.** THP1 cells were pulse-labeled with L-[<sup>35</sup>S]methionine, as described in detail previously (16). Briefly, cells were plated at a density of  $5 \times 10^6$  cells in a 60-mm dish in triplicate. 5 ng/ml of TNF $\alpha$  was added, and the cells were incubated for 72 h at 37°C. After incubation, adherent cells and cells in suspension were washed twice with methionine-free RPMI-1640 and then incubated for 2 h in 1 ml of methionine-free RPMI-1640 medium supplemented with 100  $\mu$ Ci/ml of [<sup>35</sup>S]methionine supplemented with 10  $\mu$ M unlabeled L-methionine. After incubation, the medium was collected after centrifugation to remove cells. Adherent and cells in suspension were washed twice in ice-cold phosphate-buffered saline and lysed in 2.0% SDS at 95°C for 3 min. The lysate was then diluted with lysis buffer (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 15 mM NaCl, 10 mM L-methionine, 1% Triton X-100, 1% deoxycholate, pH 7.4) to final concentration of 0.2% SDS. Cell extracts and culture media were analyzed by immunoprecipitation followed by 10% SDS-PAGE and fluorography as previously described (16).  $4 \times 10^6$  TCA-precipitable counts/min were immunoprecipitated from each cell lysate sample, and  $4 \times 10^5$  counts/min were immunoprecipitated from each medium sample. Incorporation of labeled methionine into apo E protein in THP1 cells was linear over the assay period. For quantitation, apo E bands were excised from the gel and digested in 30% H<sub>2</sub>O<sub>2</sub> for scintillation counting (16). Radioactivity in the apo E shown was normalized for rate of total protein synthesis and secretion, which was not significantly different between groups.

**RNA isolation and Northern hybridization.** Cells were seeded at a density of  $10 \times 10^6$  cells in a 100-mm dish. To isolate total cellular RNA, washed cells were solubilized in guanidine isothiocyanate as described, followed by sedimentation of the extract through CsCl (9). For some experiments, poly (A)<sup>+</sup> RNA was purified after passing total RNA through silanized oligo (dT) columns. For Northern analysis, formaldehyde-treated RNA samples were fractionated by electrophoresis in 1.0% agarose, transferred to Nytran (Schleicher & Schuell, Inc., Keene, NH), and hybridized with a labeled apo E cDNA probe as previously described (9). Radiolabeled apo E cDNA probe was prepared by random-primed synthesis using pHEA4, which is a 1.1-kb AatII-HinfI fragment that encompasses the entire protein coding region of the human apo E cDNA subcloned into pUC (9). Each reaction was carried out using 50 ng of cDNA with a minimum of  $1 \times 10^7$  cpm. To provide an internal control, Nytran membranes were stripped according to the manufacturer's instructions and reprobed with a labeled 0.7-kb PstI fragment of the cDNA for beta-actin. The *c-fos* probe, which was a 1.0-kb PstI subfragment from the *fos* cDNA and the ICAM-1 probe, which was a 1.4-kb BglII-SaII subfragment from the ICAM-1 cDNA, was labeled using the same method.

**Measurement of DNA synthesis.** Rates of DNA synthesis were assessed by determining [<sup>3</sup>H]thymidine incorporation into TCA precipitable material (17). After cultures were treated with the indicated amount of TNF $\alpha$  for 24 h, cells were pulsed with 2.5  $\mu$ Ci/ml [<sup>3</sup>H]thymidine for 2 h and then washed with RPMI-1640 five times. This was followed by five washes with ice-cold 5% TCA. Cells were then extracted in 1



**Figure 1.** TNF $\alpha$  enhances apo E mRNA expression in human monocyte/macrophages. Human monocytes (isolated by elutriation, > 95% pure) were incubated in Teflon FEP culture bottles (Fischer, Pittsburgh, PA) in 0.2% endotoxin-free BSA plus or minus TNF $\alpha$  at 10 ng/ml. After 24 h, cells were harvested for analysis by Northern hybridization. The intensity of the bands for apo E and  $\beta$ -actin (as the internal control) was determined by scanning densitometry.

ml of 0.25 N NaOH for scintillation counting. Protein was assayed using Lowry's method (18).

**Cell transfection and measurement of luciferase activity.** An apo E-luciferase chimeric construct, -2300/+24 apo E pGL2, was made using standard recombinant techniques (19). The Sma 1-AatII fragment of the apo E gene was cloned into the polylinker region of pGL-2 Basic (Promega Corp. Madison, WI). THP1 cells were transfected using a modified dextran method (20).  $1 \times 10^7$  cells were transfected with 2  $\mu$ g of -2300/+24 apo E pGL2 plasmid in 1 ml STBS buffer (25 mM Tris-Cl, 137 mM NaCl, 5 mM KCl, 0.6 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.7 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>) with 100  $\mu$ g DEAE dextran for 90 min and shocked with 10% DMSO for 5 min. After transfection, the cells were incubated in RPMI medium with 10% FBS overnight. At that time, all transfected cells were mixed in a single pool prior to being aliquoted to experimental treatments, to eliminate differential transfection efficiency as an experimental variable. The medium was then changed to RPMI with 0.2% endotoxin-free BSA, and 10 ng/ml of TNF- $\alpha$  was added. Before harvesting, the cells were left at room temperature for 20 min; the cells were then lysed, and luciferase activity was measured using luciferase assay kit and a luminometer (Enhanced Luciferase Assay Kit; Luminometer Monolight 2010; Analytical Luminescence Laboratory, San Diego, CA). Protein concentration was measured using a DC protein assay kit (Bio Rad Laboratories, Richmond, CA).

## Results

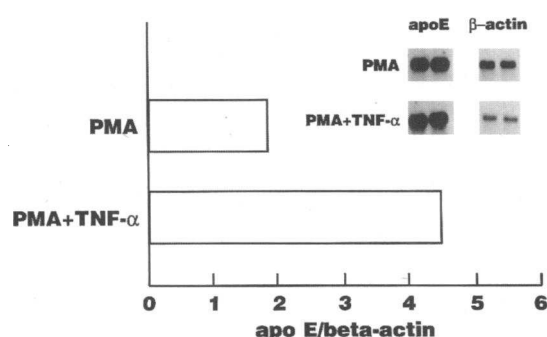
**Phenotype-specific effects of TNF $\alpha$  on human monocyte/macrophage apo E gene expression.** Fig. 1 shows the effect of TNF $\alpha$  at 10 ng/ml on apo E gene expression in freshly isolated human monocytes. A four- to fivefold increase of apo E mRNA abundance resulted from treatment with TNF $\alpha$  over a 24-h period. Apo E gene expression in more fully differentiated macrophages (after 7 d in culture) showed little response to TNF $\alpha$  at 5 ng/

**Table I. Effect of TNF $\alpha$  on apo E mRNA Expression in Differentiated Macrophages**

Cell type	apo E/ $\beta$ -actin	Fold change
THP1 cells		
24 h control	2.58	1.0
24 h + TNF $\alpha$	1.97	0.76
72 h control	2.46	1.0
72 h + TNF $\alpha$	1.57	0.64
Human monocyte-derived macrophages		
48 h control	3.88 $\pm$ 0.14	1.0
48 h + TNF $\alpha$	3.57 $\pm$ 0.40	0.92

THP1 cells in 10% serum were treated with 100 ng/ml of PMA for 3 d. At that time the medium was replaced with 0.2% endotoxin-free BSA alone or with 5 ng/ml of TNF $\alpha$  for the indicated time. Human monocytes isolated by elutriation were allowed to differentiate in serum containing medium for 7 d. At that time, the medium was replaced with 0.2% endotoxin-free BSA alone or with 5 ng/ml TNF $\alpha$  for 48 h. Cells were analyzed by Northern blot by hybridization. The intensity of the bands for apo E and  $\beta$ -actin was measured by scanning densitometry. Results for THP1 cells are the average of duplicate samples, which varied < 10%. Results from human monocyte-derived macrophage are mean $\pm$ SD of triplicate samples.

ml after a 48-h incubation period (Table I). Addition of TNF $\alpha$  to cells allowed to differentiate over shorter periods of time produced a 30–40% reduction of apo E mRNA levels (not shown). Similar phenotype-specific effects were observed in the human monocytic THP1 cell line. In this cell line, we and others have shown that treatment of THP1 monocytes with phorbol ester induced expression of the macrophage phenotype in these cells as well as in apo E gene expression (8, 9, 12). When TNF $\alpha$  was added during the initial 18 h of differentiation, apo E mRNA abundance was further increased more than two-fold by the addition of TNF $\alpha$  (Fig. 2). When TNF $\alpha$  was added to THP1 cells after differentiation had progressed for 72 h, apo



**Figure 2.** TNF $\alpha$  enhances apo E mRNA expression in early differentiating macrophages. THP1 cells in 0.2% endotoxin-free BSA were treated with 100 ng/ml of PMA alone or with 5 ng/ml of TNF $\alpha$  for 18 h. Northern blots of poly (A)<sup>+</sup> RNA for apo E and  $\beta$ -actin were processed as described in Methods. The bars show the change of apo E mRNA abundance after correcting for the  $\beta$ -actin signal.

**Table II. Dose-dependent Effect of TNF $\alpha$  on apo E mRNA Expression**

	apo E/ $\beta$ -actin	Fold change
Control	0.09	1
TNF $\alpha$ 5 ng/ml	0.32	3.6
TNF $\alpha$ 10 ng/ml	0.40	4.4
TNF $\alpha$ 15 ng/ml	0.42	4.7

20  $\mu$ g total cellular RNA, isolated from untreated cells or cells treated with the indicated concentration of TNF $\alpha$  for 24 h, was analyzed by Northern hybridization. The intensity of the bands corresponding to apo E and  $\beta$ -actin was determined by scanning densitometry. The values shown are the averages of duplicate samples, which varied < 10%.

E mRNA abundance was reduced by 24 and 36% after an additional 24 and 72 h, respectively, in TNF $\alpha$  (Table I). The results in both cell types, therefore, demonstrated a relationship between TNF $\alpha$  effect on apo E expression and phenotypic expression of monocyte/macrophage cells. Additional studies were performed to investigate further TNF $\alpha$ -mediated induction of apo E gene expression using the THP1 cell line.

*TNF $\alpha$  induces apo E gene expression in THP1 cells in a time- and dose-dependent manner.* Table II shows the results of a representative experiment to evaluate the effect of TNF $\alpha$  on apo E gene expression over a range of doses. In this experiment, apo E messenger RNA abundance increased 3.6-fold in response to 5 ng/ml TNF $\alpha$  and 4.7-fold in response to 15 ng/ml TNF $\alpha$ . The 5 ng/ml dose was chosen to evaluate the time dependency of apo E mRNA response. As shown in Table III, after a 24-h incubation in TNF $\alpha$ , a slightly greater than twofold increase in apo E mRNA abundance was detected. The greatest increase of apo E mRNA was observed when the incubation in TNF $\alpha$  was extended to 72 h, at which time a 14-fold increase in apo E messenger RNA abundance was measured in treated as compared with control cells.

*Effect of TNF $\alpha$  on apo E synthesis and secretion.* Induction of messenger RNA levels for macrophage target genes of TNF $\alpha$  are not always reflected in changes in protein synthesis and secretion (21, 22). We therefore directly evaluated the effect of TNF $\alpha$  on apo E synthesis and secretion by pulse labeling cells

**Table III. Time Course Effect of TNF $\alpha$  on apo E mRNA Expression**

Time	apo E/ $\beta$ -actin	
	Control	TNF $\alpha$
18 h	0.09	0.11
24 h	0.07	0.16
72 h	0.09	1.26

20  $\mu$ g of total cellular RNA isolated from untreated cells and cells treated with 5 ng/ml of TNF $\alpha$  for the indicated time was analyzed by Northern hybridization. The intensity of the bands corresponding to apo E and to the internal control,  $\beta$ -actin, was determined by scanning densitometry. The results shown are the averages of duplicate samples, which varied by < 10%.

Table IV. Effect of TNF $\alpha$  on apo E Synthesis and Secretion

	Medium	Cell extract
	<i>dpm</i>	
Control	265 $\pm$ 73	447 $\pm$ 69
TNF $\alpha$	1163 $\pm$ 49	822 $\pm$ 178

5  $\times$  10<sup>6</sup> THP1 cells in 60-mm dishes were incubated for 72 h in medium alone or with 5 ng/ml of TNF $\alpha$ . The cells were then labeled for 2 h with [<sup>35</sup>S]methionine to measure the rate of apo E synthesis and secretion as described in Methods. The apo E bands were excised from the gels, and radioactivity in apo E is shown in the lower panel as SEM from triplicate samples.

with [<sup>35</sup>S]methionine and quantitatively immunoprecipitating newly synthesized apo E from cell lysates and media. The results of a representative experiment are shown in Table IV. In culture media, apo E radioactivity was increased more than fourfold in response to a 72-h incubation in TNF $\alpha$ . In cell lysates, there was a 1.8-fold increase in apo E radioactivity in response to TNF $\alpha$ .

*TNF $\alpha$  induction of apo E messenger RNA abundance is associated with phenotypic modulation.* Three markers for phenotypic modulation were assessed to compare with changes in apo E mRNA expression during our experimental incubations. TNF $\alpha$ , added at 5 ng/ml for 24 h, significantly depressed DNA synthesis (14.5 $\pm$ 1.7 vs. 9.0 $\pm$ 0.7 cpm  $\times$  10<sup>3</sup>/mg protein, control versus TNF $\alpha$ ) in THP1 cells. In Table V, expression of ICAM-1 mRNA is presented as a function of TNF $\alpha$  treatment duration. This gene codes for an adhesion molecule that is a counter-receptor for LFA-1 expressed on other leukocytes; interaction between these two proteins may enhance leukocyte recruitment and communication (23). As shown, after 6 h in TNF $\alpha$ , ICAM-1 messenger RNA species remained undetectable. However, after 18 and 24 h, there was an increase in ICAM-1 messenger RNA abundance produced by TNF $\alpha$  treatment. TNF $\alpha$  treatment is also associated with a rapid and transient enhancement of *c-fos* gene expression (Table V). Levels for *c-fos* message were undetectable in untreated cells. However, these became easily detectable after 15 min in TNF $\alpha$  and remained elevated for at least 30 min. Levels of the *c-fos* message again became undetectable after 60 min of TNF $\alpha$  treatment.

*Transduction pathways for TNF $\alpha$  effect on apo E gene expression.* Many of the effects of TNF $\alpha$  have been ascribed to a second messenger pathway that uses ceramide as an important regulatory molecule (24–26). These effects can be reproduced by treating cells with cell permeable analogues of ceramide, and we used this approach to evaluate the importance of this second messenger for transducing the effect of TNF $\alpha$  on apo E gene expression. As shown in Fig. 3 (*upper panel*), C<sub>2</sub>-ceramide did not reproduce the effect of TNF $\alpha$  on apo E gene expression, nor did it potentiate the effect of TNF $\alpha$  on this gene. Similarly, C<sub>2</sub>-ceramide did not reproduce the effect of TNF $\alpha$  on ICAM-1 expression. In this case, however, the TNF $\alpha$  effect was potentiated by ceramide (Fig. 3, *middle panel*). Neither extending the incubation in C<sub>2</sub>-ceramide for up to 72 h, addition of C<sub>6</sub>- ceramide analogues, nor treatment of cells with

Table V. Time Course Effect of TNF $\alpha$  on *c-fos* and ICAM-1 mRNA Expression

<i>c-fos</i> / $\beta$ -actin	
Control	N.D.
TNF $\alpha$ 15 min	0.13
TNF $\alpha$ 30 min	0.15
TNF $\alpha$ 60 min	N.D.
ICAM-1/ $\beta$ -actin	
Control	N.D.
TNF $\alpha$ 6 h	N.D.
TNF $\alpha$ 18 h	0.41
TNF $\alpha$ 24 h	0.48

20  $\mu$ g of total cellular RNA was isolated from untreated cells or cells treated with 5 ng/ml of TNF $\alpha$  for the indicated times and analyzed by Northern blot hybridization. The intensity of the bands for ICAM-1 or *c-fos* along with  $\beta$ -actin was determined by scanning densitometry. N.D. indicates that there was no *c-fos* or ICAM-1 signal detected for that experimental condition. Values shown are the average of duplicate samples, which varied by < 10%.

sphingomyelinase to generate endogenous cellular ceramide led to induction of apo E mRNA abundance (data not shown).

TNF $\alpha$  effects can also be mediated by activation of pkC in transduction pathways separate from those involving ceramide (24–26). We therefore examined whether pathway inhibition of pkC activation during TNF $\alpha$  treatment would attenuate apo E gene response. In the experiment shown in the bottom panel of Fig. 3, treatment with TNF $\alpha$  produced a significant increase in apo E gene expression ( $P < 0.02$ ). Concurrent treatment with the pkC inhibitor H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine, completely prevented this induction however. A similar inhibition of TNF $\alpha$  stimulation was produced by using staurosporine to inhibit pkC activity (not shown).

*Effect of TNF $\alpha$  on apo E gene transcription.* We next considered the mechanism by which TNF $\alpha$  increases apo E mRNA abundance. For other macrophage genes, TNF $\alpha$  has been shown to increase mRNA abundance by stabilization of mRNA species without any change in gene transcription rate. To assess the contribution of increased apo E gene transcription, THP1 cells were transiently transfected with a chimeric construct containing apo E promoter sequences (from –2300 to +24 bp) fused to a luciferase reporter. After transfection, a single pool of cells was created to normalize transfection efficiency, and this pool was then aliquoted for incubations in TNF $\alpha$  treatment for 24 and 48 h. TNF $\alpha$  induced the expression of this chimeric construct 2.2-fold at 24 h and 2.1-fold at 48 h (Fig. 4).

## Discussion

Our studies demonstrate that treatment with TNF $\alpha$  stimulates apo E gene transcription, mRNA abundance, and apo E synthesis and secretion in monocyte/macrophage cells in a phenotype-dependent manner. Such concordant response at all major regulatory loci is not demonstrated by all macrophage targets of TNF $\alpha$  modulation. For example, for FC $\gamma$  IIb, TNF $\alpha$  has been shown to decrease protein expression but increase mRNA abun-

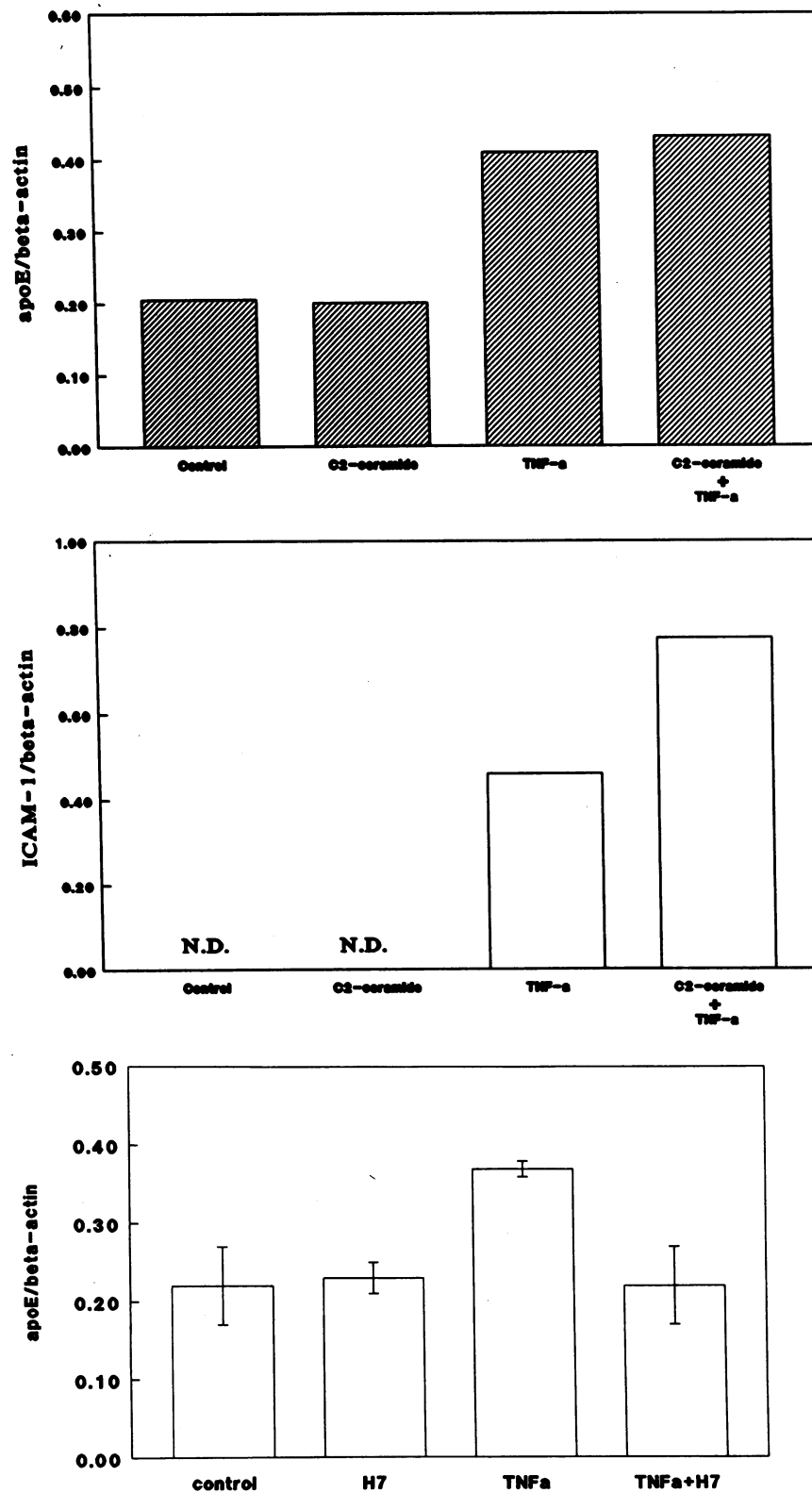


Figure 3. Transduction pathways for TNF $\alpha$  activation of apo E gene expression. Cells cultured with RPMI-1640 containing 0.05% free-endotoxin BSA were treated with 5 ng/ml of TNF $\alpha$ , 5  $\mu$ M of C<sub>2</sub>-ceramide, 10  $\mu$ g/ml of H7, or a combination of these for 24 h. Northern blots for apo E, ICAM-1, and  $\beta$ -actin were processed as described previously. The upper panel shows the effect of TNF $\alpha$  and C<sub>2</sub>-ceramide on apo E mRNA abundance after correcting for  $\beta$ -actin signal. The middle panel shows the TNF $\alpha$  and C<sub>2</sub>-ceramide effect on ICAM-1 mRNA abundance after the same correction. N.D. indicates that there was no detected signal for that experimental condition. Values shown are the mean of duplicate samples, which varied by < 10%. The lower panel shows the effect of H7 inhibition on TNF $\alpha$  stimulation of apo E mRNA abundance after correction for  $\beta$ -actin abundance. Values shown are the mean  $\pm$  SEM from triplicate samples.

dance in macrophages, and this increased mRNA abundance is due to a change in mRNA stability (21). While our studies establish that increased apo E gene transcription contributes to enhanced apo E mRNA abundance after TNF $\alpha$  treatment, a

potential role for apo E mRNA stabilization is not excluded, especially for the large increase of apo E mRNA levels measured after prolonged incubation in TNF $\alpha$  (up to a 13-fold increase after 72 h). Alternatively, inclusion of additional apo E

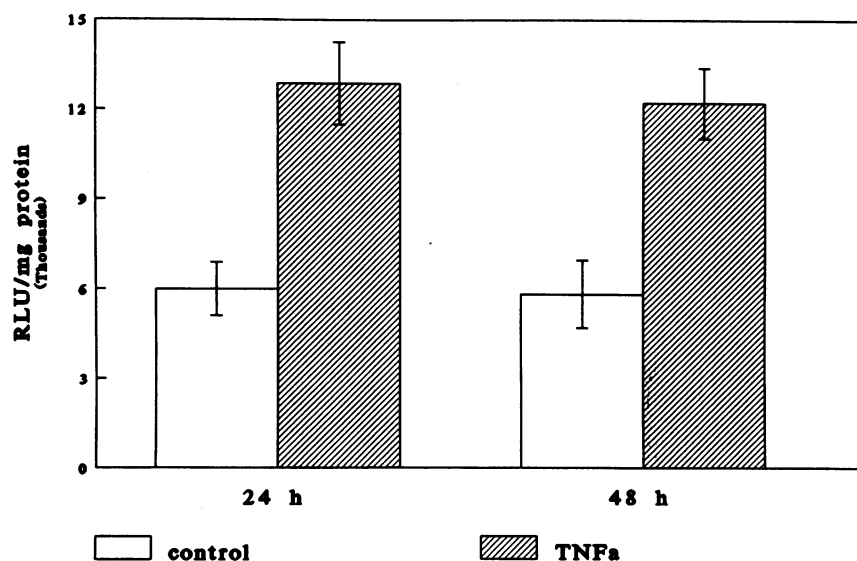


Figure 4. TNF $\alpha$  promotes the expression of the apo E gene promoter. Cells transfected with -2300/+24 apo E pGL-2 were cultured with 10 ng/ml of TNF $\alpha$  for the indicated times and then harvested for measurement of luciferase activity. All cells were derived from a single pool of transfected cells to normalize for transfection efficiency as described in Methods. The data are shown as the mean  $\pm$  SD of luciferase activity (relative light units/mg protein  $\times 10^{-6}$ ) from triplicate samples.

gene elements may be necessary to maximize the transcriptional response of chimeric constructs to TNF $\alpha$ .

Others have studied the effect of TNF $\alpha$  on apo E expression with inconsistent results. Menju et al. investigated the effect of TNF $\alpha$  on THP1 cells after these cells were treated for 4 d with PMA, 12-O-tetradecanoylphorbol-13-acetate (12). Additional treatment for 12 h in TNF $\alpha$  produced no effect on apo E mRNA abundance or protein synthesis. Zuckerman treated thioglycollate-elicited mouse peritoneal macrophages with TNF $\alpha$  for a longer period of time (48 h) and found an  $\sim 50\%$  reduction of apo E protein secretion (10). Both of these reports are in accord with our observations regarding the phenotype-specific effect of TNF $\alpha$  on apo E gene expression. Monocyte/macrophages are multipotential cells that can respond to their humoral environment in divergent ways. Phenotype-dependent modulation of apo E expression by TNF $\alpha$  is consistent with the pleiotropic effects of TNF $\alpha$  that have been reported for other macrophage targets (22, 27). Such a phenotype-dependent response to cytokine stimulation may be related to activation of endogenous cytokine synthesis and secretion by the fully differentiated and activated macrophage. For example, PMA stimulation has been shown to increase TNF $\alpha$  mRNA expression within 3 h and TNF $\alpha$  protein synthesis within 10 h in human monocytes (28). However, if endogenous synthesis of TNF $\alpha$  by differentiated macrophages accounts for the lack of apo E induction after treatment with exogenous TNF $\alpha$ , the suppression of apo E expression observed by Zuckerman et al. (10) and by us (Table I) during prolonged incubations in TNF $\alpha$  would not be expected. Alternatively, phenotype-specific effects of TNF $\alpha$  may relate to differences in cell-matrix interactions between monocyte and macrophage phenotypes. Adhesion of cells to extracellular matrix has been shown to modify their response to TNF $\alpha$  (29). Further, in the vessel wall, it is likely that the presence of other cytokines will be involved in modulating monocyte/macrophage response to TNF $\alpha$ . Interferon- $\gamma$ , for example, has been shown to be an important influence on the response of the IGF-1 expression after treatment of macrophages with TNF $\alpha$  (30).

TNF $\alpha$  treatment produced cell cycle arrest as indicated by

a markedly reduced rate of DNA synthesis. In addition, TNF $\alpha$  increased the expression of c-fos within 15 min of its addition. Rapid and transient induction of c-fos expression serves as a marker for macrophage differentiation and/or activation (31). Also, TNF $\alpha$  enhanced the expression of the ICAM-1 gene. In arterial wall plaques, ICAM-1 expression is found in arterial smooth muscle cells and macrophages (32), and it has previously been shown that TNF $\alpha$  induces the expression of ICAM-1 in arterial smooth muscle cells (33). The effect of TNF $\alpha$  on apo E gene expression cannot be generalized to all cytokines associated with monocyte/macrophage activation or differentiation. Brand et al. added PMA and interferon- $\gamma$  and observed no effect on apo E mRNA abundance in THP1 cells (34). THP1 cells have been shown to express MCSF receptors (35); however, in our laboratory, the addition of MCSF, with or without phorbol ester, to THP1 cells also had no effect on apo E mRNA abundance. This agrees with the report of Brand et al., who made a similar observation regarding the lack of MCSF effect on apo E protein synthesis and secretion in these cells (34).

Treating cells with TNF $\alpha$  has reportedly produced sphingomyelin turnover as well as endogenous cellular ceramide (24–26). Investigation of biologically important signal transduction pathways for TNF $\alpha$  has focused on the role of ceramide as a second messenger as well as on a potential role for pKc activation. There are examples of pKc activation and ceramide producing opposing effects in target monocyte/macrophage cells (26). In HL60 cells, TNF $\alpha$  leads to increased activity of mitogen-activated protein kinase and the accumulation of nuclear factor- $\kappa$ B (NF $\kappa$ B) (36, 37). Both of these effects can be reproduced by exogenously added ceramide analogues. In Jurkat cells, TNF $\alpha$  decreases DNA synthesis and increases NF $\kappa$ B accumulation (38). In these cells, ceramide inhibits DNA synthesis but does not produce NF $\kappa$ B accumulation, although it potentiates the effect of TNF $\alpha$  on NF $\kappa$ B accumulation. These observations support a role for ceramide as a second messenger molecule mediating TNF $\alpha$  effects. Recently, however, the importance of ceramide as a TNF $\alpha$  second messenger has been

questioned. For example, Betts et al. directly measured ceramide levels, and they could find no difference produced by TNF $\alpha$  treatment in HL60 cells or Jurkat cells, even though NF $\kappa$ B activation was measurable in both cell types (39). Our data demonstrate that ceramide is not involved in TNF $\alpha$  modulation of the apo E gene. Addition of cell-permeant ceramide analogues did not increase apo E messenger RNA abundance nor did it potentiate the effect of TNF $\alpha$  on apo E. We also observed that treatment of THP1 cells with sphingomyelinase, to generate endogenous cellular ceramide, did not augment apo E mRNA abundance (not shown). Inhibitors of pkC activation, however, attenuated the effect of TNF $\alpha$  on apo E gene expression, suggesting the importance of this pathway for mediating TNF $\alpha$  effects on the apo E gene. With respect to the mechanism for TNF $\alpha$  induction of apo E gene transcription, it is noteworthy that TNF $\alpha$  treatment of THP1 cells induces expression of the *c-fos* gene. The protein product of this gene is a component of the AP1 transcription complex, which we have previously shown to be important for enhanced apo E gene transcription in the monocyte/macrophage (19).

In summary, our results document a significant role for TNF $\alpha$  in modulating monocyte/macrophage apo E gene expression. TNF $\alpha$  stimulates apo E gene transcription, mRNA abundance, and protein synthesis in the monocyte/macrophage in a phenotype-specific manner. This effect is observed in monocyte cells or cells in the early stage of monocyte/macrophage differentiation. In fully differentiated/activated macrophages, TNF $\alpha$  has no effect or reduces apo E mRNA abundance. Macrophages in vessel wall lesions are derived from the migration of blood-borne monocytes that cross the endothelium and transform, over time, into fully differentiated macrophages. Phenotypic monocytes as well as monocyte/macrophages in more advanced stages of differentiation have been identified in the vessel wall intima of hyperlipemic animals (40, 41). TNF $\alpha$  produced in the vessel wall could significantly modulate the abundance of apo E derived from monocytes/macrophages that are newly recruited into the vessel wall and thereby influence the genesis of the vessel wall lesion.

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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. Newman, T. C., P. A. Dawson, L. L. Rudel, and D. L. Williams. 1985. Quantitation of apolipoprotein E mRNA in the liver and peripheral tissues of nonhuman primates. *J. Biol. Chem.* 260:2452-2457.
3. 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.
4. 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.
5. Rosenfeld, M. E., S. Butler, V. A. Ord, B. A. Lipton, C. A. Dyer, L. K. Curtiss, W. Palinski, and J. L. Witztum. 1993. Abundant expression of apoprotein E by macrophages in human and rabbit atherosclerotic lesions. *Arterioscler. Thromb.* 13:1382-1389.
6. Mazzone, T., and C. Reardon. 1994. Expression of heterologous human apolipoprotein E by J774 macrophages enhances cholesterol efflux to HDL<sub>3</sub>. *J. Lipid Res.* 35:1345-1353.
7. Werb, Z., and J. R. Chin. 1983. Endotoxin suppresses expression of apoprotein E by mouse macrophages in vitro and in culture. *J. Biol. Chem.* 258:10642-10648.
8. 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. *Biochem.* 27:2651-2655.
9. Basheeruddin, K., C. Rechteris, and T. Mazzone. 1992. Transcriptional and post-transcriptional control of apolipoprotein E gene expression in differentiating human monocytes. *J. Biol. Chem.* 267:1219-1224.
10. Zuckerman, S. H., G. F. Evans, and L. O'Neal. 1992. Cytokine regulation of macrophage apoE secretion: opposing effects of GM-CSF and TGF- $\beta$ . *Arteriosclerosis*. 96:203-214.
11. Clinton, S. K., R. Underwood, L. Hayes, M. L. Sherman, D. W. Kufe, and P. Libby. 1992. Macrophage colony-stimulating factor gene expression in vascular cells and in experimental and human atherosclerosis. *Am. J. Pathol.* 140:301-316.
12. 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.
13. Libby, P., and G. K. Hansson. 1991. Involvement of the immune system in human atherogenesis: current knowledge and unanswered questions. *Lab. Invest.* 64:5-15.
14. Barath, P., M. C. Fishbein, J. Cao, J. Berenson, R. H. Helfant, and J. S. Forrester. 1990. Detection and localization of tumor necrosis factor in human atheroma. *Am. J. Cardiol.* 65:297.
15. 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.
16. Mazzone, T., L. Pustelnikas, and C. A. Reardon. 1992. Post-translational regulation of macrophage apoprotein E production. *J. Biol. Chem.* 267:1081-1087.
17. Chait, A., and T. Mazzone. 1982. A secretory product of human monocyte-derived macrophages stimulates low density lipoprotein receptor activity in arterial smooth muscle cells and skin fibroblasts. *Arteriosclerosis*. 2:134-141.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
19. Basheeruddin, K., C. Rechteris, and T. Mazzone. 1993. Evaluation of the role of AP1-like proteins in the enhanced apolipoprotein E gene transcription accompanying phorbol ester induced macrophage differentiation. *Biochim. Biophys. Acta*. 1218:235-241.
20. Zhang, Y., M. Doerfler, T. C. Lee, B. Guillemin, and W. N. Rom. 1993. Mechanisms of stimulation of interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$  by mycobacterium tuberculosis components. *J. Clin. Invest.* 91:2076-2083.
21. Chicheportiche, Y., and P. Vassalli. 1994. Tumor necrosis factor induces a block in the cotranslation of Fc $\gamma$ RIIb mRNA in mouse peritoneal macrophages. *J. Biol. Chem.* 269:20134-20138.
22. Shieh, J.-H., R. H. Peterson, D. J. Warren, and M. A. Moore. 1989. Modulation of colony-stimulating factor-1 receptors on macrophages by tumor necrosis factor. *J. Immunol.* 143:2534-2539.
23. Springer, T. H. 1990. Adhesion receptors of the immune system. *Nature (Lond.)*. 346:425-434.
24. Kolesnick, R., and D. W. Golde. 1994. The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. *Cell*. 77:325-328.
25. Heller, R. A., and M. Kronke. 1994. Tumor necrosis factor receptor-mediated signaling pathways. *J. Cell Biol.* 126:5-9.
26. Hannun, Y. A. 1994. The sphingomyelin cycle and the second messenger function of ceramide. *J. Biol. Chem.* 269:3125-3128.
27. Witsell, A. L., and L. B. Schook. 1992. Tumor necrosis factor  $\alpha$  is an autocrine growth regulator during macrophage differentiation. *Proc. Natl. Acad. Sci. USA*. 89:4754-4758.
28. Sariban, E., K. Imamura, R. Luebbes, and D. Kufe. 1988. Transcriptional and posttranscriptional regulation of tumor necrosis factor gene expression in human monocytes. *J. Clin. Invest.* 81:1506-1510.
29. Nathan, C., and S. Michael. 1991. Cytokines in context. *J. Cell Biol.* 113:981-986.
30. Lake, F. R., P. W. Nobel, P. M. Henson, and D. W. H. Riches. 1994. Functional switching of macrophage responses to tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) by interferons: implications for the pleiotropic activities of TNF $\alpha$ . *J. Clin. Invest.* 93:1661-1669.
31. Muller, R. 1986. Cellular and viral fos genes: structure, regulation of expression and biological properties of their encoded products. *Biochim. Biophys. Acta*. 823:207-225.

32. Printseva, O. Y., M. M. Pecio, and A. M. Gown. 1992. Various cell types in human atherosclerotic lesions express ICAM-1. *Am. J. Pathol.* 140:889–896.
33. Couffinhal, T., C. Duplaa, L. Labat, J. M. D. Lamaziere, C. Moreau, O. Printseva, and J. Bonnet. 1993. Tumor necrosis factor- $\alpha$  stimulates ICAM-1 expression in human vascular smooth muscle cells. *Arterioscler. Thromb.* 13:407–414.
34. Brand, K., N. Mackman, and L. K. Curtiss. 1993. Interferon- $\gamma$  inhibits macrophage apolipoprotein E production by posttranslational mechanisms. *J. Clin. Invest.* 91:2031–2039.
35. Datta, R., K. Imamura, S. J. Goldman, A. C. Dianoux, D. W. Kufe, and M. L. Sherman. 1992. Functional expression of the macrophage colony-stimulating factor receptor in human THP-1 monocytic leukemia cells. *Blood.* 79:904–912.
36. Raines, M. A., R. N. Kolesnick, and D. W. Golde. 1993. Sphingomyelinase and ceramide activate mitogen-activated protein kinase in myeloid HL-60 cells. *J. Biol. Chem.* 268:14572–14575.
37. Yang, Z., M. Costanzo, D. W. Golde, and R. N. Kolesnick. 1993. Tumor necrosis factor activation of the sphingomyelin pathway signals nuclear factor  $\kappa$ B translocation in intact HL-60 cells. *J. Biol. Chem.* 268:20520–20523.
38. Dbaibo, G. S., L. M. Obeid, and Y. A. Hannun. 1993. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) signal transduction through ceramide. *J. Biol. Chem.* 268:17762–17766.
39. Betts, J. C., A. B. Agranoff, G. J. Nabel, and J. A. Shayman. 1994. Dissociation of endogenous cellular ceramide from NF- $\kappa$ B activation. *J. Biol. Chem.* 269:8455–8458.
40. Gerrity, R. G., H. K. Naito, M. Richardson, C. J. Schwartz. 1979. Dietary induced atherogenesis in swine. *Am. J. Pathol.* 95:775–792.
41. Gerrity, R. G. 1981. The role of the monocyte in atherogenesis. *Am. J. Pathol.* 103:181–190.