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Decreased inducibility of TNF expression in lipid-loaded macrophages

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Abstract

Background: Inflammation and immune responses are considered to be very important in the pathogenesis of atherosclerosis. Lipid accumulation in macrophages of the arterial intima is a characteristic feature of atherosclerosis which can influence the inflammatory potential of macrophages. We studied the effects of lipid loading on the regulation of TNF expression in human monocyte-derived macrophages.

Results: In macrophages incubated with acetylated low density lipoprotein (ac-LDL) for 2 days, mRNA expression of TNF in cells stimulated with TNF decreased by 75%. In cell cultures stimulated over night with IL-1β, lipid loading decreased secretion of TNF into culture medium by 48%. These results suggest that lipid accumulation in macrophages makes them less responsive to inflammatory stimuli. Decreased basal activity and inducibility of transcription factor AP-1 was observed in lipid-loaded cells, suggesting a mechanism for the suppression of cytokine expression. NF-κB binding activity and inducibility were only marginally affected by ac-LDL. LDL and ac-LDL did not activate PPARγ. In contrast, oxidized LDL stimulated AP-1 and PPARγ but inhibited NF-κB, indicating that the effects of lipid loading with ac-LDL were not due to oxidation of lipids.

Conclusions: Accumulation of lipid, mainly cholesterol, results in down-regulation of TNF expression in macrophages. Since monocytes are known to be activated by cell adhesion, these results suggest that foam cells in atherosclerotic plaques may contribute less potently to an inflammatory reaction than newly arrived monocytes/macrophages.

Background

Lipid accumulation in macrophages leads to formation of foam cells, a distinct cell type found in fatty streaks and more advanced atherosclerotic lesions. Apart from effects on cellular lipid metabolism, the effects of intracellular lipid accumulation on gene expression have not been extensively studied. Since uptake of low density lipoprotein (LDL) is subject to negative feedback control of the LDL

receptor pathway, we chose to incubate cells with acetylated LDL (ac-LDL), which is taken up by the scavenger receptors. In this way, a more efficient lipid loading is achieved.

Lipid loading with ac-LDL in phorbol myristate acetate (PMA)-pretreated THP-1 macrophages has been reported to increase production of interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) [1]. It was further shown that IL-8 mRNA is expressed in a macrophage-rich area of the human atheromatous lesion [1]. In natural killer (NK) cells, very low density lipoprotein (VLDL) and ac-LDL decreased secretion of granulocyte-macrophage colony stimulating factor (GM-CSF) [2]. In non-primed NK cells, these lipoproteins stimulated IL-8 secretion, but in NK cells primed with IL-2, secretion of IL-8 decreased, indicating that the differentiation state of the cell or other factors can influence the response of cells to lipoproteins [2]. Ac-LDL has also been demonstrated to decrease the secretion of platelet-derived growth factor from endothelial cells [3].

In order to understand the interactions between three important aspects of atherosclerosis, macrophages, lipid accumulation and inflammation, we studied the effects of ac-LDL on the expression of TNF in human monocyte-derived macrophages.

Results
Lipid loading decreases TNF expression
The effect of lipid loading on TNF protein secretion was determined in the following way: Human monocyte-derived macrophages were incubated in medium containing 5% lipoprotein-deficient serum (LPDS) with or without ac-LDL (50 μg/mL) for 2 days, washed, and incubated in serum-free medium without GM-CSF for 24 h. Immediately after the treatment with ac-LDL, IL-1β (10 ng/mL) was added to some dishes to induce secretion of TNF. Culture medium was collected and analysed for TNF by ELISA. In three separate experiments, TNF levels in culture medium were 48% ± 13% (mean ± SEM) lower in lipid-loaded cells than in control cells incubated with LPDS only (Table 1).

Next, we analysed the effect of lipid loading on TNF mRNA expression. Macrophages were loaded with lipid by incubation with ac-LDL (50 μg/mL) in medium containing 5% LPDS for 2 days. The cells were then harvested and mRNA was isolated for northern blotting. Lipid loading did not increase the very low or undetectable basal TNF mRNA levels (Figure 1, lanes 1 and 3). We also studied the effects of lipid loading on the inducibility of TNF mRNA expression. Macrophages were stimulated with TNF, which is known to stimulate its own synthesis [4]. In lipid-loaded macrophages, TNF-inducible TNF mRNA ex-

Table 1: IL-1β-induced TNF secretion is decreased in lipid-loaded macrophages

| Treatment	TNF concentration (pg/mL)		
	Experiment 1	Experiment 2	Experiment 3
LPDS	5.9	5.4	6.4
LPDS + IL-1β	14.2	165.3	23.0
Ac-LDL	5.5	5.6	5.4
Ac-LDL + IL-1β	8.4	54.5	14.6
Inhibition	**41%**	**67%**	**36%**

Macrophages were incubated for 48 h in the presence of 5% LPDS ± 50 μg/mL ac-LDL. They were then rinsed with serum-free medium, and incubated for 24 h in serum-free medium with or without IL-1β (10 ng/mL). Culture medium was collected, centrifuged at 4000 *g* for 10 min, and aliquots of the supernatant were frozen for analysis of secreted TNF by ELISA.

pression decreased by 75% (Figure 1, lanes 2 and 4), as compared to macrophages incubated in the presence of 5% LPDS only, followed by TNF stimulation.

The effect of lipid loading on transcription factors
In order to identify transcription factors involved in the regulation of TNF expression by lipid loading, we performed electrophoretic mobility shift assays. NF-κB binding activity was not substantially influenced by incubation of macrophages with ac-LDL for 48 h (Figure 2). By contrast, lipid loading diminished both basal and TNF-induced activation of AP-1 (Figure 2). Based on densitometric scanning of the autoradiograph, the decrease was 67% for basal activity and 74% for TNF-induced binding activity. Incubation with LDL also resulted in reduced AP-1 activity (Figure 3, lanes 6 and 7), but LDL was less potent than ac-LDL in this respect, most probably because LDL is not taken up as effectively as ac-LDL by macrophages. As determined by phosphorimager analysis, LDL treatment reduced AP-1 binding in unstimulated cells by 57%, and in TNF-stimulated cells by 58%. Corresponding values for ac-LDL-treated cells in this experiment were 78% and 71%, respectively, as compared to cells treated with LPDS only.

We also incubated macrophages with oxidized LDL (ox-LDL) to test if the effects of ac-LDL could be due to oxidation of LDL. Ox-LDL enhanced AP-1 binding activity (Figures 3 and 4), in accordance to our previous studies showing a similar effect in human adherent monocytes [5] and smooth muscle cells [6]. In cells not stimulated with TNF, AP-1 binding increased by 43% (Figure 3, lane 10) to 275% (Figure 4), while in TNF-stimulated cells the ox-LDL-induced increase ranged from 52% (Figure 3, lane
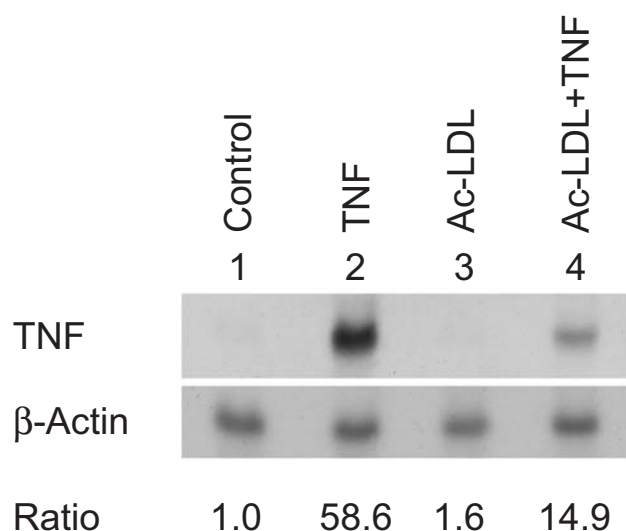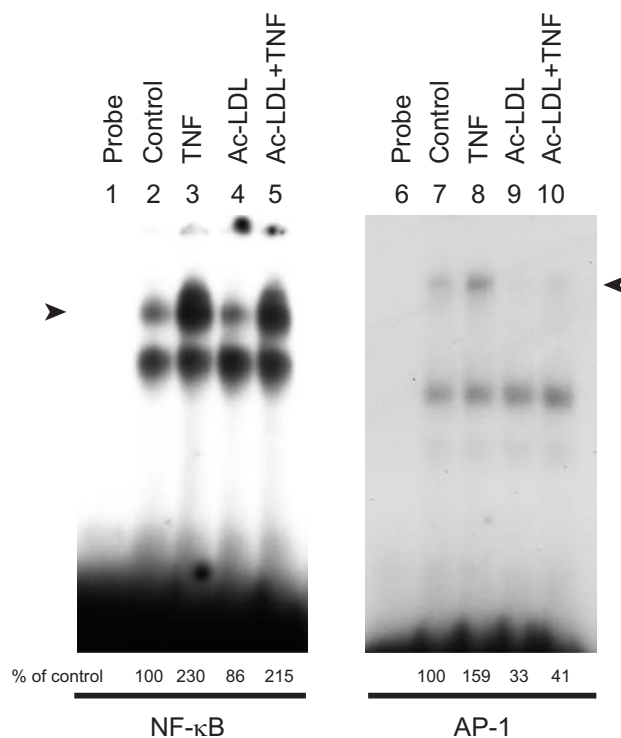
Figure 1
Decreased inducibility of TNF mRNA in macrophages incubated with ac-LDL. Macrophages were cultured for 48 h in the presence of 5% LPDS ± 50 μg/mL ac-LDL. Half of the dishes were then stimulated with TNF (10 ng/mL) for 2 h. TNF mRNA was analysed by Northern blotting, using β-actin as loading control. Lanes: 1, Control; 2, TNF; 3, ac-LDL; 4, ac-LDL + TNF. Data are representative of three separate experiments.

11) to 242% (Figure 4). When the same nuclear extracts were analysed for NF-κB binding activity, it was seen that ox-LDL inhibited both basal (69% decrease) and TNF-induced (57% decrease) DNA binding activity of NF-κB (Figure 4). We have previously demonstrated the same effect in smooth muscle cells stimulated with LPS [6]. LDL which had been stored at 4°C for 4 weeks without EDTA ('Old LDL'), had a weak inhibitory effect on NF-κB, and a weak stimulatory effect on AP-1 (Figure 4), indicating that even partially oxidized LDL stimulates AP-1. Since lipid loading with fresh ac-LDL or LDL did not inhibit NF-κB, it appears that the lipoproteins were not oxidized to a significant degree during the treatments. Taken together, the results indicate that the inhibitory effect of lipid loading on AP-1 was primarily due to uptake of non-oxidized lipoproteins.

Specificity assays demonstrated that NF-κB1 (p50) and RelA (p65) were the major subunits in NF-κB activated by stimulation with TNF (Figure 5). For AP-1, c-Jun and JunD were among the most abundant subunits (Figure 6).

Since ox-LDL is known to activate the anti-inflammatory transcription factor PPAR-γ [7], we tested whether ac-LDL treatment activates this transcription factor. While ox-LDL treatment (100 μg/mL, 2 h) stimulated PPAR-γ binding
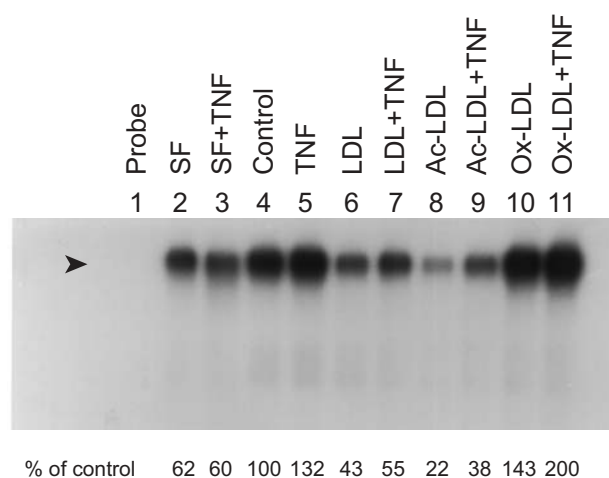

Figure 2
Lipid loading does not decrease inducibility of NF-κB but inhibits AP-1. Macrophages were cultured for 48 h in the presence of 5% LPDS ± 50 μg/mL ac-LDL. The cells were then stimulated with TNF (10 ng/mL) for 2 h. Nuclear extracts were prepared and transcription factor activities (lanes 1–5, NF-κB; lanes 6–10, AP-1) were determined in the extracts. The arrowheads indicate the positions of specific complexes. Lanes: 1 and 6, probe only; 2 and 7, control; 3 and 8, TNF; 4 and 9, ac-LDL; 5 and 10, ac-LDL + TNF. Similar results were obtained in five independent experiments.

activity, LDL and ac-LDL either had no effect or weakly suppressed PPAR-γ (Figure 7). Similar effects have been reported elsewhere [7], despite the fact that many fatty acids and fatty acid derivatives activate PPARs [8–10]. Shorter treatments (4 h) with LDL, ac-LDL, and VLDL had similar effects (data not shown) confirming the suppressive effect of lipid loading on AP-1 was not due to activation of PPAR-γ, and added further support to the notion that lipoproteins were not oxidized to any significant degree during the treatments.

Discussion
In the present study, we have shown that uptake of ac-LDL by macrophages leads to reduced inducibility of TNF expression. Suppression of cytokine expression was associated with reduced basal and stimulated DNA binding activity of AP-1, a transcription factor known to regulate TNF expression. Even though NF-κB has been shown to
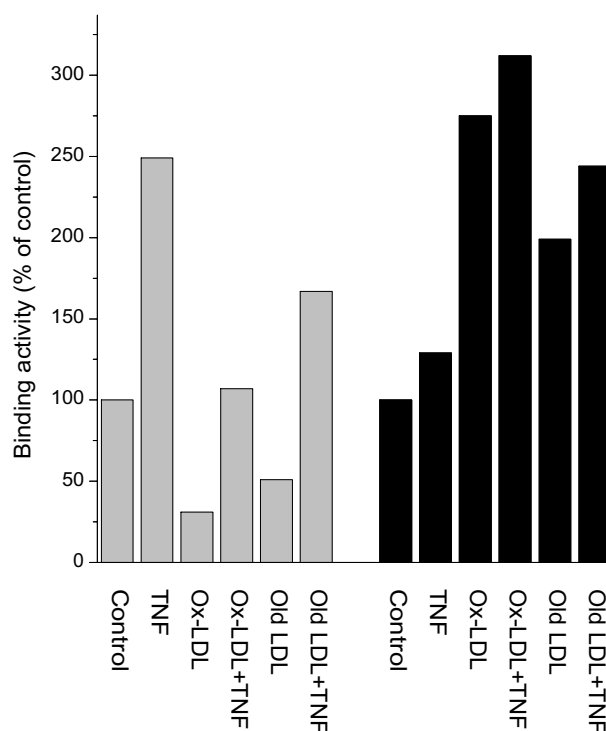
Figure 3
Comparison of the effects of different lipoproteins on AP-1. Macrophages were cultured for 48 h in serum-free medium (SF, lanes 2–3) or in the presence of 5% LPDS ± 50 μg/mL LDL or 50 μg/mL ac-LDL. Half of the dishes were then stimulated with TNF (10 ng/mL) for 2 h. Some dishes were treated for 2 h with ox-LDL (100 μg/mL) before addition of TNF. EMSA was performed as described in methods. The arrowhead indicates the position of AP-1 specific complexes. Lanes: 1, probe only; 2, SF; 3, SF + TNF; 4, Control (5% LPDS); 5, TNF; 6, LDL; 7, LDL + TNF; 8, ac-LDL; 9, ac-LDL + TNF; 10, ox-LDL; 11, ox-LDL + TNF. Data are representative of three independent experiments.

Figure 4
Effects of ox-LDL on NF-κB and AP-1 binding activity. Macrophages were incubated for 48 h in serum-free medium without GM-CSF and subsequently treated for 2 h with copper-oxidized LDL (ox-LDL, 100 μg/mL) or LDL stored for one month at 4°C without EDTA (old LDL, 100 μg/mL). Half of the cells were simultaneously stimulated with TNF (10 ng/mL). The same nuclear extracts were analysed for NF-κB (grey bars) and AP-1 (black bars) binding activity by EMSA followed by densitometry. Data are representative of two independent experiments.

regulate the expression of TNF, there are a number of reports [4,11,12] suggesting that AP-1 sites are more important for the activation of this gene. AP-1 has been implicated in the transcriptional regulation of several other cytokines as well, including IL-1β [13], IL-2 [14], IL-3 [15], IL-6 [16], colony-stimulating factor-1 [17], and transforming growth factor β1 [18]. Our results suggest that AP-1 is important for the expression of TNF, even in the presence of high levels of active NF-κB.

LDL oxidized in the presence of Cu^{2+} or Fe^{2+} has been shown to inhibit LPS or maleylated BSA-induced expression of TNF and IL-1α mRNA in mouse peritoneal macrophages [19]. In the same study, LDL and ac-LDL suppressed LPS-induced expression of inflammatory cytokines, but the authors thought that these effects could have been due to oxidation of lipoproteins. In the present study, we have shown that ox-LDL and ac-LDL have different effects on the transcription factors NF-κB, AP-1 and PPARγ, indicating that the suppression of cytokine expression by ac-LDL was not due to oxidation of lipoproteins.

Inhibition of NF-κB by oxidized lipoproteins has been demonstrated in several studies [6,20–22]. Other groups

[23–26] have reported stimulatory effects. In another study [27], ox-LDL was shown to inhibit NF-κB after long (>20 h) incubations, even though a stimulatory effect was reported for short treatments (4 h). Our data from experiments with macrophages and smooth muscle cells suggests that oxidized LDL inhibits NF-κB during short treatments as well, but we can not completely exclude the possibility that some preparations of LDL or ox-LDL might be stimulatory. Inhibition of NF-κB by ox-LDL could attenuate the inflammatory response to oxidized lipids, but it could also make cells more susceptible to apoptosis [28], which in vascular context has been shown to induce inflammation [29]. In any case, the consistent activation of AP-1 by ox-LDL in macrophages and in smooth muscle cells [6] indicates that AP-1 may in some cases be more important for the direct inflammatory effects of ox-LDL than NF-κB.
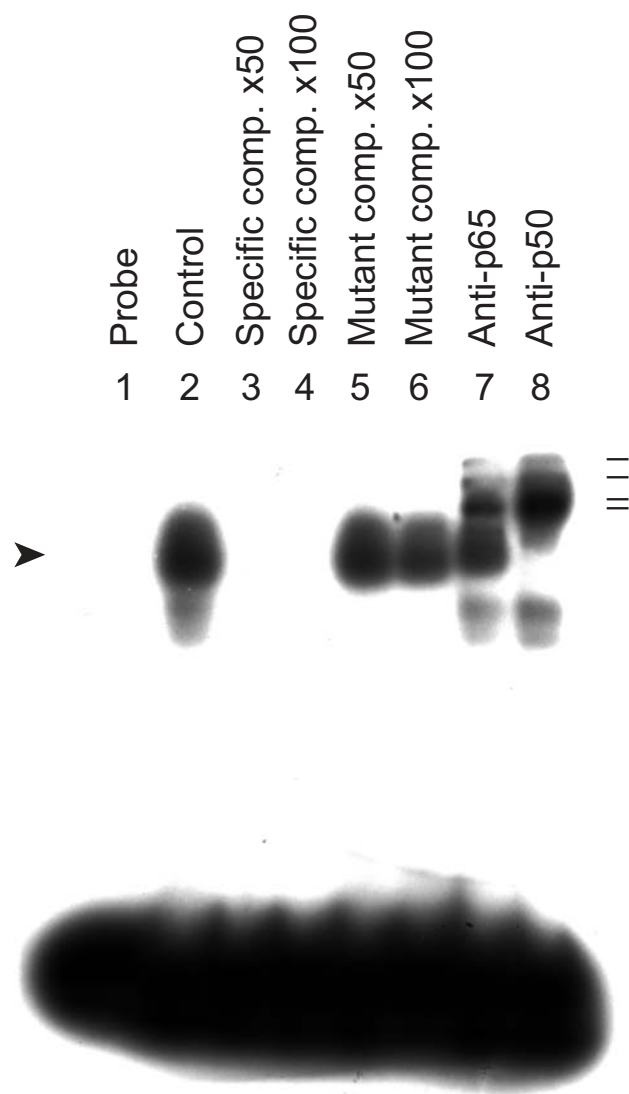
Figure 5
Specificity tests for NF-κB binding. EMSA was performed using extracts from macrophages stimulated with TNF for 2 h. Unlabeled probe in excess (as indicated below) was used as specific competitor. The mutant competitor (see Methods), had one point mutation in the NF-κB binding site. The antibodies (2 μg/sample) were added after the radioactive probe. After addition of antibodies, incubation was continued for 1 h on ice. Lanes: 1, probe only; 2, control (TNF, 10 ng/mL, 2 h); 3, specific competitor (50X excess); 4, specific competitor (100X excess); 5, mutant competitor (50X excess); 6, mutant competitor (100X excess); 7, anti-p65 (RelA); 8, anti-p50 (NFKB1). The arrowheads indicate the positions of specific complexes. The bars mark supershifted bands. Data are representative of four separate experiments.
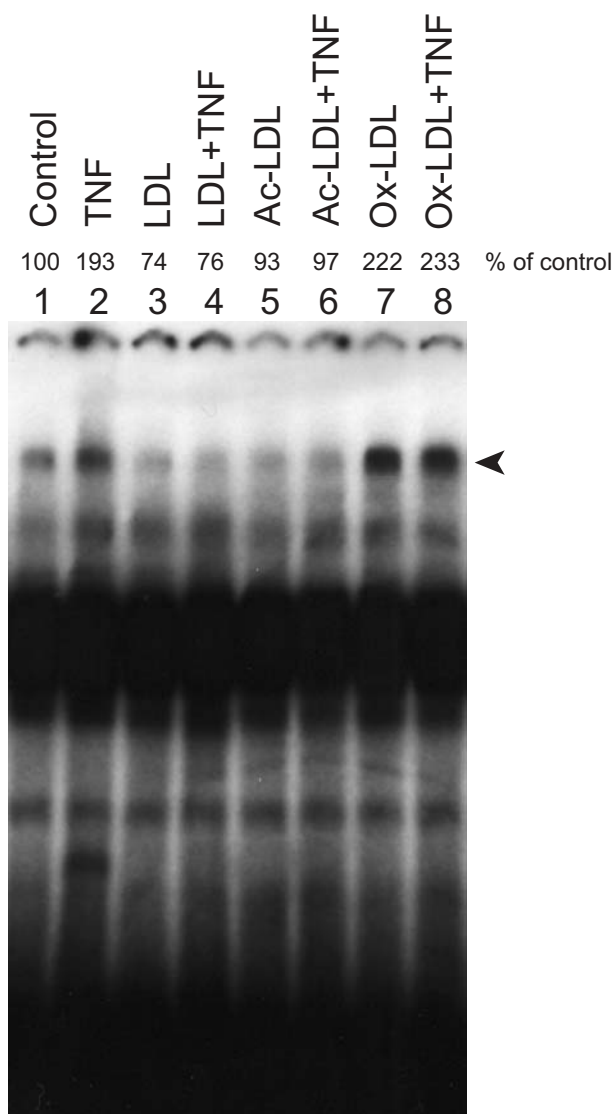

Figure 6
Specificity tests for AP-1 binding. EMSA was performed using extracts from macrophages stimulated with TNF for 2 h. Unlabeled probe in excess (as indicated below) was used as specific competitor. The mutant competitor (see Methods), had 2 point mutations in the AP-1 binding site, respectively. The antibodies (2 μg/sample) were added after the radioactive probe. After addition of antibodies, incubation was continued for 1 h on ice. Lanes: 1, probe only; 2, control (TNF, 10 ng/mL, 2 h); 3, specific competitor (20X excess); 4, specific competitor (40X excess); 5, mutant competitor (20X excess); 6, mutant competitor (40X excess); 7, anti-c-Fos; 8, anti-c-Jun (rabbit); 9, anti-c-Jun (goat); 10, anti-JunB; 11, anti-JunD. The arrowheads indicate the positions of specific complexes. The bars mark supershifted bands. Data are representative of four separate experiments.

induced TNF expression despite perturbing NF-κB activation [30], adding further support to the notion that major changes in AP-1 levels are more predictive of changes in TNF expression than minor effects on NF-κB.

Significant amounts of LDL can be taken up by macrophages without prior oxidation. In addition to uptake of native LDL, aggregated LDL and complexes of LDL with proteoglycans, matrix proteins, mast cell granules or antibodies are efficiently taken up by macrophages [31]. Furthermore, human monocyte-derived macrophages take

In a recent study on THP-1 macrophages, treatment with ox-LDL for 3 days decreased mRNA expression of NF-κB but increased that of c-*jun*[30]. Ox-LDL potentiated LPS-

Figure 7
Lipid loading does not induce activation of PPAR-γ. Macrophages were cultured for 48 h in the presence of 5% LPDS ± 50 μg/mL LDL or ac-LDL. Half of the cells were then stimulated with TNF (10 ng/mL) for 2 h. Nuclear extracts were prepared and PPAR-γ binding activities were determined in the extracts. The arrowhead indicates the position of specific complexes. Lanes: 1, control; 2, TNF; 3, LDL; 4, LDL + TNF; 5, ac-LDL; 6, ac-LDL + TNF; 7, ox-LDL; 8, ox-LDL + TNF. Similar data were obtained in three separate experiments.

up massive amounts of VLDL, which has been reported to enter the vascular wall [32,33]. Another potential source of non-oxidized lipid is the phagocytosis of cellular remains and extracellular lipid deposits. It can thus be argued that substantial amounts of non-oxidized lipoproteins are taken up by macrophages in the vascular wall. The relative contribution of such lipid uptake to foam cell formation *in vivo* is difficult to estimate. We chose ac-LDL instead of VLDL as the means to load macrophages with non-oxidized lipid, because these cells take up VLDL so extensively that long treatments would probably kill the cells as a result of lipid overload.

Recently, patients with familial hypercholesterolemia were reported to have significantly lower TNF production in blood stimulated *ex vivo* by LPS, compared to normolipidemic controls [34]. The study by Thai *et al.*[19] reported suppressive effects of ac-LDL on the mRNA expression of TNF, IL-1α, and MCP-1 in mouse peritoneal macrophages, as well as on macrophage-mediated tumor cytolysis [19]. In other studies, ac-LDL and LDL have been shown to inhibit natural killer (NK) cell function [35], and both ox-LDL and LDL decreased TNF mRNA expression in NK cells [36], providing indirect support for the concept that LDL uptake results in downregulation of the inflammatory functions of monocytes/macrophages. It is important to note that even though the immediate effect of LDL uptake seems anti-inflammatory, accumulation of lipoproteins and foam cells in the vascular wall indirectly results in tissue damage and inflammation that can weaken the atherosclerotic plaque.

Conclusions
The present study shows that the inducibility of TNF expression decreases in lipid-loaded human macrophages. Hence, uptake of LDL may have a suppressive effect on the inflammatory potential of macrophages. The results imply that foam cells in atherosclerotic plaques could contribute less potently to an inflammatory reaction than newly arrived monocytes/macrophages. Suppression of inflammatory processes by cellular lipid accumulation could play a protective role by down-regulation of the destructive activities of macrophages such as matrix degradation, production of oxidants and killing of smooth muscle cells. On the other hand, if uptake of lipids promotes differentiation of monocytes/macrophages into a more quiescent state, clearance of lipids trapped in the vascular wall could decrease.

Methods
Reagents
[γ32P]ATP (185 TBq/mmol) was from Amersham Biosciences (Buckinghamshire, UK). Antibodies against NFKB1 (p50) and RelA (p65) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The oligonucleotides and antibodies for mobility shift assays of AP-1 and NF-κB were obtained from Promega (Madison, WI) and Santa Cruz Biotechnology. TNF and IL-1β were purchased from R&D Systems (Minneapolis, MN). Ficoll-Paque and Biotrak ELISA kits for TNF were purchased from Amersham Biosciences and used according to the manufactur-

er's instructions. Nonidet P-40 was from Roche (Basel, Switzerland).

Cell culture
Human peripheral blood monocytes were isolated from buffy coats obtained from healthy blood donors. An equal volume of PBS was added to the buffy coats. An amount of dextran (6% stock solution) was then added corresponding to 10% of the volume after addition of PBS. The tubes were left to stand for 35 min, whereafter the upper phase was transferred to new tubes and centrifuged at 200 *g* for 10 min. The supernatant was discarded and the pellets resuspended in PBS. The centrifugation was repeated twice, whereafter the cells were resuspended in 30 mL PBS. The suspension was transferred to two new tubes (15 mL to each). 10 mL Ficoll-Paque was added underneath the 15 mL suspensions, and the tubes were centrifuged at 400 *g* for 35 min. Leukocytes were collected from the interface and pelleted by centrifugation at 400 *g* for 35 min. Cells were resuspended in 50 mL PBS and counted. They were centrifuged at 400 *g* for 10 min and resuspended in an appropriate volume of Macrophage-SFM (Invitrogen Life Technologies, Carlsbad, CA) culture medium supplemented with GM-CSF (10 ng/mL). 80 million cells were added to 100 mm cell culture dishes. Two hours later, the cells were rinsed three times with warm PBS containing Ca^{2+} and Mg^{2+}. Cells were then incubated over night in Macrophage-SFM culture medium supplemented with GM-CSF (10 ng/mL), transferrin (25 μg/mL) and gentamicin (50 μg/mL). The medium was changed the following day, and subsequently every third day. Seven days later, monocytes had differentiated into macrophages and experiments or pretreatments without GM-CSF were started, as indicated in the text. Cell culture and preparation of LDL were coordinated so that only fresh LDL and ac-LDL (up to two weeks old) were used in the experiments.

Preparation of nuclear extract
Cells in 100 mm plastic dishes were rinsed with ice-cold PBS and harvested in 5 mL PBS by scraping. Nuclear extracts were prepared essentially as described by Alksnis *et al*[37]. The cells were washed with 1 mL PBS and resuspended in 100 μL hypotonic buffer (10 mmol/L Hepes, pH 7.3, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol (DTT), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF)). After centrifugation, cells were lysed by resuspension in 300 μL of lysis buffer (10 mmol/L Hepes, pH 7.3, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.4% Nonidet P-40, 1 mmol/L DTT, 1 mmol/L PMSF, 1 μg/mL leupeptin, 15 μg/mL aprotinin). Following 10 min incubation at 4°C, nuclei were collected by centrifugation for 1 min at 8000 *g*. The pellets were washed once in 1 mL of 20 mM KCl buffer (20 mmol/L Hepes, pH 7.3, 22% glycerol, 20 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L PMSF, 1 μg/mL leu-

peptin, 15 μg/mL aprotinin). The isolated nuclei were resuspended in 41 μL of 20 mmol/L KCl buffer, and 39 μL of 600 mmol/L KCl buffer (20 mmol/L Hepes, pH 7.3, 22% glycerol, 0.6 mol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L PMSF, 1 μg/mL leupeptin, 15 μg/mL aprotinin) was added. Nuclear proteins were extracted by incubation on ice for 30 min. After centrifugation for 15 min at 8000 *g*, the supernatant containing nuclear proteins was transferred to a precooled microcentrifuge tube, and an aliquot of the extract was diluted 40 times with 300 mmol/L KCl buffer (mixture of those above) for protein assay. Protein concentration was determined spectrophotometrically [38] according to the following equation: Protein concentration (μg/mL) = 184 × A(230 nm) − 81.7 × A(260 nm).

Electrophoretic mobility shift assay (EMSA)
Equal amounts of protein from nuclear extracts (1–3 μg) were incubated on ice with 2 μg poly(dI-dC) and 1 μg acetylated bovine serum albumin (BSA) in binding buffer (giving the final concentrations stated below) for 10 min. The oligonucleotide probe (50000 cpm in 5 μL) was added and the reaction mixture (25 μL) was incubated for 30 min at room temperature. Final concentrations in binding reactions were as follows: 10% glycerol, 10 mmol/L Hepes (pH 7.9), 60 mmol/L KCl, 5 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L PMSF. DNA-protein complexes were separated from unbound DNA probe on native 7% polyacrylamide gels in low ionic strength buffer (22.3 mmol/L Tris, 22.3 mmol/L borate, 0.5 mmol/L EDTA, pH 8). The sequences of the double-stranded oligonucleotide probes labeled with T4 kinase and [γ³²-P]ATP were as follows: κB consensus: 5'-AGT TGA GGG GAC TTT CCC AGG C-3'; κB mutant: 5'-AGT TGA GG<u>C</u> GAC TTT CCC AGG C-3'; AP-1 consensus: 5'-CGC TTG ATG ACT CAG CCG GAA-3'. AP-1 mutant: 5'-CGC TTG ATG ACT <u>TG</u>G CCG GAA-3'. CD36 gene DR-1 site was used as EMSA probe for PPAR-γ [7]: 5'-GGG GTC AGT AAG TCA GAG GCC AGG GA-3'; PPAR-γ competitor: 5'-GGG GTC AGT AAG TCA G<u>TTTC</u>A<u>G</u>GG <u>A</u>-3'.

Preparation of LDL
Blood was drawn from healthy volunteers into citric acid-containing vials and plasma recovered by centrifugation at 1400 *g* for 20 min at +1°C. The density of the isolated plasma was adjusted to 1.019 kg/l by adding 28 μL of 0.760 mol/L (630.22 g/L) NaBr (density 1.46 g/mL at 20°C) to each mL of plasma. The plasma was centrifuged at 50000 rpm (190000 *g*) for 20 h at +1°C in Beckman Quick-Seal Bell-Top tubes in a Beckman 70 Ti rotor. Top and bottom fractions were separated with a Beckman Tube Slicer by cutting the tubes 3 cm from the top. The top fraction (VLDL and IDL) was discarded. The density of the bottom fraction was raised to 1.065 g/mL by adding 100 μL of 0.760 mol/L NaBr to each mL of the fraction. After

ultracentrifugation as above LDL was harvested as a top fraction by cutting the tubes 1 cm from the top with the tube slicer. The LDL was immediately sterile filtered (0.2 µm filter) and transferred to sterile dialysis tubing for subsequent dialysis at 4°C against sterile PBS with 0.3 mmol/L EDTA for 3 days, with two daily exchanges of the PBS. The protein content of the LDL preparation was determined after dialysis and sterile filtration as described by Lowry [39].

Acetylation of LDL
1 mL of 0.91 mol/L (100 mg/mL) N-acetylimidazole in phosphate buffer was added to 3 mL of LDL (typical protein concentration 7 mg/mL) in 100 mmol/L sodium phosphate, 100 mmol/L KCl, pH 7.4, at 0°C. After 15 min, the reaction was stopped by passing the reaction mixture over Sephadex G-50 (BIO-RAD Econo-Pac 10 DG column). The acetylated LDL was then dialyzed at 4°C against PBS, 0.3 mmol/L EDTA, for 3–5 days prior to use. Lipid uptake was confirmed in all the experiments by the clearly visible presence of large amounts of lipid in cell lysates after treatments with ac-LDL but not after control treatments without lipoproteins. Lipid uptake was quantitated by high-performance thin-layer chromatography (HP-TLC), which showed that the cellular lipid content/total protein (µg/mg) increased after treatment with ac-LDL (50 µg/mL, 48 h) as follows: cholesteryl ester 87 (ac-LDL) vs. 29 (control) (200% increase compared to control); triacylglycerol 56 vs. 24 (133% increase); unesterified cholesterol 57 vs. 51 (12% increase); phosphatidylcholine 36 vs. 36 (unchanged).

Oxidation of LDL
Copper oxidation was performed by incubation (37°C, 5% CO_2) of LDL in the presence of 5 µmol/L $CuSO_4$ for the times indicated, typically 15 h. To determine thiobarbituric acid reactive substances (TBARS), lipoprotein samples (0.1 mL) were incubated in a boiling water bath for 15 min with 0.1 mL of 1 mmol/L ferric chloride, 0.1 mL of 1 mmol/L butylated hydroxytoluene (BHT), 1.5 mL of 0.2 mol/L glycine buffer (pH 3.6) and 1.5 mL of 3.5 mmol/L thiobarbituric acid (TBA) supplemented with 0.3% SDS. After cooling to room temperature, the pink chromogen was extracted[40] with acetic acid (1 mL) and chloroform (2 mL). The optical density of the upper layer was measured at 532 nm against a water blank. 1,1,3,3-Tetraethoxypropane (TEP) was used as a standard. Values < 2 nmol TEP equivalents/mg of protein were obtained for non-oxidized LDL, while ox-LDL had values of 20 to 30 nmol TEP equivalents/mg of protein.

Northern Blotting
Poly(A⁺)RNA was isolated using the MicroFastTrack kit (Invitrogen) utilizing oligo(dT) cellulose. 2 µg of the mRNA was electrophoresed in formaldehyde/1.3% agar-ose gels and transblotted to nylon membranes. The blots were hybridized with ^{32}P-end-labeled probes with a specific activity approximately 35×10^9 cpm/µg. The cDNA probe encoding TNF was a 1196 bp fragment cleaved from a pBR322 derived vector obtained from Chiron Corporation (Emeryville, CA). Prehybridization was done at 42°C for 6 h. Hybridizations were done overnight at 42°C in a commercial hybridization solution (NorthernMax, Ambion, Austin, Texas) containing 0.1 mg/mL salmon sperm DNA and 2 ng of probe per mL. After hybridization, blots were washed twice in 2X SSPE, 0.1% SDS at room temperature for 45 min, twice in 2X SSPE, 2% SDS at 65°C for 45 min, and twice in 0.1X SSPE, 0.1% SDS at room temperature for 15 min. The membranes were then rinsed in 2X SSPE and wrapped in saran wrap before scanning using a Fujifilm BAS-1500 phosphorimager and subsequent exposure to Amersham Hyperfilm MP film with an intensifying screen at -70°C.

Authors' contributions
Ares MPS conceived of the study, carried out most of the experiments and drafted the manuscript. Stollenwerk M and Olsson A carried out some mobility shift assays and TBARS analyses. Kallin B helped performing the northern blotting. Jovinge S participated in the design of the study. Nilsson J participated in its design and coordination. All authors read and approved the final manuscript.

List of abbreviations
TNF, tumor necrosis factor

LDL, low density lipoprotein

ac-LDL, acetylated LDL

ox-LDL, oxidized LDL

AP-1, activator protein-1

NF-κB, nuclear factor-κB

PPARγ, peroxisome proliferator-activated receptor γ

IL, interleukin

MCP-1, monocyte chemotactic protein-1

VLDL, very low density lipoprotein

GM-CSF, granulocyte-macrophage colony-stimulating factor

LPDS, lipoprotein-deficient serum

Acknowledgments

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