# Native LDL potentiate TNFα and IL-8 production by human mononuclear cells

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**Abstract Native LDL (nLDL) increases expression of adhesion molecules on endothelial cells through induction of $Ca^{2+}$ mobilization. $Ca^{2+}$ mobilization is also involved in the induction of proinflammatory cytokines, important mediators involved in atherogenesis. The aim of the study was to evaluate the capacity of nLDL to affect spontaneous and lipopolysaccharide (LPS)-stimulated cytokine production. Preincubation of human peripheral blood mononuclear cells (PBMC) with nLDL for 24 h did not influence spontaneous production of tumor necrosis factor α (TNFα) or interleukin-8 (IL-8), but significantly potentiated LPS-induced production of these cytokines. nLDL preincubation of PBMC did not increase the expression of the LPS receptors Toll-like receptor-4, CD14, or CD11c/CD18. Potentiation of cytokine production by nLDL was mediated through induction of $Ca^{2+}$ mobilization, because: *a*) nLDL induced a sustained pattern of repetitive $Ca^{2+}$ transients in human PBMC; *b*) the $Ca^{2+}$ chelator fura 2-acetoxymethyl ester, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid, an intracellular $Ca^{2+}$ chelator, inhibited the potentiating effect of nLDL on LPS-induced cytokine synthesis; *c*) induction of $Ca^{2+}$ mobilization by thapsigargin potentiated LPS-induced cytokine production. nLDL are able to potentiate LPS-induced production of cytokines by human PBMC, and this effect is probably mediated through induction of $Ca^{2+}$ mobilization. This may represent an important pathogenetic mechanism in atherogenesis induced by hyperlipoproteinemia.**—Netea, M. G., B. J. Kullberg, P. N. M. Demacker, L. E. H. Jacobs, T. J. G. Verver-Jansen, A. Hijmans, L. H. J. van Tits, J. G. J. Hoenderop, P. H. G. M. Willems, J. W. M. Van der Meer, and A. F. H. Stalenhoef. **Native LDL potentiate TNFα and IL-8 production by human mononuclear cells. *J. Lipid Res.* 2002.** 43: **1065–1071.**

**Supplementary key words** tumor necrosis factor α • interleukin-8 • $Ca^{2+}$ mobilization • low density lipoprotein

Atherosclerosis is the major cause of morbidity and mortality in the Western society. There is accumulating evidence for an important role of inflammatory processes in the early phase of atherogenesis (1). The early interaction between the circulating monocytes and the endothelial cells of the intima, followed by subsequent migration of monocytes into the vessel wall where they differentiate into macrophages, is a very important initial step in the inflammatory reaction leading to atherogenesis (2). In the vessel wall, the newly-formed macrophages start to ingest biologically-modified lipid particles and transform into foam cells which constitute the first step in the formation of "fatty streaks," the precursor lesion which subsequently leads to the formation of the atherosclerotic plaque (3). Among the risk factors for the development of atherosclerosis, hyperlipoproteinemia, especially the increased circulating concentrations of LDL, is one of the most important (4, 5). Several studies have stressed the capacity of modified LDL forms such as oxidized LDL (oxLDL) and acetylated LDL (acLDL) to accumulate into and activate the macrophages, inducing inflammatory reactions in the vessel wall, which would eventually accelerate atherogenesis. Modified LDL can stimulate monocyte migration (6), expression of adhesion molecules (7), and production of proinflammatory cytokines and chemokines such as tumor necrosis factor α (TNFα), interleukin-8 (IL-8), and monocyte chemoattractant protein-1 (MCP-1) (8, 9). The induction of cytokines during the process of atherogenesis represents an important step, as these signal molecules increase the expression of adhesion molecules on endothelial cells (10), enhance the uptake of oxidized LDL through increased expression of macrophage scavenger receptors (11), regulate plaque stability (12), and induce production of endogenous growth factors that regulate cell proliferation in the arterial cell wall (13).

Abbreviations: BAPTA-AM, the $Ca^{2+}$ chelator fura 2-acetoxymethyl ester, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; IL-8, interleukin-8; LPDP, lipoprotein-depleted plasma; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells; PKC, protein kinase C; TLR4, Toll-like receptor 4; TNF, tumor necrosis factor; TNFbp, TNF binding proteins.

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In addition to the data attesting a role for modified LDL during the inflammatory processes leading to atherosclerosis, recent studies reported that native LDL (nLDL) also have important immune-stimulating properties, which may be an important factor connecting hyperlipoproteinemia with inflammation during atherogenesis. nLDL stimulates chemokine CCR2 expression on monocytes (14) and potentiates chemotaxis of human monocytes (15). Allen and colleagues have shown that nLDL induces expression of VCAM-1 and E-selectin in human vascular endothelial cells, and this is mediated through induction of $Ca^{2+}$ mobilization (16). Interestingly, it has been demonstrated that $Ca^{2+}$-dependent mechanisms are directly involved in cytokine production and release by macrophages and neutrophils (17, 18), and induction of TNF by acetylated LDL is also a $Ca^{2+}$-dependent phenomenon (8). It is therefore tempting to speculate that nLDLs are also able to potentiate spontaneous and/or stimulated production of proinflammatory cytokines by bloodstream human monocytes through $Ca^{2+}$-dependent mechanisms, contributing in this way to the development of atherosclerosis.

The aim of the present study was to evaluate the capacity of nLDL to potentiate spontaneous and lipopolysaccharide (LPS)-stimulated cytokine production by freshly isolated human peripheral blood mononuclear cells (PBMC), and to investigate the possible mechanisms responsible for these effects.

## MATERIALS AND METHODS

### Materials

LPS (*Escherichia coli* serotype O55:B5) was obtained from Sigma Chemical Co. (St. Louis, MO). Fura 2-acetoxymethyl ester (fura 2-AM), 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-AM), and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR). Thapsigargin was obtained from LC Services (Woburn, MA). Murine anti-human monoclonal antibodies were used for the FACS studies: anti-CD11c [clone SHCL3 (19)], anti-CD18 (clone AZN-L19), and anti-CD14 (RM052, Beckman Coulter, Fullerton, CA). TNF binding proteins (TNFbp) were a kind gift from Charles Dinarello (University of Colorado Health Sciences Center, Denver, CO). LDL, HDL, and lipoprotein-depleted plasma (LPDP) were isolated from freshly collected human EDTA-plasma by sequential centrifugation, as previously described (20). As oxidation of LDL has been suggested to influence cytokine production, we measured LDL oxidation by quantitating thiobarbituric acid-reactive substances (TBARS) by fluorometry (21). The TBARS assay uses the capacity of aldehydes, which are formed during LDL oxidation, to combine with thiobarbituric acid to yield a fluorochrome.

### Experimental design

Venous blood was collected by venipuncture in the cubital fossa from 11 healthy volunteers. All subjects gave informed consent prior to the study. Separation of PBMC was performed as described elsewhere (22). PBMC were washed twice in saline and suspended in culture medium (RPMI 1640 Dutch modification, ICN Biomedicals, Costa Mesa) supplemented with human serum 5%, gentamicin 1%, L-glutamine 1%, and pyruvate 1%. The cells were counted in a Coulter counter (Coulter Electronics, Mijdrecht, The Netherlands) and the number was adjusted to $5 \times 10^6$ cells/ml. $5 \times 10^5$ cells/well were incubated in 96-wells plates (Greiner, Alphen a/d Rijn, The Netherlands) in a 200 μl final volume with either lipoprotein deficient plasma LPDP (<0.1 mmol/l cholesterol) or native LDL (2.0 mmol/l cholesterol), for 24 h at 37°C. This LDL concentration is the maximal concentration we can obtain after sequential centrifugation. In a control experiment, PBMC were incubated with a similar concentration of HDL. After 24 h, the cells were divided in two subgroups. In the first subgroup, the supernatants were collected and the spontaneous cytokine production after LPDP or nLDL incubation was measured. In the second subgroup, the plates were centrifuged mildly (500 *g*, 5 min), and the cells were washed three times with LPS-free PBS. Subsequently, 200 μl culture medium or LPS (1 ng/ml) was added to the cells, and the plates were incubated for an additional 24 h at 37°C. At the end of the incubation time, the supernatants were collected and stored at −70°C.

In additional experiments, to study the role of the $Ca^{2+}$ mobilization for the LDL potentiation of cytokine production, the initial incubation of PBMC with LPDP or LDL was performed in the presence or absence of an intracellular $Ca^{2+}$ chelator (BAPTA-AM, 10 μM), which was present the entire 24 h of preincubation period. As a positive control, a $Ca^{2+}$-mobilization inducer (thapsigargine, 1 μM) was added in separate wells, without LPDP or nLDL supplementation. These compounds were washed after the initial 24 h incubation, and were not added during the subsequent 24 h LPS stimulation.

The role of protein kinase C (PKC) activation for the effects of nLDL was investigated by blocking its activity by addition of staurosporine (1 μM) to the PBMC during the 24 h incubation with LPS. In addition, we assessed whether endogenous TNF is required for the LPS-induced production of IL-8 by neutralizing endogenous TNF through addition of TNFbp (10 μg/ml) to PBMC 1 h prior to addition of the stimuli. All combinations of stimuli and agents were not toxic to the cells, as shown by trypan blue exclusion method (>95% viability of cells).

### Cytokine measurements

TNFα concentrations were determined by a specific radioimmunoassay, as previously described (23). IL-8 was measured by a commercial ELISA kit (Pelikine Compact, CLB, Amsterdam, The Netherlands).

### RT-PCR for Toll-like receptor-4

A semiquantitative RT-PCR method was used to assess the effect of LDL on the expression of Toll-like receptor-4 (TLR4) mRNA. Total RNA was isolated by the method of Chomczynski and Sacchi (24) from $1 \times 10^6$ PBMC incubated for 6 h or 24 h with either LDL or LPDP. The RNA was dissolved in RNAase-free water and its quality analyzed by agarose gel electrophoresis. For each sample, 0.5 μg of total RNA was reverse transcribed as previously described (25). Sequences of the PCR primers used for human TLR4 were: sense 5′-ATACTTAGACTACTACCTCCATG -3′ and antisense 5′-AAACTCAAGGCTTGGTAGATC -3′; and for the house keeping gene β2-microglobulin (β2m) were: sense 5′-CCAGCAGAGAATGGAAAGTC-3′ and anti-sense 5′-GATGCTGCTTACATGTCTCG-3′. Each primer pair was tested to determine the annealing temperature and the linear range of the reaction. PCR reactions consisted of 3 μl cDNA in 50 μl PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM Kcl, 1.5 mM $MgCl_2$, 0.001% gelatin) containing 100 μM dNTPs, 0.3 μM of each primer, and 1.25 units Taq polymerase. Reaction mixtures were overlaid with



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mineral oil. PCR cycles were perfomed (30 s denaturation at 92°C, 30 s annealing at 51°C, and 90 s extension at 72°C) on the Mastercycler 5330 (Eppendorf, Hamburg, Germany). The plateau phase of the β2m reaction was apparent after 30 cycles, and of TLR4 after 38 cycles. We therefore selected 26 cycles for the β2m PCR and 32 cycles for the TLR4 PCR. PCR products were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide in order to quantify the intensity of the banding pattern. Gels were scanned on a densitometer (GS-670, Biorad, Veenendaal, The Netherlands) and analyzed using Molecular Analyst Software. The amount of TLR4 mRNA in each sample was expressed semi-quantitatively as a ratio versus the amount of the housekeeping gene β2m mRNA.

### Flow cytometry analysis

PBMC from seven volunteers was isolated as described above, and after 24 h incubation with LPDP or LDL the expression of CD14, CD11c, and CD18 was analyzed by flow cytometry. The analysis was performed on a FACScan (Becton and Dickinson, Oxnard, CA) using standard immunofluorescence staining techniques. The expression of the LPS receptors on the membrane of monocytes and lymphocytes incubated with nLDL was calculated as a percentage of the expression of the respective molecule on the membrane of the cells incubated with LPDP.

### Measurements of the cytosolic free $Ca^{2+}$ concentration after nLDL incubation

Cytosolic free $Ca^{2+}$ concentrations in PBMC (after either LPDP, nLDL, or LPS had been added to the cells at the concentrations mentioned before) were measured as previously described, using 10 M fura 2-AM as $Ca^{2+}$-indicator (26). Dynamic video imaging was carried out with the Magical system and TARDIS software provided by Joyce Loebl (Tyne and Wear, UK). Fluorescence emission ratios are reported as a measure of intracellular $Ca^{2+}$ concentration $(Ca^{2+})_i$ (26).

### Statistical analysis

All cytokine stimulation experiments were performed in duplicate (in at least five volunteers), and data are given as mean ± SD. Comparisons between the groups were performed using the non-parametric Wilcoxon test. The differences were considered significant at $P < 0.05$.

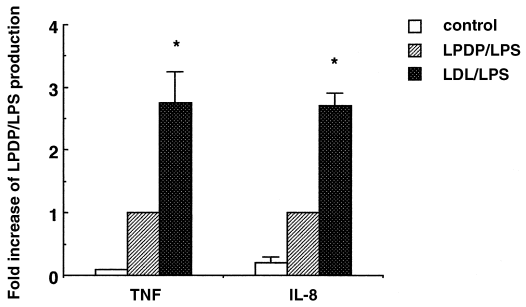


**Fig. 1.** Potentiation of lipopolysaccharide (LPS)-induced cytokine production by native LDL (nLDL). Preincubation of peripheral blood mononuclear cells (PBMC) with nLDL for 24 h potentiated the subsequent LPS-induced production of tumor necrosis factor (TNF) and interleukin-8 (IL-8), compared with lipoprotein-depleted plasm (LPDP) preincubation. Data are given as mean ± SD obtained from 11 volunteers. *$P < 0.01$.

## RESULTS

### Potentiation of cytokine production by nLDL

Preincubation of PBMC with either LPDP or nLDL for 24 h did not induce subsequent spontaneous production of TNF during the next 24 h. Similarly, subsequent spontaneous IL-8 release was also very low, although measurable, and did not differ between the cells preincubated with either LPDP or nLDL (data not shown). When the PBMC were subsequently stimulated with LPS, they produced significantly more cytokines when preincubated with nLDL than with LPDP (TNF: 369 ± 65 vs. 132 ± 23 pg/ml, $P < 0.01$; IL-8: 34.4 ± 2.1 vs. 12.7 ± 3.9 ng/ml, $P < 0.01$) (**Fig.1**). The concentrations of TBARS in the LDL preparation was below the detection limit (0.01 nmol/l) both before and after 24 h incubation with the PBMC, implying that LDL oxidation did not play a role in the effects observed on cytokine production.

To investigate whether the stimulatory effect on LPS-induced TNF and IL-8 production is specific for LDL, we preincubated cells with either HDL or LPDP in a separate experiment. There was no potentiating effect of HDL on the LPS-stimulated production of TNF and IL-8 ($P > 0.05$). Peripheral blood mononuclear cells (PBMC) consist of approximately 20% monocytes and 80% lymphocytes. In previous experiments, we have investigated the relative importance of these two cell populations for the production of TNF and IL-8, and we have shown that more than 95% of the total cytokine synthesis originates from the monocyte population (not shown).

### The effect of nLDL on LPS receptor expression

Because nLDL potentiate LPS-induced production of TNF and IL-8, we investigated whether this may be mediated through increased expression of LPS receptors on the surface of the cells incubated with nLDL. The TLR4 expression was similar in PBMC incubated with either LDL or LPDP: TLR4/β2m ratio after 6 h preincubation 0.40 ± 0.07 versus 0.44 ± 0.10; and after 24 h preincubation 0.25 ± 0.09 versus 0.33 ± 0.14, $P > 0.05$. There was a modest though significant increase in the expression of CD14 on the surface of monocytes preincubated with nLDL (119 ± 8% of the expression on monocytes incubated with LPDP, $P < 0.05$). In contrast, the expression of both CD11c (107 ± 11%, $P > 0.05$) and CD18 (97 ± 12%, $P > 0.05$), was not different between monocytes incubated with either nLDL or LPDP. CD14, CD11c, and CD18 expression on lymphocytes was low and not different between the incubations with LDL or LPDP (not shown).

### Intracellular $Ca^{2+}$ mobilization by nLDL

As it has been recently shown that nLDL is able to induce intracellular $Ca^{2+}$ transients in human vascular epithelial cells (16), we tested whether this may also be the case for PBMC. Addition of nLDL to freshly isolated PBMC caused a rapid increase in the intracellular $Ca^{2+}$ concentration (**Fig. 2A**). Interestingly, although the level of $Ca^{2+}$ gradually decreased after reaching a maximum, the majority of the cells were able to respond with repeti-



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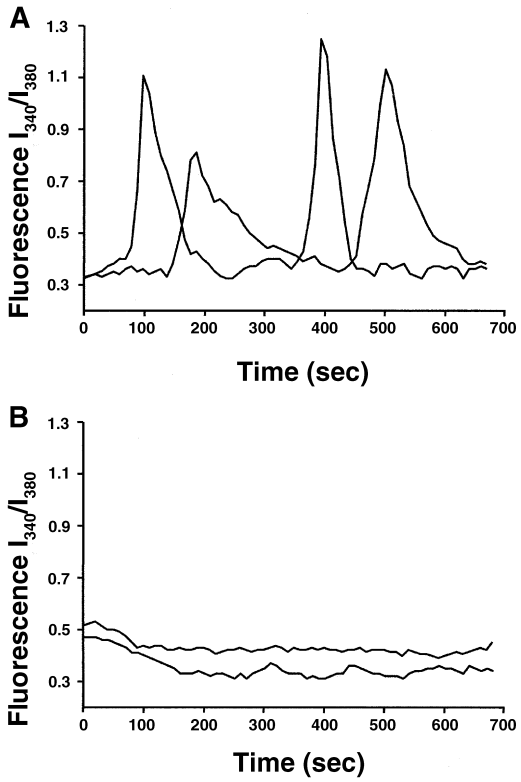


**Fig. 2.** Induction of $Ca^{2+}$ mobilization by nLDL. Stimulation of PBMC with nLDL (A) led to rapid induction of a transient increase of intracellular $Ca^{2+}$. Most of the cells responded with more than one transient, in a oscillatory pattern. No increase in intracellular $Ca^{2+}$ levels was measured after adding LPDP to the cells (B). The two lines represent fluorescence emission ratios as a measure of intracellular $Ca^{2+}$ concentrations in duplicate experiments.

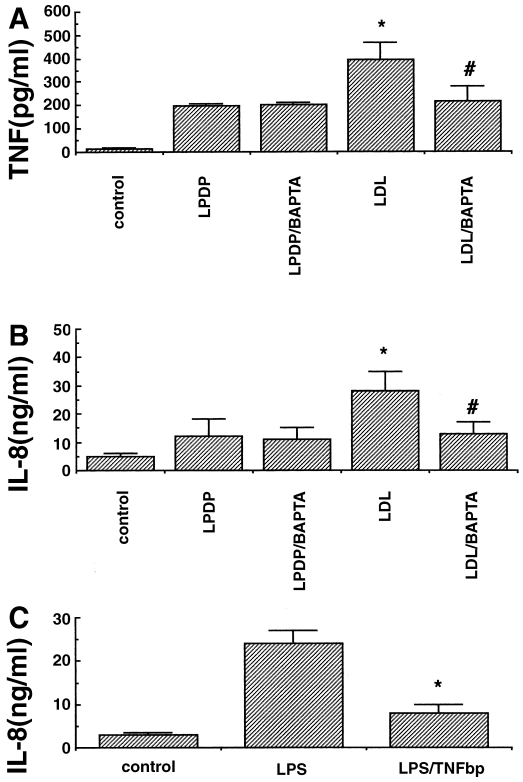


**Fig. 3.** The role of $Ca^{2+}$ on nLDL effect on TNF and IL-8 production. Addition of the $Ca^{2+}$ chelator fura 2-acetoxymethyl ester, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid during the preincubation of PBMC with nLDL, significantly reduced the potentiating effect of nLDL on LPS-induced TNF (A) or IL-8 (B) synthesis. No effect of BAPTA-AM was found when added to LPDP. Neutralization of endogenous TNF by TNF binding proteins strongly down-regulated LPS-induced IL-8 production (C). Data are given as mean ± SD obtained from five volunteers. $^{*}P < 0.05$ when compared with LPDP. $^{\#}P < 0.05$ when compared with LDL alone.

tive $Ca^{2+}$ transients, indicating the induction of a sustained pattern of $Ca^{2+}$ mobilization (Fig. 2A). In contrast, no effect was observed when LPDP was added to the cells (Fig. 2B). In additional experiments, we investigated the capacity of LPS to induce $Ca^{2+}$ transients. No effect on intracellular $Ca^{2+}$ after stimulation of PBMC with LPS was measured (not shown).

### The role of $Ca^{2+}$ mobilization for the potentiation of TNF production by nLDL

To investigate whether induction of repetitive $Ca^{2+}$ transients by nLDL leads to increased responsiveness of PBMC to LPS, we added BAPTA-AM (a known chelator of intracellular $Ca^{2+}$) to the cells during the incubation period with nLDL or LPDP. As shown in **Fig. 3A**, addition of BAPTA-AM significantly reduced the potentiating effect of nLDL on LPS-induced TNF production. In addition, thapsigargin, a specific blocker of intracellular $Ca^{2+}$ ATPase activity that increases the intracellular $Ca^{2+}$ concentration, strongly potentiated the LPS-stimulated TNF synthesis from 156 ± 37 to 1365 ± 243 pg/ml ($P < 0.02$). Similarly, potentiation of LPS-induced IL-8 synthesis was $Ca^{2+}$-dependent, and influenced by both BAPTA and thapsigargin (Fig. 3B). In contrast, blocking PKC and tyrosine kinases activity by staurosporine did not influence nLDL/LPS-induced TNF production (551 ± 129 vs. 462 ± 164 pg/ml, $P > 0.05$).

### The intermediary role of endogenous TNF for LPS-induced IL-8 production

Since TNF is known to be a potent stimulus of IL-8 synthesis, the role of endogenous TNF in the induction of IL-8 by LPS was investigated by blocking endogenous TNF with TNFbp. A 63% reduction in LPS-induced IL-8 was observed when TNFbp was added to PBMC (Fig. 3C). This implies that the potentiation of LPS-induced IL-8 production by nLDL is at least in part mediated by stimulation of endogenous TNF release.

## DISCUSSION

The results of the present study demonstrate for the first time that nLDL are able to render freshly isolated human PBMC hyperresponsive to LPS, and subsequently potentiate the cytokine production after LPS triggering. These effects are specific for LDL, as HDL did not in-



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fluence LPS-induced cytokine production. The action exerted by nLDL is mediated through induction of $Ca^{2+}$ mobilization in the cells, as stimulating or blocking intracellular $Ca^{2+}$ influxes modulates the nLDL-induced cytokine stimulation. At present, however, the mechanisms by which nLDL-induced $Ca^{2+}$ signaling exerts its potentiating effect is unknown. The possibility that nLDL-induced $Ca^{2+}$ signaling leads to upregulation of LPS receptors is unlikely, as the expression of LPS receptors on the cell membrane was only marginally influenced.

The importance of inflammatory processes for the development of atherosclerosis has been strongly underlined in the recent years (1). It has been shown that modified forms of LDL, such as oxLDL and acLDL, are able to activate immune processes by stimulating monocyte migration (6), expression of adhesion molecules (7), and production of proinflammatory cytokines and chemokines (8, 9). However, in the last few years it has become apparent that non-modified nLDL also has immune-stimulating properties, such as stimulation of adhesion molecule expression on vascular endothelial cells (16), chemokine receptor expression (14), and monocyte chemotaxis (15). In line with these studies, our report provides new information regarding the capacity of nLDL to potentiate LPS-induced production of proinflammatory cytokines such as TNF, and chemokines such as IL-8. This phenomenon may have important consequences, as IL-8 chemotaxis can be instrumental in the early phase of atherogenesis (27), and TNF participates in the atherosclerotic process through various mechanisms such as stimulation of adhesion molecule expression (10), stimulation of scavenger receptor expression and cholesterol loading (11), regulation of plaque stability (12), and stimulation of growth factors important for proliferation (13). Despite the effects of nLDL on LPS-induced cytokines, no direct effect of nLDL on cytokine production could be seen. This is in agreement with other studies that have demonstrated the absence of a direct stimulatory effect of nLDL on cytokine production (8, 9).

The capacity of nLDL to potentiate LPS-induced cytokines is highly relevant in view of the recent data implicating chronic infection of the vessel wall with the intracellular gram-negative microorganism *Chlamydia pneumoniae* in the pathogenesis of atherosclerosis. In addition to more than a dozen epidemiological studies documenting an association between infection with *C. pneumoniae* and early atherosclerosis (28), in-vitro studies have shown that *C. pneumoniae* can infect monocytes/macrophages and smooth muscle cells (29), and can induce macrophage foam cell formation (30, 31). Recently, we and others have also shown the capacity of Chlamydia-stimulation to potentiate LDL oxidation (32, 33). The multiplication of Chlamydia inside macrophages and putative chronic release of cellular components including LPS trigger the production of proinflammatory cytokines such as TNF, IL-1, and IL-6 (34, 35). Hyperresponsiveness of macrophages from hyperlipoproteinemic individuals to this stimulation may prove a highly deleterious event for the subsequent development of atherosclerosis. In this respect, we have recently reported that macrophages isolated from hyperlipoproteinemic $LDLR^{-/-}$ mice (36), as well as PBMC from homozygous patients with familial hyperlipoproteinemia (FH) (37), are also hyperresponsive to LPS stimulation.

There are several possible targets for the action of nLDL on cytokine production. Recent studies have demonstrated the pivotal role of the TLR4-MD2-CD14 receptor for LPS signaling (38). We have assessed the effects of LDL preincubation on the expression of the LPS receptors. LDL did not influence the expression of TLR4 in human PBMC, and this mechanism is therefore unlikely to account for the potentiating effects on LPS-induced production of cytokines. Our data are sustained by the recent publication of data showing that only oxLDL, but not nLDL, are able to increase expression of TLR4 (39). In vitro studies have shown that lipoproteins are essential for cell membrane expression of CD14 (40), which is the high-affinity chain of the receptor needed for the transfer of LPS to TLR4 (41). However, our experiments have demonstrated only a modest increase of 19% in the CD14 expression after incubation of cells with nLDL, which is unlikely to account for the several-fold increase in cytokine production after LPS challenge. Moreover, monocytes of familial hypercholesterolemia patients express normal amounts of CD14 (37, 42), and the increased cytokine production after LPS stimulation of macrophages isolated from $LDLR^{-/-}$ mice is CD14-independent (36). The cytokine production in macrophages from $LDLR^{-/-}$ mice is mediated through CD11c/CD18 (36), which is the second possible LPS co-receptor (43). However, this effect is not exerted at the level of CD11c expression, but at the level of intracellular pathways (36). In line with these data, incubation of human PBMC with nLDL in the present study did not lead to increased expression of either CD11c or CD18.

As the effect of nLDL on cytokine synthesis seemed to be located at the level of post-receptor pathways, our attention focused on the observation that nLDL induces intracellular $Ca^{2+}$ transients (16). As $Ca^{2+}$-dependent mechanisms are involved in cytokine production and release by macrophages and neutrophils (17, 18), it was tempting to speculate that nLDL are also able to potentiate synthesis of proinflammatory cytokines through $Ca^{2+}$-dependent mechanisms. As previously shown in vascular endothelial cells (16), we were able to document an oscillatory pattern of $Ca^{2+}$ mobilization by nLDL in human PBMC. To investigate whether this phenomenon is involved in potentiation of LPS-induced cytokine release, we sought to modulate the nLDL-induced mobilization of $Ca^{2+}$ and to assess its consequences at the level of LPS-induced cytokine production. Indeed, thapsigargin, which is a specific inducer of intracellular $Ca^{2+}$, strongly potentiated LPS-induced TNF production. Conversely, the intracellular $Ca^{2+}$ chelator BAPTA-AM reversed the stimulatory effects of nLDL on LPS-stimulated cytokine synthesis. These data strongly suggest that the effect of nLDL is mediated through mobilization of intracellular $Ca^{2+}$. This is in agreement with the data documenting the same mechanism of action in the induction of TNF by acetylated LDL



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(8), and stimulation of VCAM-1 and E-selectin by nLDL in vascular endothelial cells (16). In contrast, no effect of PKC and tyrosine kinase activation on nLDL/LPS cytokine production has been documented when the activity of these enzymes was blocked by staurosporine.

We thereafter investigated whether the signals induced by LPS also involve mobilization of intracellular $Ca^{2+}$. No $Ca^{2+}$ transients were measured after stimulation of cells with LPS, indicating that LPS stimulation of cytokines does not directly involve $Ca^{2+}$-dependent mechanisms. However, the potentiation of LPS-induced TNF and IL-8 by nLDL and thapsigargin, accompanied by sustained $Ca^{2+}$ transients, suggests that $Ca^{2+}$-dependent mechanisms potentiate the LPS signaling pathway. Recent data have demonstrated that LPS binds to the TLR4-MD2-CD14 receptor complex (38, 44), and this in turn activates an intracellular cascade involving MyD88, IRAK, TRAF6, and NF-kB translocation. The precise level of interaction between $Ca^{2+}$ and this signaling pathway remains to be elucidated. In addition to TNF, IL-8 was also found to be under control of $Ca^{2+}$-dependent pathways. Experiments involving neutralization of endogenous TNF by TNFbp show that at least part of LPS-induced IL-8 production is mediated through endogenous TNF, which is in line with the data from the literature indicating TNF as a potent IL-8 stimulus (45).

As the stimulatory action of nLDL on cytokine production has also been documented in vivo in $LDLR^{-/-}$ mice and homozygous patients with familial hypercholesterolemia (36, 37), it is unlikely that this effect is mediated through the LDL receptor. Alternatively, preliminary data from our laboratory (Netea et al., unpublished results) suggest that the intracellular events leading to an increased responsiveness to a LPS challenge are triggered by a modification of cell membrane fluidity by nLDL, as others have also suggested (46). Other yet to be answered questions regard the cascade of events downstream to $Ca^{2+}$ mobilization, and whether these events are linked to the recruitment of transcription factors such as NF-kB and/or AP-1, which have been shown to be triggered by nLDL (47, 48). The answer to these questions is crucial not only to a better understanding of the processes linking hyperlipoproteinemia and inflammation as mechanisms of atherogenesis, but also for developing new therapeutic strategies aimed to block or delay the atherosclerotic processes.

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## REFERENCES


1. Wick, G., G. Schett, A. Amberger, R. Kleindienst, and Q. Xu. 1995. Is atherosclerosis an immunologically mediated disease? *Immunol. Today.* **16:** 27–33.
2. Faruqi, R. M., and P. E. DiCorleto. 1993. Mechanisms of monocyte recruitment and accumulation. *Br. Heart J.* **69:** S19–S29.
3. Partharasathy, S. D., D. Steinberg, and J. L. Witztum. 1992. The role of oxidized low-density lipoproteins in the pathogenesis of atherosclerosis. *Annu. Rev. Med.* **43:** 219–225.
4. Ross, R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature.* **362:** 801–809.
5. Steinberg, D., and J.L. Witztum. 1990. Lipoprotein and atherogenesis. *JAMA.* **264:** 3047–3052.
6. Navab, M., S. S. Ines, S. Y. Hama, G. P. Hough, L. A. Ross, R. W. Bork, A. J. Valente, J. A. Berliner, D. C. Drinkwater, H. Laks, and A. M. Fogelman. 1991. Monocyte transmigration induced by modification of low density lipoprotein in coculture of human aortic wall cells is due to induction of MCP-1 and abolished by high density lipoprotein. *J. Clin. Invest.* **88:** 2039–2046.
7. Khan, B. V., S. S. Partharasathy, R. W. Alexander, and R. M. Medford. 1995. Modified low density lipoprotein and its constituents augment cytokine-activated vascular cell adhesion molecule-1 gene expression in human vascular endothelial cells. *J. Clin. Invest.* **95:** 1262–1270.
8. Pollaud-Cherion, C., J. Vandaele, F. Quartulli, J. Decerprit, and B. Pipy. 1998. Involvement of calcium and arachidonate metabolism in acetylated-low-density-lipoprotein-stimulated tumor necrosis factor-alpha production by rat peritoneal macrophages. *Eur. J. Biochem.* **253:** 345–353.
9. Wang, N., I. Tabas, R. Winchester, S. Ravalli, E. Rabani, and A. Tall. 1996. Interleukin 8 is induced by cholesterol loading of macrophages and expressed by macrophage foam cells in human atheroma. *J. Biol. Chem.* **271:** 8837–8842.
10. Bevilacqua, M. P. 1993. Endothelial-leukocyte adhesion molecules. *Annu. Rev. Immunol.* **11:** 767–804.
11. Li, H., M. W. Freeman, and P. Libby. 1995. Regulation of smooth muscle cell scavenger receptor expression in vivo by atherogenic diets and in vitro by cytokines. *J. Clin. Invest.* **95:** 122–133.
12. Libby, P., G. Sukhova, R. T. Lee, and Z. S. Galis. 1995. Cytokines regulate vascular functions related to stability of the atherosclerotic plaque. *J. Cardiovasc. Pharmacol.* **25 (Suppl. 2):** S9–S12.
13. Filonzi, E. L., H. Zoellner, H. Stanton, and J. A. Hamilton. 1993. Cytokine regulation of granulocyte-macrophage colony stimulating factor and macrophage colony stimulating factor production in human arterial smooth muscle. *Atherosclerosis.* **99:** 241–248.
14. Han, K. H., R. K. Tangirala, S. R. Green, and O. Quehenberger. 1998. Chemokine receptor CCR2 expression and monocyte chemoattractant protein-1-mediated chemotaxis in human monocytes. A regulatory role for plasma LDL. *Arterioscler. Thromb. Vasc. Biol.* **18:** 1983–1991.
15. Kreuzer, J., S. Denger, L. Jahn, K. Ritter, E. von Hodenberg, and W. Kubler. 1996. LDL stimulates chemotaxis of human monocytes through a cyclooxygenase-dependent pathway. *Arterioscler. Thromb. Vasc. Biol.* **16:** 1481–1487.
16. Allen, S., S. Khan, F. Al-Mohanna, P. Batten, and M. Yacoub. 1998. Native low-density lipoprotein-induced calcium transients trigger VCAM-1 and E-selectin expression in cultured human vascular enothelial cells. *J. Clin. Invest.* **101:** 1064–1075.
17. Seabra, V., R. F. Stachlewitz, and R. G. Thurman. 1998. Taurine blunts LPS-induced increases in intracellular calcium and TNFα production by Kupffer cells. *J. Leuk. Biol.* **64:** 615–621.
18. Kuhns, D. B., H. A. Young, E. K. Gallin, and J. I. Gallin. 1998. $Ca^{2+}$-dependent production and release of IL-8 in human neutrophils. *J. Immunol.* **161:** 4332–4339.
19. Schwarting, R., H. Stein, and C. Y. Wang. 1985. The monoclonal antibody aS-HCL1 (a-Leu-14) and aS-HCL3 (a-Leu-M5) allow diagnosis of hairy cell leukemia. *Blood.* **65:** 974–983.
20. Demacker, P. N. M., H. E. Vos-Janssen, A. P. Jansen, and A. Van't Laar. 1977. Evaluation of the dual precipitation method by comparison with the ultracentrifugation method for the measurement of lipoproteins in serum. *Clin. Chem.* **23:** 1238–1244.
21. Janero, D. 1990. Malondialdehyde and thiobarbituric acid reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radic. Biol. Med.* **9:** 515–540.
22. Endres, S., R. Ghorbani, G. Lonnemann, J. W. M. Van der Meer, and C. A. Dinarello. 1988. Measurement of immunoreactive interleukin-1 beta from human mononuclear cells: optimization of recovery, intrasubject consistency, and comparison with interleukin-1 alpha and tumor necrosis factor. *Clin. Immunol. Immunopathol.* **49:** 424–438.
23. Drenth, J. P. H., S. H. M. Van Uum, M. Van Deuren, G. J. Pesman, J. Van der Ven-Jongekrijg, and J. W. M. Van der Meer. 1995. Endurance run increases circulating IL-6 and IL-1ra but downregulates ex vivo TNF-α and IL-1β production. *J. Appl. Physiol.* **79:** 1497–1503.
24. Chomczynski, P., and N. Sacchi. 1987. Single step method of RNA




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isolation by acid guanidinum phenol chloroform extraction. *Anal. Biochem.* **162:** 156–159.
25. Netea, M. G., J. P. H. Drenth, N. De Bont, A. Hijmans, M. Keuter, E. Dharmana, P. M. N. Demacker, and J. W. M. Van der Meer. 1996. A semi-quantitative reverse-transcriptase polymerase chain reaction method for measurement of mRNA for TNFα and IL-1β in whole blood cultures. Its application in typhoid fever and exentric exercise. *Cytokine.* **8:** 739–744.
26. Willems, P. H. G. M., S. E. Van Emst-De Vries, C. H. Van Os, and J. J. H. H. M. De Pont. 1993. Dose-dependent recruitment of pancreatic acinar cells during receptor-mediated calcium mobilization. *Cell Calcium.* **14:** 145–159.
27. Boisvert, W. A., R. Santiago, L. K. Curtiss, and R. A. Terkeltaub. 1998. A leukocyte homologue of the IL-8 receptor CXCR-2 mediates the accumulation of macrophages in atherosclerotic lesions of LDL receptor-deficient mice. *J. Clin. Invest.* **101:** 353–363.
28. Danesh, J., R. Collins, and R. Peto. 1997. Chronic infections and coronary artery disease: is there a link? *Lancet.* **350:** 430–436.
29. Gaydos, C. A., J. T. Summersgill, N. N. Sahney, J. A. Ramirez, and T. C. Quinn. 1996. Replication of Chlamyda pneumoniae in vitro in human macrophages, endothelial cells, and aortic artery smooth muscle cells. *Infect. Immun.* **64:** 1614–1620.
30. Kalayoglu, M. V., and G. I. Byrne. 1998. Induction of macrophage foam cell formation by Chlamydia pneumoniae. *J. Infect. Dis.* **177:** 725–729.
31. Kalayoglu, M. V., and G. I. Byrne. 1998. A Chlamydia pneumoniae component that induces macrophage foam cell formation is chlamydial lipopolysaccharide. *Infect. Immun.* **66:** 5067–5072.
32. Kalayoglu, M. V., B. Hoerneman, D. LaVerda, S. G. Morrison, R. P. Morrison, and G. I. Byrne. 1999. Cellular oxidation of low-density lipoprotein by Chlamydia pneumoniae. *J. Infect. Dis.* **180:** 780–790.
33. Netea, M. G., C. A. Dinarello, B. J. Kullberg, T. Jansen, L. Jacobs, A. F. H. Stalenhoef, and J. W. M. Van der Meer. 2000. Oxidation of low-density lipoproteins by acellular components of Chlamydia pneumoniae. *J. Infect. Dis.* **181:** 1868–1869.
34. Heinemann, M., M. Susa, U. Simnacher, R. Marre, and A. Essig. 1996. Growth of Chlamydia pneumoniae induces cytokine production and expression of CD14 in a human monocytic cell line. *Infect. Immun.* **64:** 4872–4875.
35. Kaukoranta-Tolvanen, S-S. E., A-M. Teppo, K. Laitinen, P. Saikku, K. Linnavuori, and M. Leinonen. 1996. Growth of Chlamydia pneumoniae in cultured human peripheral blood mononuclear cells and induction of a cytokine response. *Microb. Pathog.* **21:** 215–221.
36. Netea, M. G., P. N. M. Demacker, B. J. Kullberg, O. C. Boerman, I. Verschueren, A. F. H. Stalenhoef, and J. W. M. Van der Meer. 1998. Increased interleukin-1a and interleukin-1β production by macrophages of low-density-lipoprotein receptor knock-out mice stimulated with lipopolysaccharide is CD11c/CD18-receptor mediated. *Immunology.* **95:** 466–472.
37. Rovers, C., M. G. Netea, P. N. M. Demacker, C. Jacobs, B. J. Kullberg, and J. W. M. Van der Meer. 1998. LPS-induced cytokine production and expression of beta-2 integrins and CD14 by peripheral blood mononuclear cells of patients with homozygous familial hypercholesterolemia. *Atherosclerosis.* **141:** 99–105.
38. Poltorak, A., X. He, I. Smirnova, M-Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler. 1998. Defective LPS signaling in C3H/HeJ and C57Bl/10ScCr mice: mutations in TLR4 gene. *Science.* **282:** 2085–2088.
39. Helen, X., P. H. Shah, E. Faure, O. Equils, L. Thomas, M. C. Fishbein, D. Luthringer, X-P. Xu, T. P. Rajavashisth, J. Yano, S. Kaul, and M. Arditi. 2001. Toll-like receptor-4 is expressed by macrophages in murine and human lipid-enriched atherosclerotic plaques and upregulated by oxidized LDL. *Circulation.* **104:** 3103–3108.
40. Esfahani, M., R. D. Bigler, J. L. Alfieri, S. Lund-Katz, J. D. Baum, and L. Scerbo. 1993. Cholesterol regulates the cell surface expression of glycophospholipid-anchored CD14 antigen on human monocytes. *Biochim. Biophys. Acta.* **1149:** 217–223.
41. Ulevitch, R. J., and P. S. Tobias. 1995. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu. Rev. Immunol.* **13:** 437–457.
42. de Bont, N., M. G. Netea, C. Rovers, T. J. Smilde, P. N. M. Demacker, J. W. M. van der Meer, and A. F. H. Stalenhoef. 1998. LPS-induced cytokine production and expression of LPS-receptors by peripheral blood mononuclear cells of patients with familial hypercholesterolemia and the effect of HMG-CoA reductase inhibitors. *Atherosclerosis.* **139:** 147–152.
43. Ingalls, R. R., and D. G. Golenbock. 1995. CD11c/CD18, a transmembrane signalling receptor for lipopolysaccharide. *J. Exp. Med.* **181:** 1473–1481.
44. Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity.* **11:** 443–451.
45. Beutler, B. E. 1992. Tumor necrosis factors: the molecules and their emerging roles in medicine. Raven Press, New York, NY.
46. Pritchard, K. A. J., S. M. Schwarz, M. S. Medow, and M. B. Stemerman. 1991. Effect of LDL on EC membrane fluidity and mononuclear cell attachment. *Am. J. Physiol.* **260:** C43–C49.
47. Zhu, Y., J. H-C. Lin, H-L. Liao, O. Friedli, L. Verna, N. W. Marten, D. S. Straus, and M. B. Stemerman. 1998. LDL induces transcription factor activator protein-1 in human endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **18:** 473–480.
48. Zhu, Y., H. Liao, J. H-C. Lin, O. Friedli, L. Verna, and M. B. Stemerman. 1998. Low-density lipoprotein augments interleukin-1-induced vascular adhesion molecule expression in human endothelial cells. (abstract) *Circulation.* **17 (Suppl.):** 592.

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