The role of lipolysis in mediating the proinflammatory effects of very low density lipoproteins in mouse peritoneal macrophages

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Abstract Hypertriglyceridemia is an important risk factor for atherosclerosis, especially in obesity. Macrophages are one of the primary cell types involved in atherogenesis and are thought to contribute to lesion formation through both lipid accumulation and proinflammatory gene expression. In this study, we sought to determine the direct impact of triglyceride (TG)-rich VLDL-induced lipid accumulation on macrophage proinflammatory processes. Incubation of mouse peritoneal macrophages with 100 µg/ml VLDL for 6 h led to 2.8- and 3.7-fold increases in intracellular TGs and FFAs, respectively ($P < 0.05$). The inflammatory proteins tumor necrosis factor-α, interleukin-1β, monocyte chemoattractant protein-1, intercellular adhesion molecule-1, matrix metalloproteinase 3 (MMP3), and macrophage inflammatory protein-1α (MIP-1α) were all upregulated by at least 2-fold ($P < 0.05$) in a dose-dependent manner in VLDL-treated macrophages. The increase in inflammatory gene expression coincided with the phosphorylation of the mitogen-activated protein kinase (MAPK) pathway members extracellular signal-regulated kinase (ERK) 1/2, stress-activated protein kinase/c-Jun NH2-terminal kinase, and p38 MAPK and was ameliorated by U0126, an inhibitor of ERK1/2. Inhibition of extracellular TG hydrolysis with tetrahydrolipstatin (Orlistat) resulted in the absence of intracellular TG and FFA accumulation and was accompanied by the amelioration of ERK1/2 phosphorylation and MIP-1α gene expression. These data indicate that VLDL hydrolysis, and the subsequent accumulation of intracellular FFAs and TGs, plays a substantive role in mediating the proinflammatory effects of VLDL. These data have important implications for the direct proatherogenic effects of VLDL on macrophage-driven atherosclerosis.—Saraswathi, V., and A. H. Hasty. The role of lipolysis in mediating the proinflammatory effects of very low density lipoproteins in mouse peritoneal macrophages. *J. Lipid Res.* 2006. **47:** 1406–1415.

Supplementary key words inflammation • gene expression • mitogen-activated protein kinases

Traditionally, LDLs have been thought to be the key plasma lipoproteins involved in atherosclerotic lesion de-

velopment. However, with the dramatic increase in the prevalence of obesity around the world, associated lipoprotein abnormalities such as hypertriglyceridemia have been brought into focus. In fact, hypertriglyceridemia is now regarded as one of the main contributing factors to the development of atherosclerotic disease (1, 2), although the exact mechanisms by which triglycerides (TGs) function in atherogenesis are unknown. TGs in fasting plasma are carried in VLDLs, and these VLDLs have been found within human and experimental atherosclerotic lesions (3, 4), providing a rationale to study their direct effects on macrophage functions such as foam cell formation and inflammation. It is well established that VLDL causes the accumulation of TGs and FFAs in macrophages (5–9). This can occur through three different mechanisms: *1)* uptake of intact VLDL particles; *2)* uptake of VLDL remnants resulting from lipoprotein lipase-mediated lipolysis; and *3)* uptake of FFAs produced by VLDL lipolysis, which can be taken into cells through both passive diffusion and receptor-mediated mechanisms. Despite these known pathways of macrophage-mediated VLDL catabolism, the consequences of VLDL-induced lipid accumulation on macrophage inflammatory processes are not known.

Macrophages not only transform into foam cells via the uptake of modified lipoproteins, they also express a battery of inflammatory genes such as cytokines, chemokines, and adhesion molecules that play pivotal roles in atherosclerotic lesion formation. It has been reported that cholesterol-loaded macrophages are an abundant source of tumor necrosis factor (TNF)-α and interleukin (IL)-6 (10). In addition, it was recently reported that VLDL po-

Abbreviations: CE, cholesteryl ester; ERK, extracellular signal-regulated kinase; IL, interleukin; LRP, low density lipoprotein receptor-related protein; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; mmLDL, minimally modified low density lipoprotein; PBMC, peripheral blood mononuclear cell; SAPK/JNK, stress-activated protein kinase/c-Jun NH2-terminal kinase; TG, triglyceride; TNF, tumor necrosis factor.

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tentiates LPS-induced TNF-α expression in human macrophages (11) and that VLDL can directly induce IL-1β and monocyte chemoattractant protein-1 (MCP-1) (12, 13).

Atherosclerosis is considered to be a chronic inflammatory disease of the vessel wall (14). Several chemokines and their receptors are important in the pathogenesis of atherosclerosis. Chemokines such as MCP-1, macrophage inflammatory protein-1α (MIP-1α), and IL-8 are reported to be expressed within atherosclerotic plaques (15). MCP-1 is one of the CC family chemokines, whose role in atherosclerosis is well known (15, 16). VLDL has been shown to induce MCP-1 in macrophages (13) and mesangial cells (17). In addition to MCP-1, there are several other CC chemokines associated with advanced atherosclerotic lesions. MIP-1α, MIP-1β, and, to a lesser extent, RANTES are expressed by activated T-cells in human plaques (18). Because the role of MIP-1α is increasingly recognized in cardiovascular diseases (19, 20), we investigated the role of VLDL-induced TG and FFA accumulation in macrophage inflammatory responses, with particular emphasis on MIP-1α.

In this study, we show that VLDL induces a dose- and time-dependent increase in the expression of several inflammatory genes, including MIP-1α. The induction of inflammation is associated with the phosphorylation of mitogen-activated protein kinase (MAPK) pathway members and is blocked by the inhibition of extracellular signal-regulated kinase (ERK) 1/2 phosphorylation. Finally, inhibition of VLDL lipolysis with tetrahydrolipstatin (Orlistat) results in a significant inhibition of VLDL-induced ERK1/2 phosphorylation and subsequent MIP-1α gene expression. Thus, our data are consistent with the hypothesis that hydrolysis of VLDL induces inflammation in macrophages via the activation of the ERK1/2 pathway.

METHODS

Materials

Antibodies against phosphorylated and unphosphorylated forms of MAPKs, β-actin, and U0126 were purchased from Cell Signaling Technology (Danvers, MA). JNK inhibitor peptide was purchased from Calbiochem (La Jolla, CA), and Orlistat was purchased at the pharmacy. SB203580 and all other chemicals were from Sigma (St. Louis, MO).

Macrophage collection

Mouse primary peritoneal macrophages. Peritoneal macrophages were collected from female C57BL/6 mice 3 days after injection of thioglycollate medium. Cells were washed and plated at 1.5×10^6 cells per well of 12-well dishes in DMEM containing 10% FBS. After incubation at 37°C for 4 h, nonadherent cells were removed, and adherent cells were incubated for an additional 48 h at 37°C before treatment. VLDL was used at the indicated concentrations in DMEM containing 5% FBS.

Human peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were obtained from human blood by Ficoll gradient centrifugation. Cells were plated in RPMI medium containing 5% FBS at a density of 1.5×10^6 cells per well in 12-well culture dishes.

Lipoproteins

Plasma VLDL (d < 1.019 g/ml) for all studies was isolated from fasted human donors by ultracentrifugation as described previously (21). After centrifugation, the lipoprotein fraction was filtered and endotoxin content in the lipoprotein preparations was tested using the LAL assay (Cambrex, Walkersville, MD). All samples were found to contain <10 pg endotoxin/mg protein. The VLDL was characterized by SDS-PAGE, lipoprotein gel electrophoresis, and fast performance liquid chromatography. It contained primarily apolipoprotein B-100 and apolipoprotein E, displayed preβ mobility, and eluted in the size range of VLDL with small amounts of intermediate density lipoprotein-sized particles. A modified Lowry assay was used to determine protein concentration of the VLDL preparations, and cells were treated with 0, 50, 100, or 150 µg/ml VLDL based upon protein concentration.

Analysis of macrophage and VLDL lipid content

Oil Red O staining. Cells were plated on eight-well chamber slides and treated with VLDL for 6 and 24 h. The cells were washed and fixed with 4% paraformaldehyde, followed by staining with Oil Red O for 4 h. In separate experiments, cells were pretreated with Orlistat followed by cotreatment with VLDL for 6 h, and lipid accumulation was monitored by Oil Red O staining.

GC-MS. Macrophage monolayers were washed extensively, and cells were harvested in PBS. Lipids were extracted from the lipoprotein fractions using the method of Folch, Lees, and Sloane Stanley (22). Individual lipid classes were separated by TLC using Silica Gel 60 A plates developed in petroleum ether-ethyl ether-acetic acid (80:20:1) and visualized by rhodamine 6G. Phospholipids, TG, and cholesteryl ester (CE) were scraped from the plates and methylated using BF_3/methanol as described by Morrison and Smith (23). The methylated fatty acids were extracted and analyzed by GC using an HP 5890 gas chromatograph equipped with a Supelcowax 10 capillary column (0.25 mm × 30 m, 0.25 µm film), a flame ionization detector, and an HP 3365 Chemstation. Fatty acid methyl esters were identified by the computer comparing the retention times with those of known standards. Odd chain fatty acids were used as internal standards to quantify the amount of lipid in the sample. Unesterified cholesterol was analyzed by the method of Rudel et al. (24). Aliquots of the total lipid extract were dried under nitrogen, dissolved in hexane, and analyzed by GC using the Hewlett Packard 5890 gas chromatograph equipped with a DB-17 column (0.53 mm inner diameter × 15 m, 1 µm film; J&W Scientific). 5α-Cholestane was added to the aliquots before drying and used as an internal standard.

RT-PCR

Total RNA was isolated from cells using TRIZOL (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RT-PCR was performed in a two-step process. First, RNA was reverse-transcribed to cDNA and amplified by PCR using random hexamers with the Reverse Transcription System kit from Promega. cDNA synthesis reactions were performed simultaneously under the same conditions for all RNA samples for each experiment. From this cDNA, PCR analyses for different genes were performed according to their optimal cycling conditions based upon the Tm of the primers. β-actin PCR was performed with each new cDNA synthesis to confirm similar starting cDNA concentrations for all PCR procedures. The sequences of the primer pairs were as described previously (25–28).

Western blotting for MAPK pathway proteins

Macrophages were collected in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1%

Nonidet P-40, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 0.5 mM PMSF. A modified Lowry protein estimation was performed, and equal concentrations of protein were electrophoresed through 4–12% SDS gels (Invitrogen), transferred to nitrocellulose membranes, and immunoblotted for proteins with appropriate antibodies. Antibodies against unphosphorylated and phosphorylated forms of ERK1/2, stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK), p38 MAPK, and β-actin were used at a 1:1,000 dilution. Detection of immunoreactive proteins was performed using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ), and signal was quantified using Quantity One software from Bio-Rad.

MAPK inhibition studies

Macrophages were preincubated in 10 μM U0126, JNK inhibitor peptide, or SB203580 for 30 min, followed by incubation with VLDL (100 μg/ml) for an additional 2 h. Cells were used for isolation of either RNA for RT-PCR or protein for Western blotting.

Inhibition of VLDL hydrolysis and intracellular TG reesterification

Macrophages were preincubated with 10 μM Orlistat for 1 h, followed by incubation with VLDL (100 μg/ml) for an additional 6 h. Cells were used for isolation of either RNA for RT-PCR or protein for Western blotting.

Statistical analysis

One-way ANOVA with the Bonferroni post hoc test was used to compare mean responses among the treatment groups. Statistical probability of $P < 0.05$ was considered significant.

RESULTS

Effect of VLDL on macrophage intracellular lipid accumulation

Primary peritoneal macrophages were collected from C57BL/6 mice and treated with 100 μg/ml VLDL for 6 or 24 h. Lipid accumulation was increased dramatically after 6 h, and an even further increase was detected after 24 h of treatment, as assessed morphologically by Oil Red O staining (**Fig. 1A**).

To determine whether the intracellular TG-fatty acid composition of the macrophages reflected the original VLDL source, we used GC-MS to analyze the percentage of each fatty acid in the original VLDL as well as in VLDL-treated macrophages (Fig. 1B). The major fatty acids in TG of the VLDL were 16:0 (26%), 18:1 (37%), and 18:2 (23%). The prevalence of 14:0, 16:1, and 18:0 were all at ~3% of total fatty acids. Other polyunsaturated fatty acids were also found in small amounts. Interestingly, the TG-
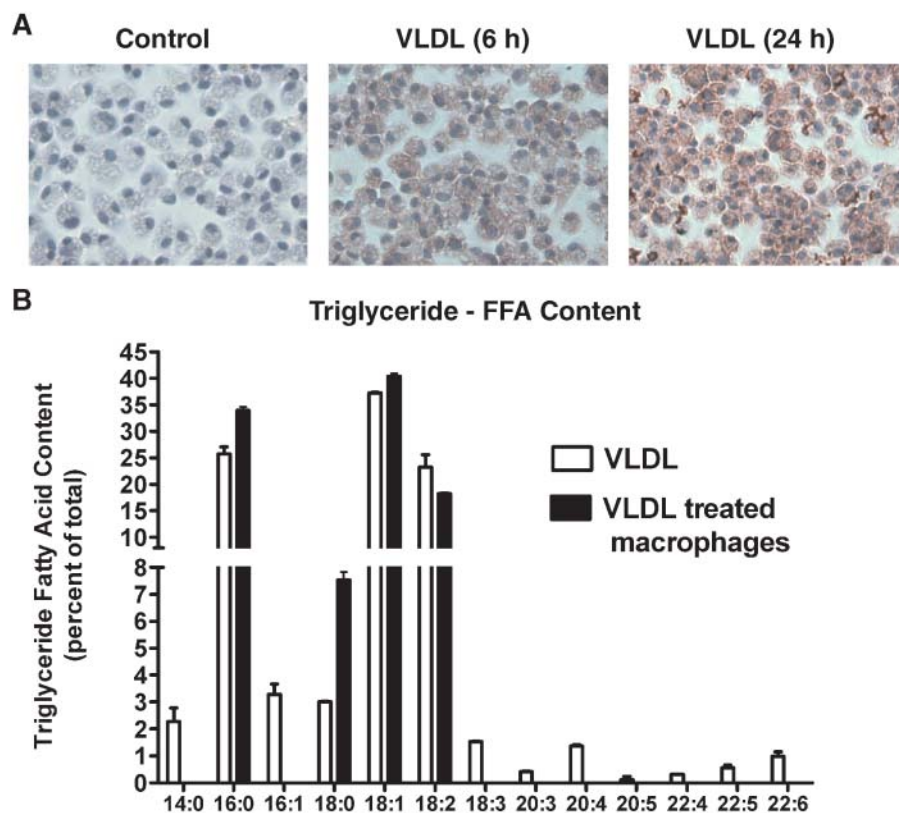

Fig. 1. Foam cell formation in VLDL-treated mouse peritoneal macrophages. Mouse peritoneal macrophages were treated with 100 μg/ml VLDL for 6 and 24 h. A: Cells were fixed, dried, and stained with Oil Red O. Magnification, 10×. B: Fatty acid composition of triglyceride (TG) from VLDL (white bars) and 6 h VLDL-treated macrophages (black bars) was analyzed from three individual samples by GC-MS as described in Methods. Data are presented as the percentage of total fatty acids (means ± SEM).

FFAs in the loaded macrophages were made up of only four types: 16:0 (34%), 18:0 (7.5%), 18:1 (40.3%), and 18:2 (18.2%). Thus, aside from a 2.3-fold increase in 18:0 (from 3% to 7%) in VLDL-treated macrophages, the concentrations of other TG-FFAs mirrored those of the original VLDL.

Induction of inflammatory gene expression in macrophages by VLDL

Macrophages treated for 6 h with physiologic concentrations of VLDL ranging from 50 to 150 μg/ml demonstrated a dose-dependent increase in the expression of inflammatory genes, such as TNF-α, IL-1β, MCP-1, intercellular adhesion molecule-1, and matrix metalloproteinase 3 (MMP-3), reaching 10.4-, 2.1-, 4.8-, 6.3-, and 2.0-fold increases in expression, respectively, in VLDL treated cells (150 μg/ml) compared with untreated cells (normalized to β-actin expression; $P < 0.05$ for all genes) (**Fig. 2**). We also observed a dose-dependent increase in MIP-1α in macrophages upon treatment with 0–150 μg/ml VLDL (**Fig. 3A, B**). Treatment of macrophages with 100 μg/ml VLDL resulted in a time-dependent increase in MIP-1α expression, which was seen as early as 2 h and was maintained for up to 24 h (Fig. 3C, D).

Treatment of undifferentiated human PBMCs with 100 μg/ml VLDL for 6 h resulted in a 1.9- ± 0.4-fold increase in TNF-α expression ($P < 0.05$); however, the expression of IL-1β and MIP-1α was not changed.

The role of VLDL-induced phosphorylation of MAPKs in MIP-1α production

Because MAPK proteins are associated with the induction of inflammatory gene expression (29), activation of MAPKs was considered one mechanism whereby VLDL could mediate its effects. All three members of the MAPK family, ERK1/2, SAPK/JNK, and p38 MAPK, were activated upon treatment with VLDL, as shown by increases in their phosphorylation at 50, 100, and 150 μg/ml VLDL

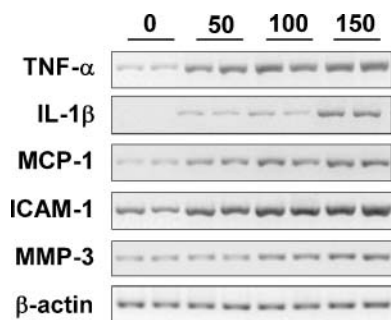

Fig. 2. Dose-dependent effects of VLDL on inflammatory genes. Mouse primary peritoneal macrophages were incubated with 0, 50, 100, or 150 μg/ml VLDL for 6 h. RNA was isolated, and the expression of the inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), and matrix metalloproteinase 3 (MMP-3) was measured by RT-PCR. β-Actin was used as a control to confirm equal RNA concentrations. Representative amplifications of duplicate samples for each gene are shown.
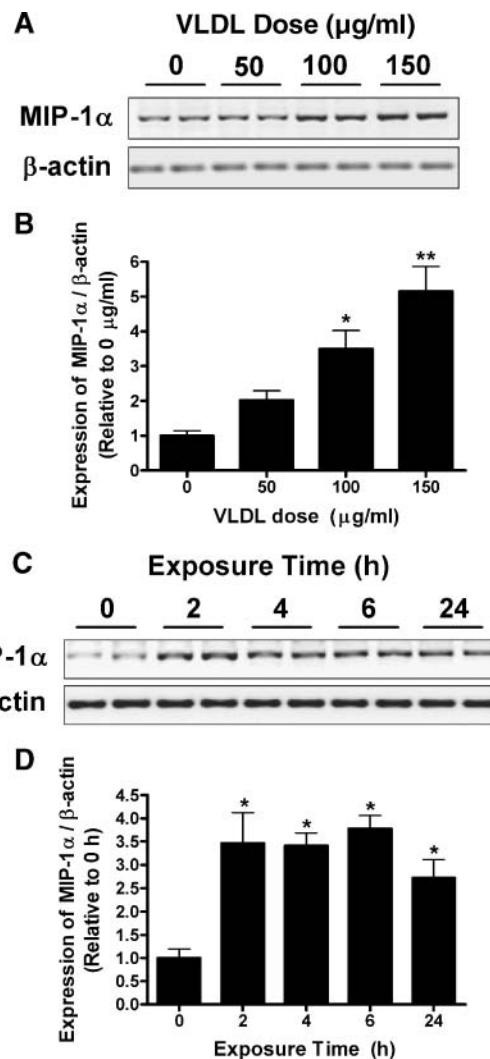

Fig. 3. Dose- and time-dependent effects of VLDL on macrophage inflammatory protein (MIP)-1α expression in mouse peritoneal macrophages. A, B: Mouse primary peritoneal macrophages were incubated with 0, 50, 100, or 150 μg/ml VLDL for 6 h. RNA was isolated, and MIP-1α gene expression was measured by RT-PCR. β-Actin was used as a control to confirm equal RNA concentrations. A: Representative amplification of duplicate samples. B: Band intensities from six samples were quantified using Quantity One software and plotted as means ± SEM. C, D: Mouse primary peritoneal macrophages were incubated with 100 μg/ml VLDL for 0, 2, 4, 6, or 24 h. C: RNA was isolated from cells and analyzed for MIP-1α expression by RT-PCR. D: Band intensities from six samples were quantified using Quantity One software and plotted as means ± SEM. * $P < 0.01$ compared with 0 μg/ml VLDL; ** $P < 0.001$ compared with 0 and 50 μg/ml VLDL.

(2.2-, 2.8-, and 2.8-fold at 150 μg/ml dose; $P < 0.05$ for all) after a 6 h treatment (**Fig. 4**).

To explore the requirement of MAPK phosphorylation for VLDL-induced MIP-1α expression, macrophages were pretreated for 1 h with MAPK inhibitors followed by treatment with VLDL (100 μg/ml) for 2 h (**Fig. 5**). U0126, an inhibitor of MEK1/2, the kinase immediately upstream of ERK1/2, suppressed the VLDL-stimulated phosphorylation of ERK1/2 (Fig. 5A, B), resulting in a reduction of VLDL-induced MIP-1α expression (Fig. 5C, D). However,
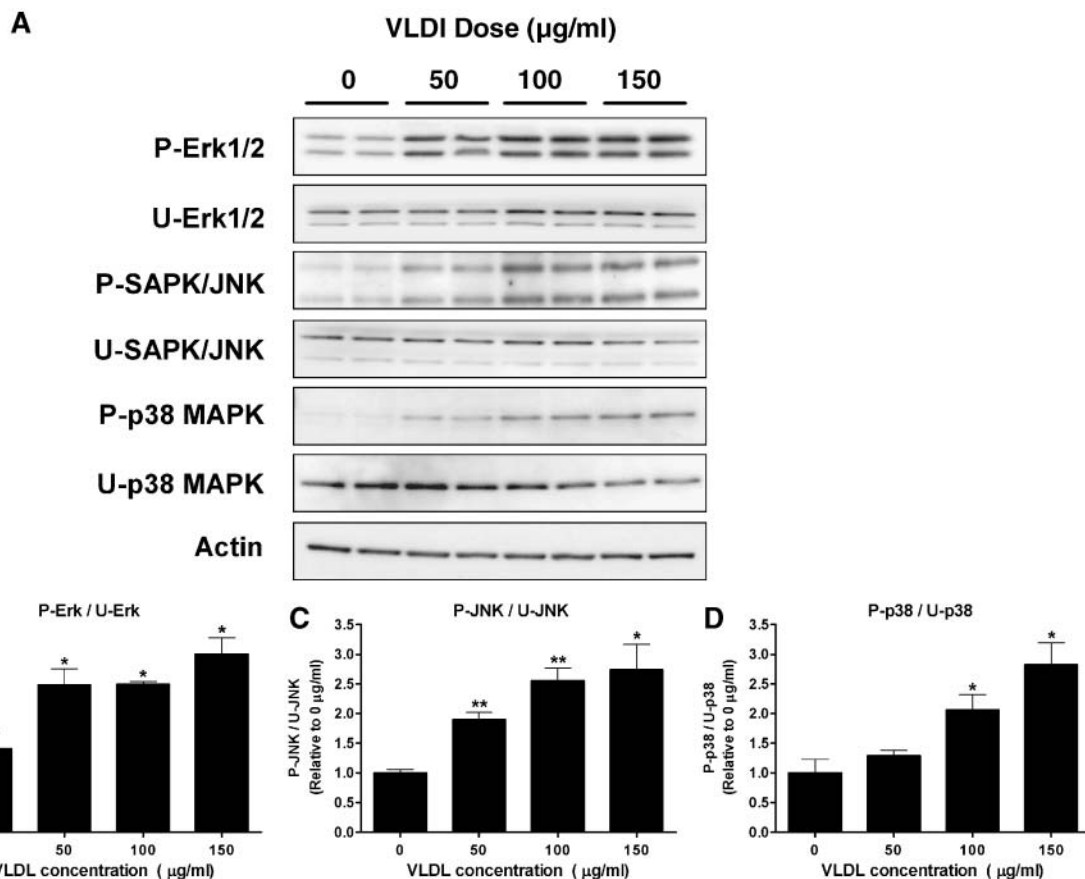
Fig. 4. Analysis of VLDL-induced phosphorylation of mitogen-activated protein kinases (MAPKs). Mouse primary peritoneal macrophages were treated for 6 h with increasing concentrations of VLDL (0–150 µg/ml), and cellular lysates were collected. Western blotting was performed for the phosphorylated (P) and unphosphorylated (U) forms of extracellular signal-regulated kinase (ERK) 1/2, stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK), and p38 MAPK. A: Representative duplicates of samples are as indicated. B–D: Band intensities of phosphorylated and unphosphorylated forms from six individual samples were quantified and plotted as fold increase of the ratio P/U over untreated samples (means ± SEM). * $P < 0.05$, ** $P < 0.005$ compared with 0 µg/ml VLDL.

treatment of macrophages with VLDL in the presence of JNK inhibitor peptide or SB203580, specific inhibitors of SAPK/JNK and p38 MAPK, respectively, did not block VLDL-induced MIP-1α expression (data not shown).

Effects of inhibition of lipolysis on macrophage lipid accumulation and inflammation

Orlistat is an inhibitor of extracellular lipases and has been shown to inhibit the accumulation of TG in macrophages after incubation with VLDL (30). We sought to analyze the effect of lipase inhibition on TG and FFA accumulation and to determine the downstream consequences relating to inflammation. Primary mouse peritoneal macrophages were pretreated with 10 µM Orlistat for 1 h, followed by coincubation with VLDL for an additional 6 h (**Fig. 6**). Oil Red O staining of macrophages revealed an absence of lipid accumulation in Orlistat-treated cells (Fig. 6A). Quantitatively, there was a 2.8-fold increase in intracellular TG, from 15.6 to 44.2 µg/mg protein, in VLDL-treated cells ($P < 0.05$), which was reduced to 7.6 µg/mg protein in VLDL + Orlistat-treated cells. FFA accumulation in VLDL-treated cells was increased by 3.7-fold compared with untreated cells (16.3 and 4.4 µg/

mg protein, respectively; $P < 0.005$) and was also blocked by Orlistat treatment (4.2 µg/mg protein). CEs were below the level of detection in control cells, and the addition of Orlistat did not alter levels in VLDL-treated cells. Phospholipid and unesterified cholesterol levels were not influenced by VLDL or by Orlistat treatment (Fig. 6B).

To determine whether VLDL hydrolysis is required for macrophage activation, we analyzed ERK1/2 phosphorylation and MIP-1α expression in Orlistat-treated cells. VLDL-induced ERK1/2 phosphorylation and MIP-1α expression were both reduced significantly, but not normalized, by pretreatment with Orlistat (**Fig. 7**).

DISCUSSION

We have shown that TG-rich VLDL directly promotes the accumulation of TGs and FFAs in macrophages, with a concomitant increase in MAPK activation and inflammatory gene expression. Inhibition of lipoprotein lipase results in a complete absence of lipid accumulation within macrophages, resulting in the attenuation of the inflammatory effects of VLDL. Our data demonstrate that incu-
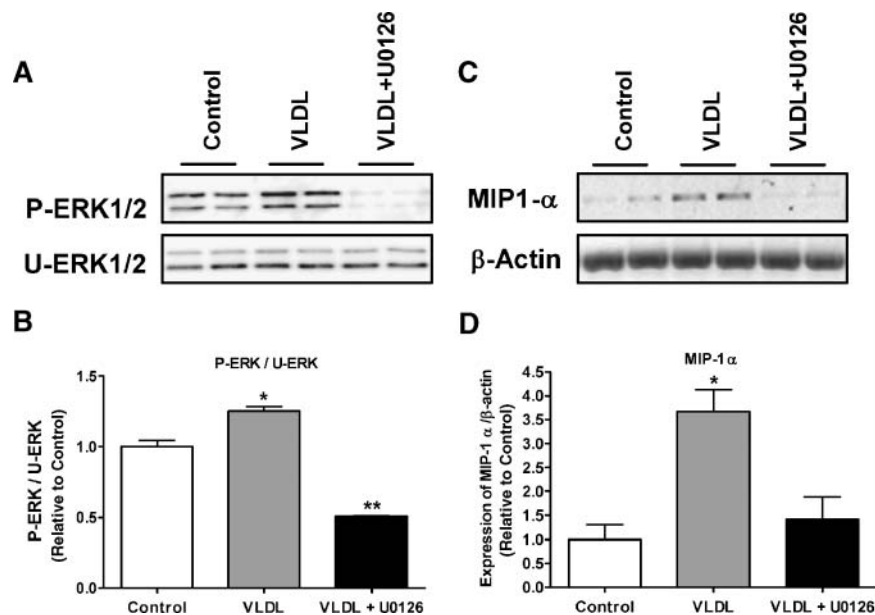
Fig. 5. Impact of ERK1/2 inhibition on MIP-1α production in VLDL-stimulated macrophages. Mouse primary peritoneal macrophages were pretreated with U0126 (10 μM), an inhibitor of ERK1/2 phosphorylation, for 1 h and then stimulated with VLDL (100 μg/ml) for 2 h. A: Lysates from cells treated with U0126 were immunoblotted with antibodies against phosphorylated (P) and unphosphorylated (U) forms of ERK1/2. B: Quantification of phosphorylated and unphosphorylated forms of the proteins was performed using Quantity One software. Data are expressed as means ± SEM of the phosphorylated/ unphosphorylated bands relative to vehicle-treated cells. C, D: RNA was isolated from cells and analyzed for MIP-1α mRNA expression level by RT-PCR. Data are expressed as means ± SEM of MIP-1α/β-actin compared with vehicle-treated cells from six separate samples. * $P < 0.01$ compared with controls and cells treated with VLDL + U0126; ** $P < 0.001$ compared with cells treated with VLDL.

bation of macrophages with VLDL promotes intracellular FFA and TG accumulation and leads to the activation of MAPK pathways, resulting in inflammatory gene expression. Furthermore, we provide evidence that inhibition of VLDL lipolysis attenuates this process.

Macrophage foam cells are a prominent cellular component of the atherosclerotic plaque in all stages of its development (31). Our findings show that VLDL induces pronounced increases in the TG and FFA contents of macrophages (Figs. 1, 6). These findings are in agreement with previous reports suggesting that incubation of macrophages with TG-rich chylomicron remnants and VLDL promotes TG and FFA accumulation to a greater extent (32) than CEs, which are known to accumulate upon acetylated and oxidized LDL treatment (9, 32).

There are at least three mechanisms that can contribute to the accumulation of TGs and FFAs in VLDL-treated macrophages. First, holo-VLDL particle uptake can occur via receptor-mediated mechanisms. Although modified lipoprotein uptake occurs via scavenger receptors, the most likely receptor for VLDL uptake is the low density lipoprotein receptor-related protein (LRP). Second, lipolysis of VLDL can lead to the uptake of VLDL remnants by receptor-mediated mechanisms (33). Third, FFA generated during the lipolysis of VLDL can also be brought into the cells via receptors or by passive diffusion (34). Macrophages are known to secrete active lipoprotein lipase (35), and lipoprotein lipase activity has been shown

to be upregulated by exposure to VLDL (8, 30). Our data showing the absence of TG and FFA accumulation in macrophages treated with the lipoprotein lipase inhibitor Orlistat are in agreement with other reports (30, 33, 36) and implicate VLDL remnant and/or FFA uptake as the primary mechanisms by which VLDL induces macrophage foam cell formation.

Macrophages not only convert to foam cells within atherosclerotic lesions, they also contribute to lesion formation by secreting inflammatory cytokines and chemokines that direct and amplify local immune response in the lesions. Yet, few studies have focused on the potential of VLDL to stimulate inflammation in macrophages. One study demonstrated that VLDL in hypertriglyceridemic patients augments TNF-α production in whole blood samples ex vivo (37). Stollenwerk et al. (11, 12) have reported that VLDL potentiates LPS-induced TNF-α in THP-1 cells. They have also shown that VLDL directly induces IL-1β expression in human monocyte-derived macrophages. In addition, Wang et al. (13) have shown that VLDL induces macrophage MCP-1 expression. Our current data suggest that VLDL independently induces a strong and direct proinflammatory response in macrophages by stimulating the expression of MIP-1α and other inflammatory cytokines. In addition, our study showing the upregulation of only TNF-α in undifferentiated PBMCs provides evidence that inflammatory responses in undifferentiated monocytes and differentiated macrophages are different.
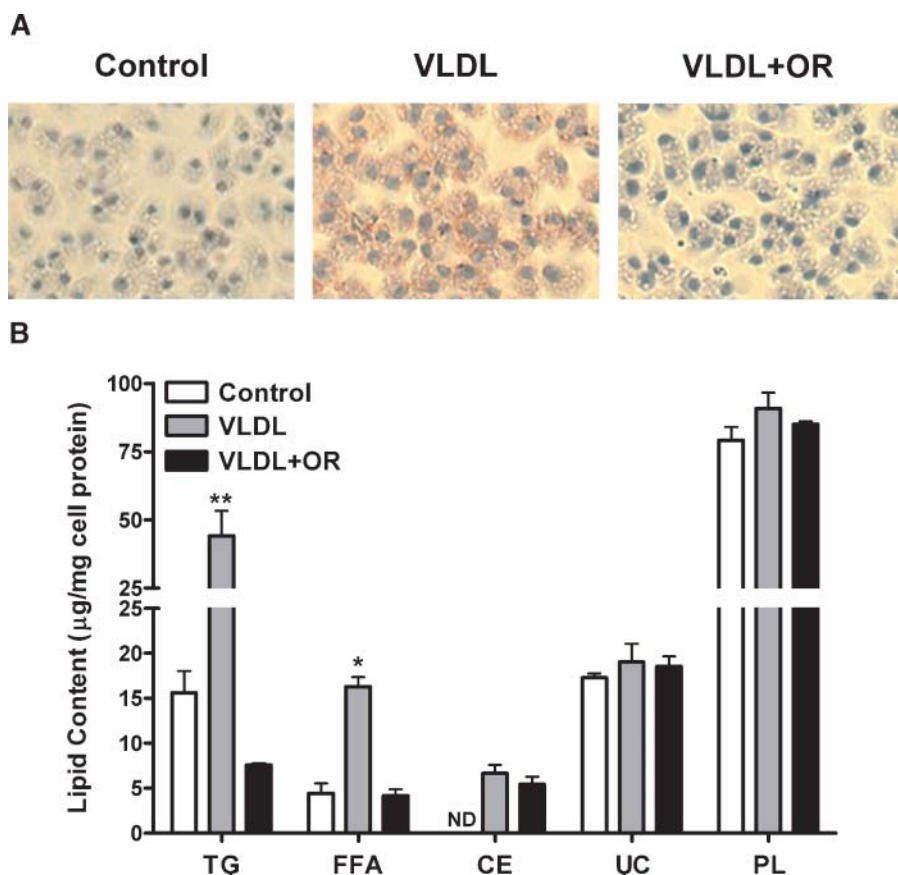
Fig. 6. Impact of extracellular lipolysis on macrophage lipid accumulation. Primary peritoneal macrophages were pretreated for 1 h with Orlistat (OR), an inhibitor of lipoprotein lipase, before incubation with 100 μg/ml VLDL for an additional 6 h. A: Cells were stained with Oil Red O to detect neutral lipids. B: Cell lysates were analyzed for lipid content by GC-MS as described in Methods. Data are presented as means ± SEM of three individual samples. ND, not detected. * $P < 0.001$, ** $P < 0.01$ compared with control and VLDL + OR.

Further studies are required to determine the bases of these differences.

MIP-1α is a CC chemokine that is increasingly recognized as an inflammatory mediator that has a critical role in the development of atherosclerotic lesions (15, 38). Increased plasma levels of MIP-1α have been reported in hypertriglyceridemic mice (39). In addition, an increase in MIP-1α mRNA has been reported in aortas of apolipoprotein E-deficient mice fed a high-cholesterol diet, providing further evidence for the involvement of MIP-1α in atherosclerosis (40). T-cells have been reported to be the major source of MIP-1α in arterial lesions (18); however, in the present study, we show increased expression of MIP-1α in TG-loaded macrophages, suggesting that the foam cells may also act as a source of this chemokine within the vessel wall.

We next examined the signaling pathways that led to the induction of MIP-1α in VLDL-stimulated macrophages. It has been reported that MAPKs play a key role in the induction of cytokine production in macrophages and also in the proliferative effect of oxidized LDL on macrophages (41–44). Our data suggest that ERK1/2 but not SAPK/JNK or p38 MAPK is involved in the induction of the inflammatory response by VLDL in macrophages.

This is in accordance with another report showing that ERK1/2 activation is involved in upregulating inflammatory pathways in minimally oxidized LDL-loaded macrophages (45). The mechanism by which the incubation of macrophages with VLDL leads to MAPK activation remains uncertain. It is possible that the uptake of FFAs released as a result of the LPL-mediated hydrolysis of VLDL initiates downstream signaling events leading to the phosphorylation of MAPK family members. This is supported by our data that inhibition of VLDL lipolysis, resulting in the absence of FFA and TG accumulation, blunts ERK1/2 activation. An alternative mechanism relates to receptor-mediated endocytosis of VLDL remnants. One of the receptors involved in VLDL uptake in macrophages is LRP. Not only does LRP mediate lipoprotein endocytosis, it has also been shown to modulate the MAPK signaling cascade in other cells, such as osteoblasts and neurons (46–48). In our study, inhibition of VLDL lipolysis by Orlistat resulted in the absence of FFA and TG accumulation and blunted ERK1/2 activation and MIP-1α expression. These data provide evidence that the intracellular accumulation of FFAs and TGs activates these events. However, the role of receptor-mediated signal transduction cannot be ruled out, because Orlistat
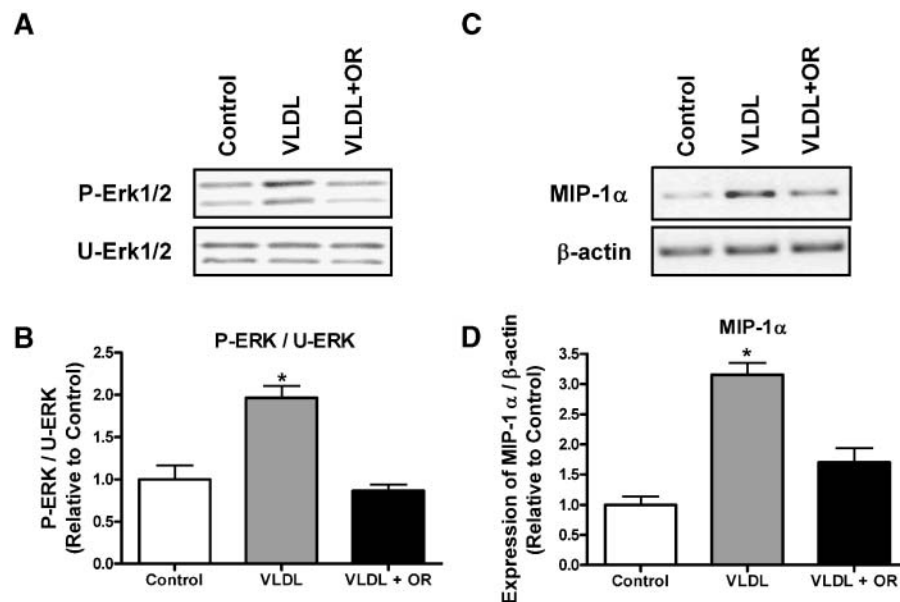
Fig. 7. Effect of OR treatment on ERK1/2 phosphorylation and MIP-1α expression in VLDL-treated cells. Primary peritoneal macrophages were pretreated for 1 h with 10 μM Orlistat (OR) before incubation with 100 μg/ml VLDL for an additional 6 h. A: Protein was prepared from cell lysates, electrophoresed through 4–12% gradient gels, and blotted for phosphorylated (P) and unphosphorylated (U) forms of ERK1/2. Representative samples from two experiments performed in duplicate are shown. B: Quantification of phosphorylated and unphosphorylated forms of the proteins was performed using Quantity One software. Data are expressed as means ± SEM of the phosphorylated/unphosphorylated bands relative to control cells from four separate samples. C: RNA was isolated and used for RT-PCR analysis of MIP-1α and β-actin. Representative samples from two experiments performed in triplicate are shown. D: Band intensities from six samples were quantified using Quantity One software and plotted as means ± SEM. * $P < 0.01$ compared with control and VLDL + OR.

inhibition of VLDL hydrolysis resulted in only a partial inhibition of MIP-1α. Further investigation is required to understand the involvement of receptor-mediated signaling in these events. However, we can conclude that hydrolysis of VLDL particles plays a crucial role in inducing macrophage inflammatory pathways. Our data on the role of VLDL hydrolysis in macrophage foam cell formation and inflammation support other studies showing that macrophage lipoprotein lipase activity potentiates atherosclerotic lesion formation in mice (49, 50).

The results we obtained regarding MAPK activation and inflammatory gene expression in VLDL-treated macrophages are similar to what has been reported regarding minimally modified low density lipoprotein (mmLDL) activation of macrophages. As mentioned above, Miller et al. (45) have reported that mmLDL induces an inflammatory response in macrophages via the activation of ERK1/2. In contrast to our results demonstrating that MIP-1α expression is increased for as long as 24 h of VLDL treatment, mmLDL treatment induced MIP-2 for <3 h. These authors also report that Toll-like receptor 4 was required for MIP-2 expression, whereas cytokines such as MCP-1, TNF-α, and IL-6 were induced by mmLDL in a Toll-like receptor 4-independent manner. Further studies are necessary to determine whether similar mechanisms are involved in mediating the proinflammatory response stimulated by VLDL.

In summary, this study provides strong evidence that VLDL potently induces TG and FFA accumulation as well as inflammatory cytokine and chemokine production in macrophages. The stimulatory effect of VLDL on macrophage inflammatory responses is mediated by the activation of the ERK1/2 pathway and, in part, requires the lipolysis of VLDL particles. Thus, our studies have important implications regarding mechanisms by which TG-rich VLDL is a direct proatherogenic stimulus in human atherosclerotic disease.

REFERENCES

1. Moller, D. E., and K. D. Kaufman. 2005. Metabolic syndrome: a clinical and molecular perspective. *Annu. Rev. Med.* **56:** 45–62.

2. Fruchart, J. C., M. C. Nierman, E. S. Stroes, J. J. Kastelein, and P. Duriez. 2004. New risk factors for atherosclerosis and patient risk assessment. *Circulation.* **109:** III15–III19.

3. Proctor, S. D., and J. C. Mamo. 1998. Retention of fluorescent-labelled chylomicron remnants within the intima of the arterial wall—evidence that plaque cholesterol may be derived from postprandial lipoproteins. *Eur. J. Clin. Invest.* **28:** 497–503.

4. Rapp, J. H., A. Lespine, R. L. Hamilton, N. Colyvas, A. H. Chaumeton, J. Tweedie-Hardman, L. Kotite, S. T. Kunitake, R. J. Havel, and J. P. Kane. 1994. Triglyceride-rich lipoproteins isolated by selected-affinity anti-apolipoprotein B immunosorption from human atherosclerotic plaque. *Arterioscler. Thromb.* **14:** 1767–1774.

5. Bates, S. R., P. L. Murphy, Z. C. Feng, T. Kanazawa, and G. S. Getz. 1984. Very low density lipoproteins promote triglyceride accumulation in macrophages. *Arteriosclerosis.* **4:** 103–114.

6. Huff, M. W., A. J. Evans, C. G. Sawyez, B. M. Wolfe, and P. J. Nestel. 1991. Cholesterol accumulation in J774 macrophages induced by triglyceride-rich lipoproteins. Comparison of very low density lipoprotein from subjects with type III, IV, and V hyperlipoproteinemias. *Arterioscler. Thromb.* **11:** 221–233.

7. Gianturco, S. H., S. A. Brown, D. P. Via, and W. A. Bradley. 1986. The beta-VLDL receptor pathway of murine P388D1 macrophages. *J. Lipid Res.* **27:** 412–420.

8. Jong, M. C., W. L. Hendriks, L. C. van Vark, V. E. Dahlmans, J. E. Groener, and L. M. Havekes. 2000. Oxidized VLDL induces less triglyceride accumulation in J774 macrophages than native VLDL due to an impaired extracellular lipolysis. *Arterioscler. Thromb. Vasc. Biol.* **20:** 144–151.

9. Batt, K. V., M. Avella, E. H. Moore, B. Jackson, K. E. Suckling, and K. M. Botham. 2004. Differential effects of low-density lipoprotein and chylomicron remnants on lipid accumulation in human macrophages. *Exp. Biol. Med. (Maywood).* **229:** 528–537.

10. Li, Y., R. F. Schwabe, T. Devries-Seimon, P. M. Yao, M. C. Gerbod-Giannone, A. R. Tall, R. J. Davis, R. Flavell, D. A. Brenner, and I. Tabas. 2005. Free cholesterol-loaded macrophages are an abundant source of TNF-alpha and interleukin-6. Model of NF-kappaB- and MAP kinase-dependent inflammation in advanced atherosclerosis. *J. Biol. Chem.* **280:** 21763–21772.

11. Stollenwerk, M. M., A. Schiopu, G. N. Fredrikson, W. Dichtl, J. Nilsson, and M. P. Ares. 2005. Very low density lipoprotein potentiates tumor necrosis factor-alpha expression in macrophages. *Atherosclerosis.* **179:** 247–254.

12. Stollenwerk, M. M., M. W. Lindholm, M. I. Porn-Ares, A. Larsson, J. Nilsson, and M. P. Ares. 2005. Very low-density lipoprotein induces interleukin-1beta expression in macrophages. *Biochem. Biophys. Res. Commun.* **335:** 603–608.

13. Wang, G. P., Z. D. Deng, J. Ni, and Z. L. Qu. 1997. Oxidized low density lipoprotein and very low density lipoprotein enhance expression of monocyte chemoattractant protein-1 in rabbit peritoneal exudate macrophages. *Atherosclerosis.* **133:** 31–36.

14. Ross, R. 1999. Atherosclerosis—an inflammatory disease. *N. Engl. J. Med.* **340:** 115–126.

15. Sheikine, Y., and G. K. Hansson. 2004. Chemokines and atherosclerosis. *Ann. Med.* **36:** 98–118.

16. Nelken, N. A., S. R. Coughlin, D. Gordon, and J. N. Wilcox. 1991. Monocyte chemoattractant protein-1 in human atheromatous plaques. *J. Clin. Invest.* **88:** 1121–1127.

17. Lynn, E. G., Y. L. Siow, and K. O. 2000. Very low-density lipoprotein stimulates the expression of monocyte chemoattractant protein-1 in mesangial cells. *Kidney Int.* **57:** 1472–1483.

18. Wilcox, J. N., N. A. Nelken, S. R. Coughlin, D. Gordon, and T. J. Schall. 1994. Local expression of inflammatory cytokines in human atherosclerotic plaques. *J. Atheroscler. Thromb.* **1 (Suppl. 1):** 10–13.

19. Frangogiannis, N. G., and M. L. Entman. 2004. Targeting the chemokines in myocardial inflammation. *Circulation.* **110:** 1341–1342.

20. Dol, F., G. Martin, B. Staels, A. M. Mares, C. Cazaubon, D. Nisato, J. P. Bidouard, P. Janiak, P. Schaeffer, and J. M. Herbert. 2001. Angiotensin AT1 receptor antagonist irbesartan decreases lesion size, chemokine expression, and macrophage accumulation in apolipoprotein E-deficient mice. *J. Cardiovasc. Pharmacol.* **38:** 395–405.

21. Farkas, M. H., L. L. Swift, A. H. Hasty, M. F. Linton, and S. Fazio. 2003. The recycling of apolipoprotein E in primary cultures of mouse hepatocytes. Evidence for a physiologic connection to high density lipoprotein metabolism. *J. Biol. Chem.* **278:** 9412–9417.

22. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226:** 497–509.

23. Morrison, W. R., and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. *J. Lipid Res.* **53:** 600–608.

24. Rudel, L. L., K. Kelley, J. K. Sawyer, R. Shah, and M. D. Wilson. 1998. Dietary monounsaturated fatty acids promote aortic atherosclerosis in LDL receptor-null, human apoB100-overexpressing transgenic mice. *Arterioscler. Thromb. Vasc. Biol.* **18:** 1818–1827.

25. Choi, C. Y., J. Y. Kim, Y. S. Kim, Y. C. Chung, K. S. Hahm, and H. G. Jeong. 2001. Augmentation of macrophage functions by an aqueous extract isolated from *Platycodon grandiflorum. Cancer Lett.* **166:** 17–25.

26. Yang, J., P. J. Lindsberg, V. Hukkanen, R. Seljelid, C. G. Gahmberg, and S. Meri. 2002. Differential expression of cytokines (IL-2, IFN-gamma, IL-10) and adhesion molecules (VCAM-1, LFA-1, CD44) between spleen and lymph nodes associates with remission in chronic relapsing experimental autoimmune encephalomyelitis. *Scand. J. Immunol.* **56:** 286–293.

27. Getchell, T. V., N. K. Subhedar, D. S. Shah, G. Hackley, J. V. Partin, G. Sen, and M. L. Getchell. 2002. Chemokine regulation of macrophage recruitment into the olfactory epithelium following target ablation: involvement of macrophage inflammatory protein-1alpha and monocyte chemoattractant protein-1. *J. Neurosci. Res.* **70:** 784–793.

28. Haro, H., H. C. Crawford, B. Fingleton, J. R. MacDougall, K. Shinomiya, D. M. Spengler, and L. M. Matrisian. 2000. Matrix metalloproteinase-3-dependent generation of a macrophage chemoattractant in a model of herniated disc resorption. *J. Clin. Invest.* **105:** 133–141.

29. Su, B., and M. Karin. 1996. Mitogen-activated protein kinase cascades and regulation of gene expression. *Curr. Opin. Immunol.* **8:** 402–411.

30. Milosavljevic, D., A. Kontush, S. Griglio, G. Le Naour, J. Thillet, and M. J. Chapman. 2003. VLDL-induced triglyceride accumulation in human macrophages is mediated by modulation of LPL lipolytic activity in the absence of change in LPL mass. *Biochim. Biophys. Acta.* **1631:** 51–60.

31. Brown, M. S., and J. L. Goldstein. 1983. Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Annu. Rev. Biochem.* **52:** 223–261.

32. Gianturco, S. H., W. A. Bradley, A. M. Gotto, Jr., J. D. Morrisett, and D. L. Peavy. 1982. Hypertriglyceridemic very low density lipoproteins induce triglyceride synthesis and accumulation in mouse peritoneal macrophages. *J. Clin. Invest.* **70:** 168–178.

33. Palmer, A. M., E. Nova, E. Anil, K. Jackson, P. Bateman, E. Wolstencroft, C. M. Williams, and P. Yaqoob. 2005. Differential uptake of subfractions of triglyceride-rich lipoproteins by THP-1 macrophages. *Atherosclerosis.* **180:** 233–244.

34. Kamp, F., W. Guo, R. Souto, P. F. Pilch, B. E. Corkey, and J. A. Hamilton. 2003. Rapid flip-flop of oleic acid across the plasma membrane of adipocytes. *J. Biol. Chem.* **278:** 7988–7995.

35. Braun, J. E., and D. L. Severson. 1992. Regulation of the synthesis, processing and translocation of lipoprotein lipase. *Biochem. J.* **287:** 337–347.

36. Ishibashi, S., N. Yamada, H. Shimano, N. Mori, H. Mokuno, T. Gotohda, M. Kawakami, T. Murase, and F. Takaku. 1990. Apolipoprotein E and lipoprotein lipase secreted from human monocyte-derived macrophages modulate very low density lipoprotein uptake. *J. Biol. Chem.* **265:** 3040–3047.

37. Mohrschladt, M. F., A. W. Weverling-Rijnsburger, F. H. de Man, D. J. Stoeken, A. Sturk, A. H. Smelt, and R. G. Westendorp. 2000. Hyperlipoproteinemia affects cytokine production in whole blood samples ex vivo. The influence of lipid-lowering therapy. *Atherosclerosis.* **148:** 413–419.

38. Moos, M. P., N. John, R. Grabner, S. Nossmann, B. Gunther, R. Vollandt, C. D. Funk, B. Kaiser, and A. J. Habenicht. 2005. The lamina adventitia is the major site of immune cell accumulation in standard chow-fed apolipoprotein E-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **25:** 2386–2391.

39. Zhao, L., M. P. Moos, R. Grabner, F. Pedrono, J. Fan, B. Kaiser, N. John, S. Schmidt, R. Spanbroek, K. Lotzer, et al. 2004. The 5-lipoxygenase pathway promotes pathogenesis of hyperlipidemia-dependent aortic aneurysm. *Nat. Med.* **10:** 966–973.

40. Veillard, N. R., S. Steffens, F. Burger, G. Pelli, and F. Mach. 2004. Differential expression patterns of proinflammatory and antiinflammatory mediators during atherogenesis in mice. *Arterioscler. Thromb. Vasc. Biol.* **24:** 2339–2344.

41. Means, T. K., R. P. Pavlovich, D. Roca, M. W. Vermeulen, and M. J. Fenton. 2000. Activation of TNF-alpha transcription utilizes distinct

MAP kinase pathways in different macrophage populations. *J. Leukoc. Biol.* **67:** 885–893.

42. Valledor, A. F., M. Comalada, J. Xaus, and A. Celada. 2000. The differential time-course of extracellular-regulated kinase activity correlates with the macrophage response toward proliferation or activation. *J. Biol. Chem.* **275:** 7403–7409.

43. Deigner, H. P., and R. Claus. 1996. Stimulation of mitogen activated protein kinase by LDL and oxLDL in human U-937 macrophage-like cells. *FEBS Lett.* **385:** 149–153.

44. Senokuchi, T., T. Matsumura, M. Sakai, T. Matsuo, M. Yano, S. Kiritoshi, K. Sonoda, D. Kukidome, T. Nishikawa, and E. Araki. 2004. Extracellular signal-regulated kinase and p38 mitogen-activated protein kinase mediate macrophage proliferation induced by oxidized low-density lipoprotein. *Atherosclerosis.* **176:** 233–245.

45. Miller, Y. I., S. Viriyakosol, D. S. Worrall, A. Boullier, S. Butler, and J. L. Witztum. 2005. Toll-like receptor 4-dependent and -independent cytokine secretion induced by minimally oxidized low-density lipoprotein in macrophages. *Arterioscler. Thromb. Vasc. Biol.* **25:** 1213–1219.

46. Lutz, C., J. Nimpf, M. Jenny, K. Boecklinger, C. Enzinger, G. Utermann, G. Baier-Bitterlich, and G. Baier. 2002. Evidence of functional modulation of the MEKK/JNK/cJun signaling cascade by the low density lipoprotein receptor-related protein (LRP). *J. Biol. Chem.* **277:** 43143–43151.

47. Grey, A., T. Banovic, Q. Zhu, M. Watson, K. Callon, K. Palmano, J. Ross, D. Naot, I. R. Reid, and J. Cornish. 2004. The low-density lipoprotein receptor-related protein 1 is a mitogenic receptor for lactoferrin in osteoblastic cells. *Mol. Endocrinol.* **18:** 2268–2278.

48. Qiu, Z., B. T. Hyman, and G. W. Rebeck. 2004. Apolipoprotein E receptors mediate neurite outgrowth through activation of p44/42 mitogen-activated protein kinase in primary neurons. *J. Biol. Chem.* **279:** 34948–34956.

49. Babaev, V. R., M. B. Patel, C. F. Semenkovich, S. Fazio, and M. F. Linton. 2000. Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis in low density lipoprotein receptor-deficient mice. *J. Biol. Chem.* **275:** 26293–26299.

50. Babaev, V. R., S. Fazio, L. A. Gleaves, K. J. Carter, C. F. Semenkovich, and M. F. Linton. 1999. Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis in vivo. *J. Clin. Invest.* **103:** 1697–1705.