Oxidatively Modified Low Density Lipoprotein (LDL) Inhibits TLR2 and TLR4 Cytokine Responses in Human Monocytes but Not in Macrophages

Received for publication, November 4, 2011, and in revised form, April 27, 2012 Published, JBC Papers in Press, May 21, 2012, DOI 10.1074/jbc.M111.320960

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Background: The role of oxidatively modified LDL (oxmLDL) in atherosclerotic inflammation is controversial.

Results: Endotoxin-free oxmDL fails to enhance mononuclear phagocyte inflammatory responses but suppresses IL-1β, TNF, and IL-6 when combined with endotoxin or PamCSK4.

Conclusion: OxmLDL suppresses proinflammatory responses.

Significance: Although LDL preparations have been linked to inflammatory responses, endotoxin-free, oxidatively modified LDL inhibits monocyte Toll ligands.

Inflammation characterized by the expression and release of cytokines and chemokines is implicated in the development and progression of atherosclerosis. Oxidatively modified low density lipoproteins, central to the formation of atherosclerotic plaques, have been reported to signal through Toll-like receptors (TLRs), TLR4 and TLR2, in concert with scavenger receptors to regulate the inflammatory microenvironment in atherosclerosis. This study evaluates the role of low density lipoproteins (LDL) and oxidatively modified LDL (oxmLDL) in the expression and release of proinflammatory mediators IL-8, IL-6, IL-1β, TNFα, and IL-8 in human monocytes and macrophages. Although standard LDL preparations induced IL-8 and IL-6 production, this inflammatory effect was eliminated when LDL was isolated under endotoxin-restricted conditions. However, when added with TLR4 and TLR2 ligands, this low endotoxin preparation of oxmLDL suppressed the expression and release of IL-1β, IL-6, and TNFα but surprisingly spared IL-8 production. The suppressive effect of oxmLDL was specific to monocytes as it did not inhibit LPS-induced proinflammatory cytokines in human macrophages. Thus, TLR ligand contamination of LDL/oxmLDL preparations can complicate interpretations of inflammatory responses to these modified lipoproteins. In contrast to providing a proinflammatory function, oxmLDL suppresses the expression and release of selected proinflammatory mediators.

Coronary artery disease, characterized by the development of atherosclerotic plaques, leads to acute coronary syndromes such as unstable angina, myocardial infarction, and death due to arterial occlusion (1, 2). Increased circulating concentrations of cholesterol, transported in the blood by low density lipoproteins (LDL), are a major risk factor for coronary artery disease. The high levels of circulating cholesterol-rich LDL particles are trapped in the arterial intima and are modified by peroxidases such as myeloperoxidases and lipoxygenases, reactive oxygen species, phenoxyl radicals, and peroxynitrites to generate oxidatively modified LDL (oxmLDL) (3). These oxmLDL particles bind to scavenger receptors on the surface of the monocytes and macrophages in the arterial intima and promote the formation of the atherosclerotic plaque.

In the macrophage, the uptake of oxmLDL is facilitated by CD36 and SR-A (4). CD36, first described as the "glycoprotein IV" observed on the surface of platelets, is the receptor for thrombospondin-1 (5). Its expression has been observed on the surface of other mammalian cell types: mononuclear phagocytes, dendritic cells, microglia, adipocytes, hepatocytes, myocytes, and epithelia. Recently, CD36 has been shown to be a key co-receptor with TLR2 for certain bacterial lipopolysaccharides (6–8). Because CD36 recognizes oxmLDL, it has been suggested to be able to induce sterile inflammation as well (9). To investigate the direct role that LDL plays in the inflammation associated with atherosclerosis, we utilized an in vitro model to study LDL/oxmLDL recognition by human mononuclear phagocytes as an inducer of proinflammatory cytokines. We focused attention on IL-6, IL-1β, TNF, IL-8, and a new member of the IκB family (IxkBγ) that has been shown to regulate a number of inflammatory cytokines and proteins of relevance to atherosclerosis (IL-6, IL-12, IL-18, GM-CSF, lipocalin-2, endothelin-1, macrophage receptor with collagenous structure (MARCO), and ghrelin) as surrogates of the early events in LDL/oxmLDL rec-
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Oxidized LDL (oxmLDL) is produced as a result of cholesterol oxidation and plays a role in atherosclerosis. However, the molecular mechanisms of oxmLDL in innate immune responses remain unclear. In this study, we investigated the effect of oxmLDL on proinflammatory responses in murine macrophages and human monocytes.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—CD14 magnetic selection beads were purchased from Miltenyi Biotec. The reagents were obtained from the following sources: RPMI 1640 (Cellgro and Invitrogen), fetal bovine serum (Atlanta Biologicals), and penicillin-streptomycin (Invitrogen). LPS E. coli strain 0111:B4 was purchased from Alexis Biochemicals. PamCSK4 was purchased from EMC microcollections GmbH. The actin monodonal clone C4 was purchased from MP Biomedicals. FITC-conjugated anti-human CD14 antibody was purchased from eBioscience. Rabbit antiserum against IκBζ and pro-IL-1β was generated in our laboratory using recombinant protein expressed in *Escherichia coli* as described previously (11). The lactate dehydrogenase assay was performed using the cytotoxicity detection kit from Roche Applied Science.

Cell Culture—Human peripheral blood mononuclear cells were isolated by Histopaque density gradient centrifugation from fresh source leukocytes from the American Red Cross. Monocytes were isolated by CD14-positive magnetic selection. Macrophages were matured in Teflon containers by plating peripheral blood mononuclear cells (2 × 10⁶/ml) in RPMI 1640 supplemented with 20% human AB serum for 6 days. After 6 days, the harvested cells were extensively washed, and macrophages were isolated by adherence. Monocytes (2 × 10⁶/ml) and macrophages (1 × 10⁶/ml) were plated in RPMI 1640 supplemented with 10% FBS and 1% penicillin streptomycin. Monocytes were stimulated with LPS (100 and 10 ng/ml) or PamCSK4 (50 and 5 ng/ml) for the times indicated. Macrophages were matured in Teflon containers by plating peripheral blood mononuclear cells (2 × 10⁶/ml) in RPMI 1640 supplemented with 20% human AB serum for 6 days. After 6 days, the harvested cells were extensively washed, and macrophages were isolated by adherence. Monocytes (2 × 10⁶/ml) and macrophages (1 × 10⁶/ml) were plated in RPMI 1640 supplemented with 10% FBS and 1% penicillin streptomycin. Monocytes were stimulated with LPS (100 and 10 ng/ml) or PamCSK4 (50 and 5 ng/ml) for the times indicated. Macrophages were stimulated with LPS (500 ng/ml) for the times indicated.

**LDL Isolation and Oxidation**—LDL was isolated from the plasma of fasting, healthy volunteer donors by sequential preparative ultracentrifugation technique using a 120.2 TL rotor in an Optima TL Beckman ultracentrifuge (Beckman Instruments) as demonstrated previously (12). LDL was dialyzed overnight into PBS at 4 °C. Protein estimation of the isolated LDL was performed by Lowry’s assay. LDL (100 μg/ml) was oxidized in the presence of Cu²⁺ (5 μM) at room temperature, and the oxidation was monitored overnight by measuring the A₂₃₄. Samples were then stored at 4 °C and used within 3 h for the preparation of LDL low in endotoxin, the glassware was soaked in E-Toxa-Clean (Sigma-Aldrich) overnight. The glassware was washed in endotoxin-free water (HyClone), dried, and autoclaved. The preparation was performed in a tissue culture hood with precautions taken for minimal endotoxin contamination with the usage of pyrogen-free tubes and tips. For dialysis, endotoxin-free PBS (Cellgro) and γ ray sterilized dialysis cassettes (Thermo Fisher Scientific) were used.

Flow Cytometry—Human monocytes (5 × 10⁶/ml) and macrophages (7 × 10⁶/ml) were prepared, stained with FITC-conjugated anti-human CD36 antibody or allophycocyanin-conjugated anti-human CD14 antibody, and analyzed by flow cytometry analysis as described previously (13).

Quantitative PCR—Monocytes were stimulated for the times indicated, and total RNA was extracted and converted to cDNA as described previously (14). Primers specific for IκBζ, IL-1β, IL-6, IL-8, TNFα, peroxisome proliferator-activated receptor γ (PPARγ), IL-1RII, IL-10, IL-1RN, and mannose receptor C type 1 (MRC-1) were used for analyzing mRNA expression. Gene expression was normalized to two housekeeping genes, GAPDH and CAP-1.

Preparation of Cell Lysates and Immunoblotting—Cells were lysed, and cell lysates were prepared as described previously (14). Total protein was estimated using Lowry’s assay (Bio-Rad), and equal protein (10–20 μg) was loaded per lane of NuPAGE 4–12% Bis-Tris gel (Invitrogen). The separated proteins were transferred onto PVDF membranes, which were blocked with 10% nonfat milk. Blocked membranes were blotted overnight at 4 °C with appropriate primary antibody followed by secondary antibody and visualization by ECL (GE Healthcare).

**RESULTS**

Monocyte Induction of IκBζ and Cytokines in Response to LDL and oxmLDL—Oxidized LDL activates proinflammatory cytokines by signaling through CD36 in concert with TLR2 (6, 8). Many of these cytokines (most notably IL-6) are regulated by the IκBζ member, IκBζ (10). To study the effects of LDL and oxmLDL on cytokine release, native LDL was isolated and modified to oxmLDL using Cu²⁺ (5 μM) and then incubated with fresh human monocytes for 3 and 6 h. As expected, standard preparations of LDL and oxmLDL induced the rapid induction of IκBζ (Fig. 1, A and B). The IκBζ expression assayed by densitometry was significantly higher (p < 0.05) upon treatment with oxmLDL as compared with controls (Fig. 1B). Additionally, this enhanced IκBζ expression was also followed by increased IL-6 and IL-8 release (Fig. 1C).

Because endotoxin contamination is possible with standard LDL isolation procedures, the endotoxin level in these LDL and oxmLDL preparations was determined by limulus amebocyte lysate assay and found to be between 15 and 90 endotoxin units/ml. To attempt to discriminate between the effect of LDL and contaminating endotoxin, LDL was therefore purified to achieve low endotoxin levels (<0.125 enzyme units/ml) and incubated with monocytes for 3 and 6 h. Although background
IxkBζ expression was detectable (Fig. 1, D and E), stimulation with native LDL and oxmLDL did not enhance expression of IxBζ. In agreement with the absent IxBζ activation, IL-6 and IL-8 expression was not significantly induced by these preparations of native LDL and oxmLDL. (Fig. 1F). These observations suggest that the expression of proinflammatory cytokines and related proteins is not due to LDL or oxmLDL.

Effect of LDL Purification Procedure on Oxidative Modifications—To rule out the possibility that the purification procedure might interfere with the oxidation of LDL, we evaluated native LDL and oxmLDL prepared by the standard and the low endotoxin methods for electronegativity (a measure of oxidative modifications (3)) (Fig. 2A). As observed, the electrophoretic mobility of oxmLDL was increased in both preparations of oxmLDL, suggesting no differences in oxidative modifications. Similarly, the peroxide content between the two different preparations as determined by thiobarbituric acid reactive substances assay and cumene peroxide assay was not affected by the mode of LDL preparation (Fig. 2, B and C). Taken together these findings suggest that differences in IxBζ and cytokine expression between standard and low endotoxin LDL/oxmLDL preparations are not due to the differences in oxidative modifications.

Monocyte Response to the Different Forms of Copper-oxidized LDL—Oxidation of native LDL with Cu2⁺ yields LDL with variable levels of oxidation, namely, mMLD, oXLDL, and oxmLDL, that are known to migrate with different electronegativities (supplemental Fig. S1) (3). To test whether these different forms of oxidized LDL might differentially activate proinflammatory responses, monocytes were incubated with these different forms of oxidized LDL. As observed, the expression of IL-6 and IL-8 was unaffected as compared with the effect in response...
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FIGURE 2. Effect of LDL preparation modality on oxidative modifications. LDL and oxmLDL were prepared in the standard or the stringent manner. A, samples were analyzed for their electronegative content by electrophoretic mobility. B and C, the different samples were also analyzed for the total peroxide content using the thiobarbituric acid reactive substances (TBARS) assay (B) and cumene peroxide assay (C). The electrophoretic mobility blot is representative of three independent experiments. The bar values for the thiobarbituric acid reactive substances assay are represented as mean ± S.E. for three independent experiments, and the bar values for the cumene peroxide assay are represented as mean ± S.E. for four independent experiments.

to LPS (positive control) (Table 1). This suggested that none of the Cu\(^{2+}\)-oxidized LDL forms alone are able to activate significant cytokine responses in human monocytes.

**Monocyte Responses Suppressed by oxmLDL when Combined with LPS**—Because the monocyte expression of IkB\(\zeta\), IL-6, and IL-8 protein occurred only with LDL and oxmLDL preparations that contained detectable endotoxin contamination, the mononuclear response to the purer forms of LDL and oxmLDL was studied in the presence of LPS. As observed previously, IkB\(\zeta\) expression was undetectable in the samples treated with LDL or oxmLDL alone (Fig. 3A). Upon treatment with LPS, the expression of IkB\(\zeta\) and pro-IL-1\(\beta\) increased as has been reported (11), but did not change with the combination of LDL/oxmLDL with LPS. However, unlike the effect on IkB\(\zeta\), pro-IL-1\(\beta\) expression dramatically decreased upon combining oxmLDL with LPS.

Additionally, the expression of IL-6, TNF\(\alpha\), and IL-1\(\beta\) followed the same pattern of expression as pro-IL-1\(\beta\), with a 2–10-fold decrease in cytokine expression upon treatment with the combination of oxmLDL and LPS (\(p < 0.05\)) (Fig. 3B). However, IL-8 responded differently in that IL-8 expression was not suppressed by the combination stimulus of oxmLDL and LPS. Thus, oxmLDL selectively down-regulates specific proinflammatory responses when recognized in combination with the TLR4 ligand, LPS.

**Gene Expression in Monocytes in Response to LDL and oxmLDL in the Presence of LPS**—To characterize the cytokine suppression mediated by oxmLDL, we measured the cytokine mRNA levels in monocytes treated with preparations of LDL and oxmLDL in the presence and absence of added LPS. Akin to IkB\(\zeta\) protein expression, LDL did not further augment LPS-induced IkB\(\zeta\) gene expression. The gene expression of IL-1\(\beta\) increased upon treatment with LPS, which remained unchanged with the combination of LDL and LPS. However, the combination of oxmLDL and LPS decreased the expression of IL-1\(\beta\), most significantly at 6 h (\(p < 0.05\)) (Fig. 4).

In contrast to the down-regulation of IL-6 and TNF\(\alpha\) expression at the protein level, there was no significant change in their gene expression upon treatment with oxmLDL and LPS. The gene expression of IL-8 mimicked its protein pattern upon treatment with LPS alone, which remained unchanged by treatment with the combination of LDL and LPS. However, treatment with oxmLDL and LPS significantly increased the gene expression of IL-8. This is in contrast to the protein expression of IL-8, which remained unaffected in response to the combination of oxmLDL and LPS. This result suggests that oxmLDL in combination with LPS decreases the gene expression of the proinflammatory gene IL-1\(\beta\) but paradoxically enhances the gene expression of IL-8.

In an attempt to understand the possible mechanisms involved in oxmLDL-mediated suppression of proinflammatory gene expression, we also analyzed the gene expression of candidate anti-inflammatory genes: IL-1RII, IL-10, IL-1RN, and MRC-1, as well as PPAR\(\gamma\), the negative regulator of NF\(\kappa\)B (supplemental Fig. S2). Although oxmLDL strongly suppressed the LPS effects on IL-10 and IL-1RN, it also enhanced the expression of IL-1RII and PPAR\(\gamma\). These changes in the gene expression patterns of different pro- and anti-inflammatory genes suggested that oxmLDL induces a unique monocyte phenotype in response to endotoxin challenge.

**Macrophages Immune to Suppressive Effects of oxmLDL**—As no significant response was observed in monocytes in response

**TABLE 1**

| Samples          | IL-6 (ng/ml) | IL-8 (ng/ml) |
|------------------|--------------|--------------|
| Control          | 0.05 ± 0.03  | 0.04 ± 0.03  |
| Native LDL       | 0.04 ± 0.03  | 0.02 ± 0.01  |
| Native LDL + Cu\(^{2+}\) (5 \(\mu\)M) | 0.02 ± 0.00 | 0.03 ± 0.01 |
| mmLDL            | 0.03 ± 0.00  | 0.02 ± 0.00  |
| oxmLDL           | 0.02 ± 0.00  | 0.00 ± 0.00  |
| oxmLDL 100 ng/ml| 3.4 ± 0.25   | 3.7 ± 0.33   |
| LPS (10 ng/ml)   | 25 ± 7       | 25 ± 7       |
| LPS 10 ng/ml     | 4.9 ± 0.9    | 16 ± 1.5     |
to low endotoxin preparations of oxmLDL, we turned to human macrophages, which utilize the scavenger receptor CD36 to take up oxmLDL in the process of becoming foam cells (18, 19). Human monocyte-derived macrophages were treated with low endotoxin preparations of LDL or oxmLDL alone or in combination with LPS for 3, 6, and 24 h. IκBζ and pro-IL-1β protein expression was induced by LPS but not by LDL or oxmLDL. Combining LDL or oxmLDL with LPS added no further response (Fig. 5A). The expression of IL-6, IL-8, and TNFα mimicked the protein expression of IκBζ and pro-IL-1β (Fig. 5B). The combination stimulus of LPS with either LDL or oxmLDL also showed no change in the expression of IL-6, IL-8, and TNFα. This was in contrast to the effect of oxmLDL on LPS-treated monocytes, where a decrease in expression of IL-1β, IL-6, and TNFα was observed.

Because our experiments showed no significant response of human monocytes and macrophages to LDL and oxmLDL, the surface expression of the scavenger receptor CD36 was confirmed in the different cell populations (20, 21) (supplemental Fig. S3). Both populations of cells, human monocytes and macrophages, demonstrated similar relative mean fluorescence intensities for CD36. Additionally, both cell types expressed CD14, although the relative mean fluorescence intensities of CD14 expression were 2-fold higher in the monocytes than in...
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FIGURE 4. Effect of oxmLDL on monocyte mRNA expression. Monocytes (2 × 10⁶/ml) were treated with LDL and oxmLDL (25 μg/ml) in the presence and absence of LPS (100 and 10 ng/ml) for the indicated times. The cells were lysed, and total RNA was extracted, converted to cDNA, and analyzed for iκBα, IL-1β, IL-6, IL-8, and TNFα gene expression by quantitative PCR. The bar values are represented as mean ± S.E for four experiments. ** represents p < 0.05.

the macrophages. This experiment suggests that the lack of a proinflammatory response to oxmLDL was not due to lack of CD36 expression.

Monocyte Response to oxmLDL in the Presence of TLR2 Agonist—oxmLDL has been reported to activate proinflammatory responses in concert with TLR2 (22), and the scavenger receptor CD36 is a co-receptor for TLR2 (6, 7). Therefore, to evaluate the ability of oxmLDL to function in the presence of a TLR2-specific ligand, freshly isolated CD14⁺ human monocytes were incubated with oxmLDL and the TLR2 ligand PamCSK4 either alone or in combination with LPS for 6 h. The expression of iκBα and pro-IL-1β was insignificant in response to LDL and oxmLDL (Fig. 6A). As expected, PamCSK4 (Pam) increased the expression of iκBα and pro-IL-1β (10, 23). However, the TLR2-dependent PamCSK4 effect on iκBα and pro-IL-1β was not augmented by the addition of LDL, mimicking the LDL- and TLR4-dependent LPS response (Fig. 3). Indeed, the expression of iκBα as well as pro-IL-1β was suppressed in response to the combination stimulus of oxmLDL and PamCSK4.

The expression of IL-6, IL-8, IL-1β, and TNFα followed a similar pattern of expression as iκBα and pro-IL-1β, with no change in the protein expression upon treatment with LDL or oxmLDL alone, but an increase upon treatment with PamCSK4 (Fig. 6B). This IL-6, IL-8, IL-1β, and TNFα response was not affected by native LDL provided with PamCSK4, but was down-regulated 2–5-fold in response to treatment with oxmLDL and PamCSK4 (p < 0.05). The addition of LPS to the PamCSK4, although enhancing overall cytokine responses, showed a similar pattern of oxmLDL-induced cytokine suppression (p < 0.05) except for IL-8 (p = not significant) akin to the previous observation in human monocytes in response to oxmLDL and LPS. Thus, oxmLDL also suppresses human monocyte responses to both TLR2 and TLR4 ligands.

DISCUSSION

Atherosclerotic plaques represent focal areas of inflammation characterized by subintimal accumulations of monocytes that become “foamy” macrophages by taking up lipids (2, 24–27). oxmLDL, which is critically important in the pathogenesis of plaque formation, binds to the scavenger receptor, CD36, on monocytes and macrophages inducing foam cell formation that have been linked to the pathogenesis of plaque formation (20, 28–34). The binding of oxmLDL to CD36 occurs in concert with the TLRs, notably TLR2 and TLR4, which have been linked to the downstream signal transduction and activation of the proinflammatory response (6, 7, 21, 35–40). Additionally, bacterial infections due to Chlamydia pneumoniae and Porphyromonas gingivalis activate TLR2 and TLR4, thereby inducing proatherogenic immune responses (41–43). These studies strongly implicate the involvement of pathogen recognition pathways of innate immune sensors in the development of atherosclerotic plaques.

This study evaluates the role of LDL and oxmLDL in the development of proinflammatory response in human monocytes and macrophages. Although the expression of the proinflammatory mediators iκBα, IL-6, and IL-8 in human monocytes was significant in response to the standard preparation of oxmLDL, limulus amebocyte lysate assays discovered a significant level of endotoxin contamination in these standard samples. Because plasma lipoproteins have been noted to serve as transport vehicles for TLR ligands such as endotoxin, to eliminate the contamination concern, preparations of LDL and oxmLDL were created under stringent conditions (9). Human monocytes treated with the low endotoxin preparations of LDL and oxmLDL showed no significant change in expression of iκBα, IL-6, and IL-8 as compared with untreated controls. Furthermore, we confirmed that this dramatic difference between the two preparations of LDL/oxmLDL toward the expression of
proinflammatory mediators was not a consequence of differences in the electronegative and peroxide content of the two preparations. This suggested that endotoxin contamination accounted for the true nature of oxmLDL effects on the expression of proinflammatory mediators and cytokines (Fig. 1).

Additionally, other intermediate forms of LDL (mmLDL, oxLDL, and oxmLDL), when prepared in a stringent manner, also failed to induce cytokine release. These results further suggest the possibility that many previous studies linking different forms of oxidized LDL to proinflammatory cytokines may have been confounded by indirect TLR activation rather than a direct response of these modified lipoproteins binding to its receptor.

However, it is conceivable that effects of oxidized LDL on inflammation may represent a cooperative effect between the oxidized lipoproteins and pathogen-associated molecular patterns. Therefore, having ruled out an effect of oxmLDL alone, we turned to study the monocytic response to the combination stimulus of oxmLDL with the TLR4 ligand, LPS. The effect of TLR4 stimulation by LPS in monocytes has been previously documented (11, 44). To our surprise, the combination of oxm-LDL and LPS dramatically down-regulated the expression of pro-IL-1β and the release of IL-6, IL-1β, and TNFα. Measurement of lactate dehydrogenase release confirmed that this suppression in cytokine release was not associated with cell death (supplemental Fig. S4). Of note, prior studies in murine systems also reported an oxmLDL-mediated immune suppression (45, 46). Additionally, the protein expression of IκBα and the release of IL-8 remained unaffected, further validating the lack of cell toxicity in response to the combination of oxmLDL and LPS.

Because cytokine release was not affected in samples treated with LPS and Cu²⁺ alone (data not shown), we eliminated the possibility that this down-regulation was a byproduct of Cu²⁺ present in the oxmLDL.

Although the detailed mechanisms responsible for the suppressive effects of oxmLDL are not included in the present study, the results provide some important clues. Firstly, evaluation demonstrated that the suppressive effect of combination stimulus of oxmLDL and LPS on IL-1β release was a consequence of down-regulation of IL-1β gene expression. Also, it appears that IL-6 and TNFα are also likely regulated by a decrease in mRNA responses (although not reaching the statistical significance seen with IL-1β). Evidence exists that oxidized phospholipids and oxysterol content of oxidized LDL down-regulate LPS-induced NFκB-mediated responses, thus leading to the hypothesis that lipid oxidation products may promote the shift from an acute inflammatory response to a chronic state (47, 48). Thus, it is conceivable that suppression of IL-1β, IL-6, and TNFα is a consequence of a similar mechanism.

Secondly, the suppression of mRNA levels is not global; IκBα mRNA levels remain unchanged, whereas IL-8 mRNA expression increases in response to the combined LPS oxmLDL stimulation. Enhanced expression of IL-8 in response to oxmLDL has been reported in endothelial cells and macrophages (49–51). Nevertheless, this increased mRNA expression of IL-8 did not lead to a concomitant increase in the protein expression of IL-8. This discrepancy in the gene and protein expression levels for IL-8 suggests a disconnect in the post-transcriptional regulation of IL-8 mRNA upon treatment with the combination of LPS and oxmLDL. Thus, taken as a whole, the differences in mRNA and protein levels between IL-8 and other cytokines suggest that the suppressive effect of oxmLDL is not due to a global inhibition of endotoxin detection by monocytes.
The effect of oxmLDL on cytokine responses was less dramatic in mature macrophages. Macrophages, like monocytes, failed to respond to purified oxmLDL. In contrast to the inhibition seen with monocytes, combining oxmLDL with LPS did not suppress the expression of $\beta$-actin, pro-IL-1$\beta$, IL-6, or TNF$\alpha$ in human macrophages. This observation was true despite similar surface expression of CD36 as well as TLR4 in the two cell populations (52). This suggests that the lack of inhibition in human macrophages may be a consequence of regulatory changes in gene expression associated with differentiation from monocyte to macrophage. In this regard, in agreement with our findings, a recent study has demonstrated the lack of significant changes in the LPS-induced proinflammatory responses mediated by oxmLDL-treated proinflammatory M1 macrophages (53). The same study also reports anti-inflammatory M2 macrophages as the predominant cells that take up oxidized LDL and progress to inflammatory M1 macrophages upon subsequent exposure to LPS, consequently expressing proinflammatory cytokines.

Although the occurrence of a similar switching event mediated by the combination of oxmLDL and LPS in our system is subject to future study, the phenotype of human monocytes treated with oxmLDL and LPS in our study suggests their commitment to a unique macrophage lineage. Signaling mediated by oxidized LDL in monocytes has been shown to activate the expression of PPAR$\gamma$, a negative regulator of proinflammatory signaling that has been shown to direct human monocytes into alternative M2 macrophages (54, 55). Although we confirm an increase in PPAR$\gamma$ expression system (supplemental Fig. S2), the evaluation of other M2-specific genes revealed inconsistent changes. IL-1RII followed the PPAR$\gamma$ pattern, but IL-10 and IL-1RN were actually suppressed, and MRC-1 demonstrated no significant change in response to oxmLDL. Taken as a whole, this expression pattern suggests that despite the lack of a con-

FIGURE 6. Monocyte response to oxmLDL in the presence of TLR2 agonist. Monocytes ($2 \times 10^5$/ml) were treated for 6 h with LDL and oxmLDL (25 $\mu$g/ml) in the presence and absence of PamCSK4 (Pam) (5 and 50 ng/ml, respectively) or PamCSK4 with LPS (100 ng/ml). A, cell lysates immunoblotted for $\beta$-actin, pro-IL-1$\beta$, and actin expression. B, protein expression for IL-6, IL-8, and TNF$\alpha$ as analyzed by ELISA. The immunoblots are representative of three independent experiments, and the bar values display the mean ± S.E. for three independent experiments (the bar values for the low dosage PamCSK4 treatment average two experiments). Vertical lines have been inserted to indicate repositioned gel lanes. * and # represent nonspecific bands. ** represents $p < 0.05$. 

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certed commitment to a classic M2 macrophage phenotype, human monocytes upon treatment with oxmLDL generally exhibit a unique anti-inflammatory phenotype.

Because the oxmLDL receptor, CD36, is better characterized as a modulator of TLR2 function as compared with TLR4, the TLR2 ligand PamCSK4 was also tested for potential synergy with oxmLDL (6, 8). Although PamCSK4 in combination with oxmLDL consistently suppressed the expression of proinflammatory mediators as well as IκBζ and IL-8, oxmLDL suppressed the induction of IL-1β, IL-6, and TNFα but not IκBζ and IL-8 when PamCSK4 was combined with LPS (Fig. 6). The rescue of the expression of IκBζ and IL-8 by the addition of LPS to oxmLDL and PamCSK4 suggests that signaling mediated by TLR4 overcomes the suppressive effect of oxmLDL on PamCSK4-induced expression of IκBζ and IL-8. These findings imply that the suppressive effect of oxmLDL may be more specific to TLR2 signaling events, consistent with the proven cooperativity between CD36 and TLR2 (6, 8).

In conclusion, our studies demonstrate that LDL and oxmLDL are unable to elicit an active proinflammatory response from human monocytes and macrophages when prepared in a low endotoxin environment. Moreover, we demonstrate that oxmLDL suppresses the LPS-induced expression and release of specific proinflammatory mediators and cytokines. Furthermore, the suppressive effect of oxmLDL for the expression and release of proinflammatory cytokines and mediators was more specific to TLR2 signaling as compared with TLR4 signaling. Thus, our findings not only contradict the popular concept of proinflammatory signaling mediated by oxmLDL but also suggest that oxmLDL cooperates negatively with TLR ligands for specific human monocyte proinflammatory responses.

Acknowledgments—We thank Freweine Berke, Jennifer Hollyfield, and Amy Gross for technical assistance through the course of this study. We also thank the Dorothy M. Davis Heart and Lung Research Institute (DHLRI) Flow Cytometry and Cell Sorting Analysis Core for assistance with the flow cytometry experiments.

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