Lipopolysaccharide-induced gene expression in murine macrophages is enhanced by prior exposure to oxLDL

Mathijs Groeneweg,† Edwin Kanters,† Monique N. Vergouwe,*, Hilde Duerink,*, Georg Kraai,† Marten H. Hofker,*, and Menno P. J. de Winther†,*

Department of Molecular Genetics,* Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, The Netherlands; and Department of Molecular Cell Biology and Immunology,† Vrije Universiteit Medical Center, Amsterdam, The Netherlands

Abstract  Uptake of modified lipoproteins by macrophages results in the formation of foam cells. We investigated how foam cell formation affects the inflammatory response of macrophages. Murine bone marrow-derived macrophages were treated with oxidized LDL (oxLDL) to induce foam cell formation. Subsequently, the foam cells were activated with lipopolysaccharide (LPS), and the expression of lipid metabolism and inflammatory genes was analyzed. Furthermore, gene expression profiles of foam cells were analyzed using a microarray. We found that prior exposure to oxLDL resulted in enhanced LPS-induced tumor necrosis factor (TNF) and interleukin-6 (IL-6) gene expression, whereas the expression of the anti-inflammatory cytokine IL-10 and interferon-β was decreased in foam cells. Also, LPS-induced cytokine secretion of TNF, IL-6, and IL-12 was enhanced, whereas secretion of IL-10 was strongly reduced after oxLDL preincubation. Microarray experiments showed that the overall inflammatory response induced by LPS was enhanced by oxLDL loading of the macrophages. Moreover, oxLDL loading was shown to result in increased nuclear factor-kB activation. In conclusion, our experiments show that the inflammatory response to LPS is enhanced by loading of macrophages with oxLDL. These data demonstrate that foam cell formation may augment the inflammatory response of macrophages during atherogenesis, possibly in an IL-10-dependent manner.—Groeneweg, M., E. Kanters, M. N. Vergouwe, H. Duerink, G. Kraai, M. H. Hofker, and M. P. J. de Winther. Lipopolysaccharide-induced gene expression in murine macrophages is enhanced by prior exposure to oxLDL. J. Lipid Res. 2006. 47: 2259–2267.

Supplementary key words  cytokines • inflammation • modified LDL • lipopolysaccharide • RAW 264.7

Macrophages play a key role in atherosclerotic lesion development (1). The onset of atherosclerosis involves attraction of monocytes to the vessel wall at local sites of endothelial dysfunction. The monocytes subsequently migrate through the endothelium and differentiate into macrophages. These macrophages take up modified LDL particles, leading to the formation of foam cells, the first hallmark of an atherosclerotic lesion. Further progression of atherosclerosis involves the attraction of additional monocytes, influx of T-cells, proliferation and migration of smooth muscle cells, and processes like cell death by apoptosis or necrosis. Atherogenesis is accompanied by the local production and secretion of inflammatory mediators, for which the macrophage is a major source.

The main characteristic of foam cell formation is the accumulation of cholesteryl esters (CEs) from LDL taken up by the macrophages. In vitro, several forms of modified LDL are being studied; including copper-oxidized LDL (oxLDL), acetylated LDL, and aggregated LDL. When administered to macrophages, these forms of modified LDL are taken up and mainly stored as free cholesterol and CE, resulting in the formation of foam cells (2, 3).

Incubation of macrophages with low concentrations of oxLDL protects cells from apoptosis and induces cell proliferation (4–6). In contrast, high concentrations and long incubation times of oxLDL will induce cell death (5). The effects of oxLDL on the inflammatory capacity of macrophages are less clear. It is generally assumed that during foam cell formation, macrophages lose their ability to elicit a strong inflammatory response upon stimulation; however, results have not been consistent in different publications (7). Effects of oxLDL are dependent on intensity of LDL oxidation (8–10) and the amount of oxLDL applied to the cells. Low concentrations are thought to enhance the inflammatory response of macrophages after activation, whereas higher concentrations may repress the inflammatory response (7).

In our study, we were interested in the effects of oxLDL loading on lipopolysaccharide (LPS)-induced gene ex-
pression and on the inflammatory response of murine macrophages. To follow the transition of macrophages to foam cells and the effect of modified LDL on the inflammatory properties of macrophages, we performed real-time PCR and microarray experiments on murine bone marrow-derived macrophages (BMMs). Our results indicate that oxLDL loading of macrophages caused changes in cytokine gene expression in BMMs. In particular, oxLDL preincubation mainly enhanced the effects of LPS activation coinciding with an enhanced activation of nuclear factor-κB (NF-κB) and a downregulation of interleukin-10 (IL-10) and interferon-β (IFN-β).

MATERIALS

LDL isolation and modification

Serum from fresh blood (0.5 l, obtained from ‘De Stichting Sanquin Bloedvoorziening’) was obtained for LDL isolation, and LDL was oxidized by CuSO₄ as described previously (11). The LDL and oxLDL were stored for up to 2 weeks under N₂ at 4°C. Endotoxin concentration of all samples was below 5 pg/ml, as tested in a Limulus Amabocyte Lysate assay (HyCult Biotechnology b.v., The Netherlands).

Cell culture and stimulation

BMMs were obtained from C57/Bl6 mice as described previously (12, 13). After differentiation, cells were cultured in R10 (RPMI 1640 medium, containing 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM l-glutamine, and 10 mM Hepes) supplemented with 15% L-929 cell-conditioned medium (14). RAW 264.7 cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml), l-glutamine (2 mM) and β-mercaptoethanol (0.1%). Experiments were performed in this same medium with the same setup as described for the BMMs. Cells were stimulated using 10 ng/ml LPS for 3 h. Cells were incubated with 25 µg/ml lipoprotein for the indicated times.

Lipid extraction and high-performance TLC

The BMMs were lifted from the bacterial plastic 6-well plates, suspended in 200 µl PBS, and frozen at −20°C. The cells were thawed and repeatedly passed through a syringe to fully lyse the cells. Cell lysate containing 200 µg protein was added to a total volume of 800 µl MilliQ. Chloroform (300 µl) and internal standard (20 µg cholesterol acetate/ml chloroform) were added, and the mixture was centrifuged at 3,000 rpm. The chloroform fraction was isolated, dried at 50°C under N₂, and stored at −20°C. High-performance TLC (HPTLC) plates (Alltech/Applied Science) were activated overnight at 130°C. The plates were prerun with a 2:1 methanol-chloroform mixture, dried at 130°C, prerun with 10% NaOH in methanol, and activated overnight at 130°C. Lipid samples were dissolved in 50 µl chloroform and slowly applied to the HPTLC plate. The run was performed with a hexane-diethylether-2-propanol mixture (90:10:5). After the run, the plates were dried at 130°C and incubated in staining reagent (H₂SO₄-dissolved MnCl₂ in 50% methanol) for 20 s, and incubated at 130°C for 30 min.

RNA isolation and quantitative PCR analysis

Total RNA was isolated from BMM using Tri-Reagent from Sigma according to the manufacturer’s recommendation. RNA concentration was measured using the Biorad Smartspec 3000 photospectrometer. Total RNA (1 µg) was used to obtain cDNA. Oligo-dT primers and RevertAid M-MuLV reverse transcriptase (Fermentas) were used according to the manufacturer’s recommendation. cDNA (10 ng) was used for TaqMan analysis with either the quantitative PCR (qPCR) Mastermix Plus Kit (Eurogentec) with 300 nM primer and 200 nM probe or the qPCR Mastermix Plus Kit for SYBR Green (Eurogentec) with 300 nM primer. Analysis was performed on an ABI Prism 7700 sequence detector. The housekeeping genes cyclophilin A and β-actin were used to correct for RNA concentration differences between the samples. Primer sequences can be obtained upon request.

ELISA

ELISAs were performed according to the manufacturer’s protocol. ELISA plates were obtained from NUNC (Denmark). Antibodies were detected by incubation with Ortho Phynyl Diamine (OPD)/H₂O₂, and H₂SO₄ was added to stop the reaction. Absorbance was measured at 490 nm in a BENCH-MARK microplate reader (BIORAD).

Microarrays

Cy 3- or Cy 5-labeled cDNA (1 µg) was hybridized to slides containing National Institute on Aging mouse 15K v3 cloneset. Slides were obtained from The University Health Network in Toronto, Canada (15–17) (http://www.microarrays.ca). A detailed description of the procedure can be found online in the supplemental material.

Luciferase activity

BMM from NF-κB reporter mice (18) (a generous gift from Drs. H. Carlsen and R. Blomhoff, University of Oslo, Norway) or RAW 264.7 cells stably transfected with the 3xNF-κB-luc plasmid (18) were incubated for 0, 1, 12, 24, and 48 h with 25 µg/ml oxLDL and subsequently activated with 10 ng/ml LPS. Cells were lysed using cell culture lysis buffer (Promega) for 20 min, after which 10 µl cell lysate was added to 50 µl luciferin. Luciferase activity was measured using the Lumac Biocounter M1500 luminometer.

Statistics

All data are expressed as a mean ± SEM of triplicate measures. Statistical analysis was performed using the Student’s t test, one-way ANOVA or two-way ANOVA when applicable, using GraphPad PRISM software. Significance is depicted in the respective figures. P values smaller than 0.05 were considered significant.

RESULTS

Analysis of foam cell formation

BMMs were incubated with oxLDL for 1, 12, 24, and 48 h to induce different stages of foam cell formation, then the cells were activated by LPS for an additional 3 h. Because we did not observe significant differences in gene expression patterns between 24 and 48 h of macrophage exposure to oxLDL, the 48 h incubation time point was omitted in the following experiments (data not shown). Cholesterol accumulation in response to oxLDL loading was monitored by lipid analysis using HPTLC. Free cholesterol concentration remained at the same level throughout the experiments.
(data not shown), whereas the amount of intracellular CEs strongly increased over a period of 24 h (Fig. 1A), indicating accumulation and processing of cholesterol in the BMMs. Cell death during oxLDL and LPS treatment was quantified by sub-G1 analysis. During oxLDL loading, the percentage of dead cells did not increase, and LPS stimulation for 3 h also did not have an effect on cell death (data not shown). qPCR experiments were performed to assess the metabolic responses of the BMMs to oxLDL. ABCA1, CD36, and SR-A expression increased after oxLDL administration (Fig. 1B–D). LPS stimulation further increased SR-A expression.

Inflammatory response of the macrophages

To analyze inflammatory responses, several genes were analyzed by qPCR. Interestingly, the expression level of tumor necrosis factor (TNF) and IL-6 after LPS stimulation was increased 2–3-fold by preincubation with oxLDL (Fig. 2A, B). Conversely, the induction of the anti-inflammatory cytokine IL-10 was strongly repressed in the cells after preincubation with oxLDL (Fig. 2C). Similarly, IL-12 was also decreased but only after 24 h oxLDL incubation (Fig. 2D). MCP1 and iNOS expression was induced after LPS stimulation but was not strongly affected by preloading with oxLDL (data not shown). These data show that oxLDL has both repressive and stimulating effects on LPS-induced inflammatory genes.

Because one of the major transcription factors involved in inflammatory gene expression is NF-κB, we focused on NF-κB-dependent gene expression. To investigate the involvement of NF-κB in the modulation of LPS-induced gene expression by oxLDL, an NF-κB-dependent luciferase assay was used. NF-κB activation was measured using BMMs from NF-κB reporter mice (18). After 24 h of oxLDL loading, there was a significant increase in LPS-induced luciferase activity compared with LPS-activated cells without oxLDL preloading (Fig. 2E). OxLDL incubation without LPS activation did not induce luciferase activity (Fig. 2E).

To examine whether the enhancement of the LPS-induced gene expression by oxLDL was specific for oxLDL, we also tested the expression of these genes in cells treated with native LDL. However, LPS-induced gene expression of several genes tested was not modulated by native LDL (data not shown).

Cytokine secretion in macrophages

To confirm the results obtained from qPCR experiments, we measured cytokine secretion to the medium after LPS activation. BMMs were treated for 1.5, 3, 6, and
24 h with 10 ng/ml LPS after preincubation with 25 μg/ml oxLDL for 24 h or without preincubation. The concentrations of TNF, IL-6, IL-12, and IL-10 in the medium were measured using an ELISA. In line with the qPCR experiments, TNF and IL-6 secretion upon LPS treatment was enhanced by oxLDL preincubation (Fig. 3A, B), whereas IL-10 secretion was reduced (Fig. 3C). Interestingly, whereas the expression of IL-12 was reduced in LPS-activated BMMs preincubated with oxLDL compared with LPS-activated BMMs (Fig. 2D) that were not preincubated with oxLDL, IL-12 secretion was enhanced by oxLDL preincubation (Fig. 3D). Other times of oxLDL loading (i.e., 3 h and 48 h) gave similar results with respect to an enhancement of TNF and IL-6 induction and a repression of IL-10 induction by LPS (data not shown).

Microarray analysis
To examine the general effect of oxLDL preincubation on LPS-induced gene expression in an unbiased way, the macrophages were further investigated via microarray analysis. We used a design proposed by Yang and Speed (19) and Churchill (20), in which every oxLDL loading time point was compared with the previous, resulting in data showing a relative change of expression in comparison to the previous condition, giving a cumulative expression pattern for each gene (see supplementary Fig. I). In our data analysis, we used genes that were at least 1.5-fold up- or downregulated in at least one lipid-loaded condition. In total, the number of genes that met these criteria was 377. Many genes differentially regulated by LPS were influenced by oxLDL loading. Interestingly, genes upregulated by LPS mainly became further upregulated by oxLDL preincubation, whereas genes downregulated by LPS mainly became further downregulated by oxLDL preincubation (Table I). This indicates that oxLDL mainly enhances the gene expression changes induced by LPS stimulation. To analyze the function of the genes found, genes were annotated using Gene Ontology (21) and the description at the National Center for Biotechnology Information’s Entrez Gene (http://www.ncbi.nlm.nih.gov). Based on this annotation, we did not readily recognize genes known to be involved in lipid metabolism or inflammation. Please see http://www.jlr.org for genes found to be differentially expressed, their gene ontology, and the fold change in expression (also see supplemental Table I).

Effects of oxLDL loading on IL-10 target genes
Previous studies have shown that oxLDL loading highly affects the production of IFN-β through inhibition of IFN-β transcription (22). In addition, the induction of IFN-β by LPS has been shown to be dependent on autocrine effects of secreted IL-10 (23). We could indeed show that oxLDL loading of BMMs reduced the induction of IFN-β gene expression by LPS by approximately 50% (Fig. 4A). Not only was IFN-β expression repressed by oxLDL preincubation, IFN-β secretion was also reduced. BMMs were preincubated with oxLDL for 24 h and subsequently activated with LPS for the indicated times. IFN-β secretion was inhibited by oxLDL compared with cells that were
not preincubated, independent of LPS activation time (Fig. 4B).

To further investigate the effects of oxLDL on other IL-10-dependent genes, we analyzed additional genes that are known to be dependent on autocrine IL-10 action: IL-1 receptor antagonist (IL-1RA) and inducible NO synthase (NOS2 or iNOS) (23). Both were strongly induced by LPS (Fig. 4C, D), and this induction was indeed repressed by 24 h oxLDL preloading. Furthermore, we analyzed the IL-10-dependent genes Arginase 1 and Arginase 2. However, Arginase 1 was not induced by 3 h of LPS but was repressed by oxLDL loading. Arginase 2 showed an inhibition similar to that of IFN-γ, IL-1RA, and iNOS, but this effect did not reach statistical significance (data not shown). Taken together, these data indicate that the reduced LPS induction of IL-10 in oxLDL-treated macrophages may have autocrine effects on genes that are dependent on IL-10 secretion by the macrophage.

TABLE 1. Effects of oxLDL on LPS regulated genes

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total LPS-regulated Genes</th>
<th>0–1 H oxLDL</th>
<th>1–12 H oxLDL</th>
<th>12–24 H oxLDL</th>
<th>24–48 H oxLDL</th>
<th>Net Total Regulated Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genes upregulated by LPS</td>
<td>123</td>
<td>40</td>
<td>34*</td>
<td>7</td>
<td>39*</td>
<td>42* Up</td>
</tr>
<tr>
<td>Genes downregulated by LPS</td>
<td>126</td>
<td>23</td>
<td>16</td>
<td>23*</td>
<td>14</td>
<td>9 Down</td>
</tr>
</tbody>
</table>

Genes changed by LPS activation of bone marrow-derived macrophages were selected, and the effect of oxLDL preincubation on these genes was followed through time. The upper part of the table represents the genes upregulated by LPS, and the lower part of the table represents the genes downregulated by LPS. LPS, lipopolysaccharide; oxLDL, oxidized LDL.

*Significantly more genes in the up- or downregulated group compared with the down- or upregulated group of the same time point as tested with a two-sided χ² test; *P < 0.05. Total regulated genes depict the net effect of the time course.
Effects of oxLDL on LPS-treated RAW 264.7 macrophages

To confirm the results we obtained in BMMs and to extend our studies to an alternative source of macrophages, experiments were performed in the murine macrophage cell line RAW 264.7. These cells were incubated with or without oxLDL for 24 h and then activated with LPS. CD36 was increased by oxLDL loading (Fig. 5A), as a hallmark of foam cell formation. Because the major effects in BMMs were observed on TNF and IL-10, we analyzed these genes in the RAW cells. TNF induction was increased approximately 2-fold in cells that had been loaded with oxLDL for 24 h (Fig. 5B), whereas IL-10 expression was strongly repressed by the oxLDL loading (Fig. 5C). These data confirm our findings in BMMs and show that the inflammation-modulating effects observed for BMMs also hold true in the RAW 264.7 cell line. Finally, we could show that an NF-κB reporter stably transfected in RAW cells was also more induced by LPS after oxLDL loading, also confirming our findings in BMMs (Fig. 5D).

DISCUSSION

In the present study, we examined the effect of oxLDL loading on the inflammatory capacity of macrophages. We show that LPS-induced TNF and IL-6 expression and secretion were upregulated, whereas IL-10 expression and secretion were downregulated by oxLDL preincubation. Moreover, using microarray analysis, we found that the overall LPS-induced gene expression patterns were mainly enhanced by oxLDL preloading. These results indicate a shift toward a proinflammatory phenotype that may highly affect atherosclerotic lesion development (1, 24).

Previous studies have assessed the effects of modified lipids on macrophage inflammatory responses. In contrast to our finding that LPS-induced gene expression patterns were enhanced by prior exposure to oxLDL, a study done by Ohlssen et al. (25) showed that oxLDL loading reduced NF-κB activation and subsequent TNF and IL-1β induction in human primary macrophages. Mikita et al. (26) recently performed a large-scale microarray analysis experiment on the human monocyte/macrophage cell line THP-1. They showed that 72 h of oxLDL loading strongly affects and mainly attenuates the LPS-induced inflammatory gene expression of macrophages and also inhibits NF-κB DNA binding. Interestingly, several major NF-κB-related proinflammatory genes, such as TNF, IL-1β, and IL-8, were strongly upregulated in these experiments (26), similar to the regulation of TNF and IL-6 in our experiment. The enhancement of LPS-induced gene expression patterns has also previously been shown by others. Brand et al. (27) have described oxLDL enhancement of LPS-

Fig. 4. Interleukin-10 (IL-10) target genes are downregulated by oxLDL. A: BMMs were incubated for 24 h with or without oxLDL and subsequently activated with LPS, and interferon-β (IFN-β) expression was quantified. B: BMMs were incubated for 24 h with oxLDL and subsequently activated by LPS for the indicated times (hours). OxLDL loading caused a decrease in IFN-β secretion, independent of the duration of LPS activation, as tested by two-way ANOVA (P < 0.05). C–D: BMMs were incubated for 24 h with or without oxLDL and subsequently activated with LPS, and the expression of the IL-10 target genes IL-1RA and NOS2 was quantified. Gene expression was corrected for cyclophillin expression. A, C, and D: *P < 0.05. Error bars represent SEM.
induced TF expression in human monocytes. It should be noted however, that they did not find this for TNF expression, indicating diverse effects on different signaling pathways.

A possible mechanism through which oxLDL could influence the inflammatory response is apoptosis. OxLDL may affect the macrophages either by inducing apoptosis as a result of the oxidation level of the lipoprotein (4, 9) or by inhibiting the uptake of apoptotic cells by macrophages, thereby prolonging the inflammatory response, as has been described by Kahn et al. (28). However, we did not see major changes in apoptosis in our experiments, and therefore the contribution of apoptotic macrophages to our observed phenotype is probably very limited.

To control the inflammatory response, inflammatory activation pathways are strongly regulated by autocrine feedback mechanisms. When activated, macrophages induce both pro- and anti-inflammatory cytokines that can affect other cells as well as the macrophages themselves. A major anti-inflammatory cytokine produced by macrophages is IL-10. IL-10 has been shown to inhibit activation of macrophages through autocrine mechanisms (29, 30). Moreover, IL-10 has been shown to be important in atherosclerosis; an IL-10 knockout mouse model shows severely increased atherosclerosis (31–33). We now show that oxLDL loading of macrophages strongly represses the induction of IL-10, and this may have major implications for the inflammatory properties of macrophages during atherogenesis. The inhibitory effect of oxLDL on LPS-induced IL-10 expression can also be observed in the downregulation of the IL-10-dependent, LPS-induced expression of IFN-β, IL-1RA, and iNOS2 (23). This indicates that oxLDL loading indeed disrupts autocrine IL-10 signaling. The inhibition of the IL-10 pathway by oxLDL loading may lead to reduced autocrine deactivation of macrophages, giving the proinflammatory phenotype we observe in our experiments (30).

IFN-β expression, like that of the genes mentioned earlier, is also IL-10 dependent (23). Activation of macrophages by LPS resulted in a strong induction of IFN-β expression. This induction was reduced after oxLDL loading of murine macrophages, which is in line with the downregulation of the other IL-10-dependent genes as well as with previous findings in THP-1 cells showing reduced levels of IFN-β expression and secretion upon oxLDL loading of THP-1 macrophages (22). Earlier studies have shown an important role for IFN-β and the IFN-β receptor (i.e., IFN-α/β R) in autocrine regulation of macrophage responses (34, 35). Thus, IFN-β would be an interesting target for further research in foam cell formation and atherosclerotic plaque development.

There may be many different forms of modified LDL present in atherosclerotic lesions. Our Cu²⁺-oxidized LDL is an often-used model substance for physiological modified LDL. However, it contains many different modifications of the lipid moiety and of the apolipoprotein B protein.

**Fig. 5.** OxLDL-induced gene expression in RAW 264.7 macrophages. Cells were incubated with 25 μg/ml oxLDL for 24 h and afterwards stimulated with LPS. Left sides of the graphs indicate cells that were not stimulated with LPS; right sides of the graphs indicate cells that were stimulated with LPS. A–C: Q-PCR analysis of genes induced by oxLDL and LPS. D: An NF-κB-dependent luciferase assay was used to investigate the effects of oxLDL on LPS-induced NF-κB activation; *P < 0.05. Error bars represent SEM.
component of the LDL particle. Certain modified phospholipids, such as oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (oxPAPC), have been described as modulating the inflammatory response. Not only is oxPAPC found in atherosclerotic lesions (36, 37), modified phospholipids have also been shown to be capable of affecting endothelial cell activation (38, 39). Future investigations may focus on the question of which components of oxLDL exert the inflammation-enhancing effect we observe.

Understanding the mechanisms underlying foam cell formation and the effects on inflammation and the early onset of atherosclerosis remains important for understanding the development of atherosclerotic plaques. Our results show an increased inflammatory response to stimuli during the oxLDL-induced transition from macrophage to foam cell, which could contribute to enhanced inflammation in the vessel wall. Gaining more understanding of the molecular mechanisms behind the onset and progression of atherosclerosis both in vivo and in vitro is essential for the development of novel drugs for the prevention and treatment of atherosclerosis.

This work was supported by the Dutch Organization for Scientific Research [Grants NWO 902-26-194 (M.H.), NWO 906-02-075 (M.P.J.d.W.), and VIDI 917.66.329 (M.P.J.d.W.)] and the European Union [European Vascular Genomics Network Grant LSHTM-CT-2003-503254 (M.H. and M.P.J.d.W.).

REFERENCES


