

Oxidized Low Density Lipoprotein Exposure Alters the Transcriptional Response of Macrophages to Inflammatory Stimulus*

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Macrophage-derived foam cells in atherosclerotic lesions are generally thought to play a major role in the pathology of the disease. Because macrophages play a central role in the inflammatory response, and the atherosclerotic lesion has features associated with chronic inflammatory settings, we investigated foam cell inflammatory potential. THP-1-derived macrophages were treated with oxidized low density lipoprotein (OxLDL) for 3 days to lipid load the macrophages and establish a foam cell-like phenotype. The cells were then activated by treatment with lipopolysaccharide (LPS), and RNA was harvested at 0, 1, and 6 h after LPS addition. RNA from treated and control cells was hybridized to microarrays containing ~16,000 human cDNAs. Genes that exhibited a 4-fold or greater increase or decrease at either 1 or 6 h after LPS treatment were counted as LPS-responsive genes. Employing these criteria, 127 LPS-responsive genes were identified. Prior treatment of THP-1 macrophages with OxLDL affected the expression of 57 of these 127 genes. Among these 57 genes was a group of chemokine, cytokine, and signal transduction genes with pronounced expression changes. OxLDL pretreatment resulted in a significant perturbation of LPS-induced NF κ B activation. Furthermore, some of the OxLDL effects appear to be mediated by the nuclear receptors retinoid X receptor and peroxisomal proliferator-activated receptor γ because pretreatment of THP-1 macrophages with ligands for these receptors, followed by LPS treatment, recapitulates the OxLDL plus LPS results for several of the most significantly modulated genes.

Atherosclerotic lesions have many features common to chronic inflammatory diseases. These features include local enrichment of inflammatory cells, persistent cell damage, changes in the extracellular matrix, increased cell proliferation, and fibrosis (1). In nascent arterial lesions, changes in surface endothelium occur due to the entry and oxidation of lipids (2). The location of such lesions is usually at major arterial branches, where endothelial cell shapes are nonuniform, and shear stress from blood flow is irregular (3). Resultant endothelial changes include increased expression of adhesion proteins and the secretion of chemoattractant molecules. This in turn leads to the recruitment, binding, and subsequent entry of monocytes and lymphocytes into subendothelial

spaces. (1). In the early stages of the atherosclerotic lesion, lipid-engorged macrophages, or foam cells, appear to be most prominent (4). As the lesion progresses, a necrotic core of lipid, oxidized lipid, and other cell debris accumulates. Increases in extracellular matrix production result in the formation of a fibrous cap or plaque that walls off the necrotic core from the endothelium (2, 5). In time, increased lesion size and local proliferation of smooth muscle cells can cause the plaque to protrude into the lumen. Weakening events, possibly associated with the local inflammatory response, can lead to plaque rupture, thrombosis, and the ensuing myocardial infarction or stroke (5, 6).

During this complex process, the foam cell macrophage is generally thought to play a major role in the pathology of the disease (2, 4). Foam cell macrophages comprise the major volume of the early lesion or “fatty streak,” and they are enriched in late-stage lesions as well (1). Macrophages carry out several critical functions that are likely to impact the development of atherosclerosis. As scavenger cells, they rid the body of dead cells and cell debris. As antigen-presenting cells, they perform an “early warning” function that helps mobilize additional members of the immune system in the case of infection. In addition, macrophages secrete cytokines and chemokines that direct and amplify the local immune response once it is triggered. Altered or unregulated control of these processes in the foam cell would likely have profound effects on the recruitment and behavior of macrophages and lymphocytes within the atherosclerotic lesion.

Previous *in vitro* studies involving foam cell macrophages have observed changes in gene expression and/or cellular response that suggest that excess lipid loading can modulate macrophage inflammatory potential (7–10). *In situ* studies of human and animal atherosclerotic lesions have identified the increased expression of several chemokine, tissue-remodeling, and lipid metabolism genes (1, 11–13). *In vivo*, infectious agents like cytomegalovirus and *Chlamydia pneumoniae* have been linked to atherosclerosis (14, 15). In addition, antibodies to OxLDL¹ epitopes and increases in circulating pro-inflammatory cytokines have been observed to correlate with vascular disease (16–18). These and other studies implicate a role for activated macrophages and other inflammatory cells in the development and progression of atherosclerosis.

In an attempt to address the question of whether inflamma-

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¹ The abbreviations used are: OxLDL, oxidized low density lipoprotein; PMA, 12-O-tetradecanoylphorbol 13-acetate; RT-PCR, reverse transcription-polymerase chain reaction; LPS, lipopolysaccharide; PPAR γ , peroxisomal proliferator-activated receptor γ ; RXR, retinoid X receptor; LXR, liver X receptor; 15dPGJ2, 15-deoxy-12,14-prostaglandin J2; 22RHC, 22-R-hydroxycholesterol; 9cisR, 9-cis-retinoic acid; NF κ B, nuclear factor κ B; TNF- α , tumor necrosis factor α 59 IL, interleukin.

tory gene expression programs are altered in the foam cell macrophage, we have carried out the following study. PMA-differentiated THP-1 cells were treated with OxLDL for 3 days to lipid load the macrophages and establish a foam cell-like phenotype. The cells were then activated by treatment with LPS followed by large scale gene expression analysis using DNA microarrays. The LPS effects on transcription were determined by comparing gene expression at 1 and 6 h after LPS stimulation with that of unstimulated cells. The effects of OxLDL pretreatment on LPS-induced transcription were determined through matched time point comparisons between OxLDL-treated and untreated cells. The results of these and other accompanying studies indicate that pretreatment of THP-1 macrophages with OxLDL significantly alters inflammatory gene expression programs initiated after LPS stimulation. In addition, our data suggest that OxLDL modulation of inflammatory signaling is partially mediated through altered regulation of NF κ B signaling and activation of the nuclear receptors RXR and PPAR γ .

MATERIALS AND METHODS

Cell Culture—Human THP-1 cells (ATCC 10801) were grown in RPMI 1640 medium containing 10% fetal bovine serum (v/v), 0.45% glucose (w/v), 10 mM Hepes, 1 mM sodium pyruvate, 1×10^{-5} M β -mercaptoethanol, 100 units/ml penicillin, and 100 μ g/ml streptomycin. For OxLDL loading experiments, cells were seeded at a density of 1×10^6 cells/ml in medium containing PMA (Research Biochemical International, Natick, MA) at 1×10^{-7} M for 24 h. The medium was then replaced by culture medium with or without 100 μ g/ml human oxidized low density lipoprotein (prepared by CuSO₄ oxidation of low density lipoprotein; Intracel Corp., Rockville, MD) for 3 days. The cells were then treated with 0.5 μ g/ml LPS to activate the macrophages. RNA, supernatants, and cell extracts were harvested at the indicated times and assayed as described in individual experiments. For experiments investigating the involvement of the nuclear receptors PPAR γ , LXR, and RXR in modulating inflammatory gene expression, PMA-differentiated THP-1 cells were incubated with either 3 μ M 15-deoxy-12,14-prostaglandin J₂ (15dPGJ₂), 0.75 μ M 22-(R)-hydroxycholesterol (22RHC), or 0.5 μ M 9-*cis*-retinoic acid (9cisR) or left untreated for 2 days followed by stimulation with 0.5 μ g/ml LPS to activate the macrophages. RNA was harvested at the times indicated and assayed as described in individual experiments.

Microarray Probing and Data Analysis—Total RNA extraction was performed using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's instructions. Poly(A)⁺ RNA was purified using Poly(A)Tract mRNA isolation systems (Promega, Madison, WI) and labeled with Cy3 and Cy5 fluorescent dyes for microarray hybridization on UniGEM-V2 and H-GEM1 (Incyte Genomics, Palo Alto, CA) as described elsewhere (19). A given gene was analyzed if at least one of its readings had a signal to background ratio of 2.5 or more, a signal intensity above 250 units for one or both dyes, and a spot size of at least 40% of the spotted area. These criteria assured that the signal level was sufficiently high above background to be reliably read and that the reading was not the result of nonuniform noise on spotted DNA sites. The identity of spotted DNA was verified by polymerase chain reaction, with a failure in polymerase chain reaction testing resulting in the exclusion of the spot measurement. Of the ~16,000 human cDNAs that were probed, 1190 genes showed 2-fold or greater differential expression at 1 and/or 6 h due to LPS treatment as compared with time 0. Of these 1190 genes, 127 genes were 4-fold or greater regulated and are described in this study. These 127 genes were clustered into four groups based on expression pattern, and each gene was highlighted if it was 2-fold or greater changed due to pretreatment with OxLDL. These latter values were measured in paired time point probings (*i.e.* +OxLDL, 1 h LPS/-OxLDL, 1 h LPS and +OxLDL, 6 h LPS/-OxLDL, 6 h LPS).

mRNA Quantitation—Real-time quantitative RT-PCR was performed on ABI Prism Sequence Detection System 5700 (PE Applied Biosystems, Foster City, CA). A set of primers was designed for each gene using Primer Express (PE Applied Biosystems). DNA sequence was obtained from GenBankTM, and amplicons of 100–200 base pairs with T_m between 68 °C and 85 °C were selected. RT-PCR reactions utilizing 0.1–1 μ g of DNase-treated total RNA were performed under conditions recommended by the manufacturer. A dissociation curve was generated at the end of the polymerase chain reaction cycle to verify

that a single product was amplified. A standard curve for each amplicon was obtained using serial dilutions of total RNA prepared from THP-1 cells. The results from triplicate (for \pm OxLDL, +LPS sample RNAs) or duplicate (\pm nuclear receptor ligands, +LPS sample RNAs) polymerase chain reactions for a given gene at each time point were used to determine mRNA quantity relative to the corresponding standard curve. The relative mRNA quantity for a given gene measured from a single reverse transcription reaction was divided by the value obtained for either glyceraldehyde-3-phosphate dehydrogenase or ribosomal protein S9 to correct for fluctuations in input RNA levels and varying efficiencies of reverse transcription reactions. The differential expression curves obtained using either of these genes as the correction factor consistently showed the same pattern.

Enzyme-linked Immunosorbent Assay Measurements—PMA-differentiated THP-1 cells were treated with or without OxLDL for 3 days as described above. The cells were then treated with LPS, and culture media were collected at 0, 3, 6, 9, and 18 h after the addition of LPS. Supernatants were then assayed by enzyme-linked immunosorbent assay for the presence of IL-1 β using reagents and protocols of the supplier (R&D Systems).

Electrophoretic Mobility Shift Analysis Measurements—Nuclear extract preparations, generation of probes, and DNA binding conditions for gel shift mobility assays followed standard protocols that have been described previously (20). The following oligonucleotide and complement were used to generate the NF κ B-specific probe: 5'-AGTTG-AGGGGAC'TTCCAGGC-3'.

RESULTS AND DISCUSSION

Microarray Study Design—PMA-differentiated THP-1 cells were treated with OxLDL for 3 days to lipid load the macrophages and establish a foam cell-like phenotype. Control cells were maintained in culture for the same length of time without OxLDL. The foam cell phenotype was determined by increased oil red-o staining and increased mRNA levels for CD36, adipophilin, PPAR γ , and other foam cell marker genes (9, 12, 13, 21). The OxLDL-pretreated and control cells were then activated by treatment with LPS, and RNA was harvested at 0, 1, and 6 h after LPS addition to generate six unique samples (Scheme 1). These six samples were then compared in two ways using DNA microarrays. LPS effects on transcription were determined in the non-OxLDL-treated cells by comparing 1 h and 6 h RNA samples to time 0 levels (data designated -Ox, 1hr LPS/-Ox, 0hr and -Ox, 6hr LPS/-Ox, 0hr; Figs. 1–4). LPS effects on transcription in the OxLDL-pretreated cells were similarly determined (data designated +Ox, 1hr LPS/+Ox, 0hr and +Ox, 6hr LPS/+Ox, 0hr; Figs. 1–4). These procedures identified the total number of LPS-regulated genes for these cells. LPS-responsive genes were tabulated (Figs. 1–4) if they exhibited a 4-fold or greater change in message levels at either 1 or 6 h after LPS treatment. This cutoff was chosen to select only those genes most affected by LPS treatment.

The effects of OxLDL pretreatment on this group of LPS-responsive genes was determined through an additional set of hybridization experiments. In these experiments, matched time point comparisons were made between RNA from -OxLDL- and +OxLDL-pretreated cells after LPS treatment to directly measure the effects of OxLDL on LPS-responsive genes (data designated +Ox, 1hr LPS/-Ox, 1hr LPS and +Ox, 6hr LPS/-Ox, 6hr LPS; Figs. 1–4). This second set of hybridizations to determine the effects of OxLDL on LPS-responsive genes was required because only comparisons between two RNA populations hybridized to a single chip can be made using Incyte microarrays. Thus, LPS responsiveness was determined through the first set of hybridizations, and OxLDL effects were determined through the second set of hybridizations. Inspection of the column data for LPS response in control and OxLDL-pretreated cells shows that interchip comparative differences (for example, -Ox, 1hr LPS/-Ox, 0hr versus +Ox, 1hr LPS/+Ox, 0hr values, Figs. 1–4) were often highly concordant with the values observed in the single chip, matched time point measurements.

SCHEME 1. Illustration of the experiment design used to generate the RNA samples used in the microarray study.

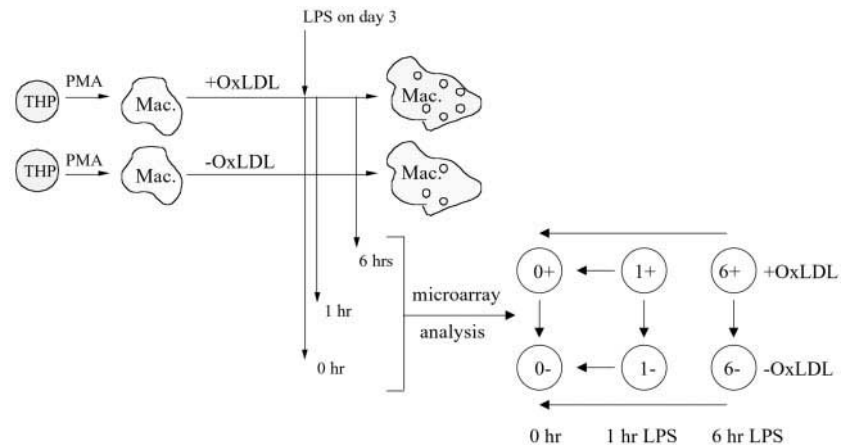
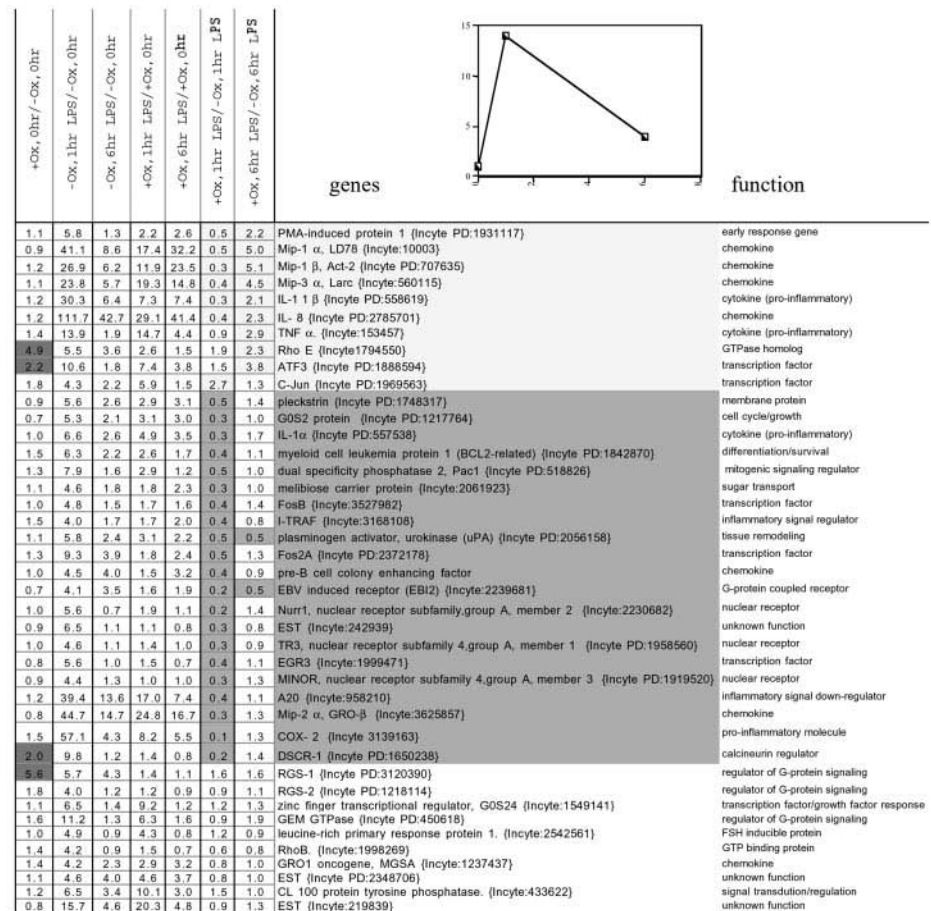


FIG. 1. Microarray data. Gene expression values for LPS-activated genes in the $-OxLDL$ - and $+OxLDL$ -treated THP-1 cells were organized into four groups based on the LPS-induced expression patterns observed in the control ($-OxLDL$) cells. Group 1 includes 41 LPS-activated genes that exhibit peak up-regulation at 1 h. LPS effects on transcription were determined in the $-OxLDL$ -treated cells by comparing 1 h and 6 h RNA samples with time 0 levels (data designated $-Ox, 1hr LPS/-Ox, 0hr$ and $-Ox, 6hr LPS/-Ox, 0hr$). LPS effects on transcription in $+OxLDL$ -treated cells were similarly determined (data designated $+Ox, 1hr LPS/+Ox, 0hr$ and $+Ox, 6hr LPS/+Ox, 0hr$). OxLDL effects on LPS-responsive genes were determined through matched time point comparisons of RNA from $-OxLDL$ - and $+OxLDL$ -treated cells after the addition of LPS (data designated $+Ox, 1hr LPS/-Ox, 1hr LPS$ and $+Ox, 6hr LPS/-Ox, 6hr LPS$). The matched time point values are highlighted in *light gray* if OxLDL pretreatment results in a 2-fold or greater up-regulation of the control LPS response and in *medium gray* if OxLDL pretreatment results in a 2-fold or greater down-regulation of the control LPS response. Genes modulated by OxLDL before LPS treatment have time 0 values ($+Ox, 0hr/-Ox, 0hr$) marked in *dark gray*. The graph at the top of the figure illustrates the general expression pattern of genes in this group for the control cells (x axis, hours after LPS treatment; y axis, fold induction).



A gene was considered to be affected by OxLDL pretreatment if there was a 2-fold or greater difference in the LPS response of the gene compared with the control matched time point value. A 2-fold cutoff has been shown to be reliable and reproducible using Incyte Genomics hybridization methodology (22). Employing the above criteria, 127 genes that were regulated 4-fold or greater by LPS were identified (108 were up-regulated, and 19 were down-regulated). Of these 127 genes, 56 genes were modulated by OxLDL pretreatment. The majority of these OxLDL effects were suppressive, resulting in diminished LPS-induced gene expression. However, several chemokine and pro-inflammatory cytokine genes were further up-regulated by the combination of OxLDL plus LPS treatment.

Overview of Gene Expression Patterns Observed for LPS-treated Control Cells—To study the effects of OxLDL pretreat-

ment on LPS-regulated gene expression, we first analyzed the expression patterns of LPS-responsive genes in cells not exposed to OxLDL. 1 h and 6 h LPS treatment times were chosen to observe both early and late transcription events. Four main gene expression patterns or groups were observed for these LPS-treated cells ($-Ox, 1hr LPS/-Ox, 0hr$ and $-Ox, 6hr LPS/-Ox, 0hr$ values). Group 1 (Fig. 1) includes 41 LPS-activated genes that exhibit peak up-regulation at 1 h. To meet the criteria for inclusion in group 1, a gene must be 4-fold or greater up-regulated at 1 h with diminished up-regulation at 6 h. Thus, genes in this group exhibit early but transient LPS-activated expression. Group 2 (Fig. 2) consists of 14 LPS-activated genes that begin up-regulation at 1 h but are further increased at 6 h. Genes in group 2 are at least 2-fold up-regulated at 1 h and 4-fold or greater up-regulated at 6 h. As

FIG. 2. Microarray data. Group 2 includes 14 LPS-activated genes that are at least 2-fold up-regulated at 1 h and 4-fold or greater up-regulated at 6 h. Definitions of column data headings, value highlighting, and illustrative graph are as stated in the legend to Fig. 1.

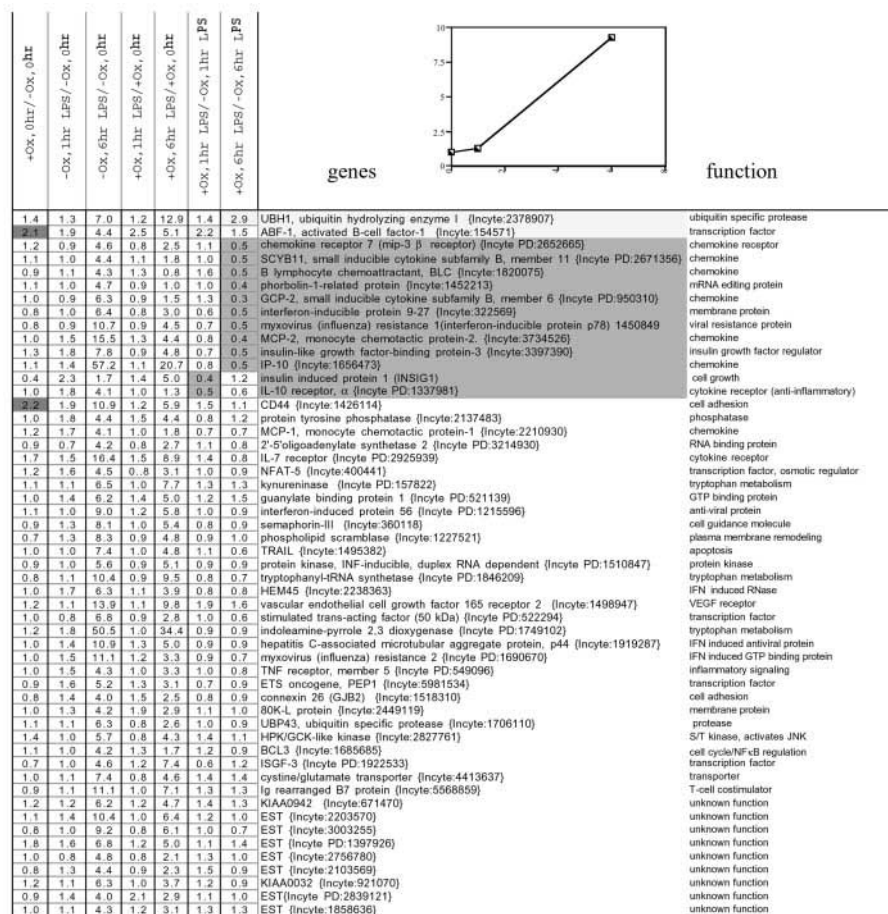
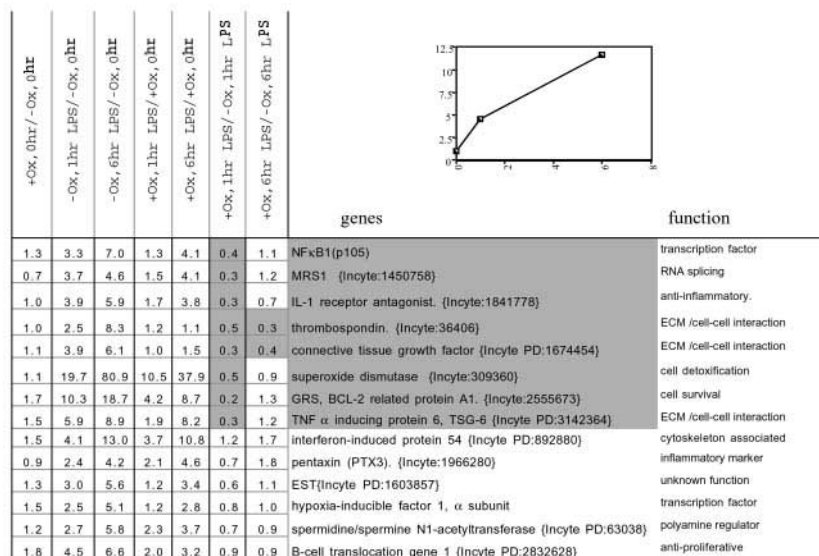


FIG. 3. Microarray data. Group 3 includes 53 genes that are less than 2-fold changed at 1 h and 4-fold or greater induced at 6 h. Definitions of column data headings, value highlighting, and illustrative graph are as stated in the legend to Fig. 1.

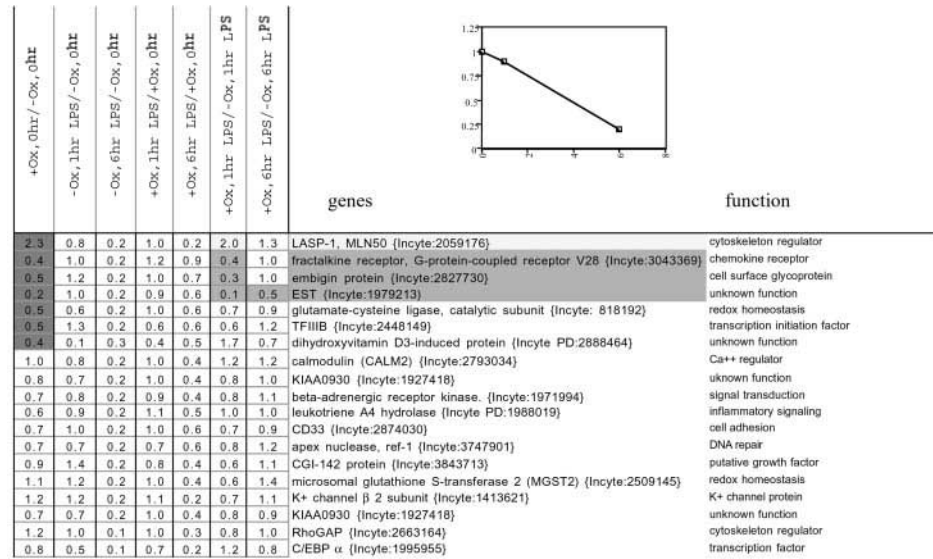
such, these genes exhibit both an early and increasing expression pattern over the 6-h time course of LPS treatment. In group 3 (Fig. 3), there are 53 genes that are up-regulated at 6 h but not at 1 h. Group 3 genes are less than 2-fold changed at 1 h and 4-fold or greater induced at 6 h. Thus, genes in this group are late up-regulated genes. Finally, in group 4 (Fig. 4), there are 19 genes that are 4-fold or greater down-regulated at 1 and/or 6 h. The OxLDL effects on genes contained within these individual expression groups are described below.

OxLDL Effects on LPS-responsive Genes in Groups 1–4—The effect of OxLDL treatment on LPS-regulated gene expression

was measured by comparing OxLDL-treated samples with untreated samples at 1 and 6 h. 1 h and 6 h matched pair values (*i.e.* +Ox, 1hr LPS / -Ox, 1hr LPS and +Ox, 6hr LPS / -Ox, 6hr LPS, Figs. 1–4) are highlighted in *light gray* if OxLDL pretreatment results in a 2-fold or greater up-regulation of the control LPS response. The matched paired values are highlighted in *medium gray* if OxLDL pretreatment results in a 2-fold or greater down-regulation of the control LPS response. Genes modulated by OxLDL before LPS treatment have time 0 values (+Ox, 0hr / -Ox, 0hr) marked in *dark gray*.

As the highlighted values in Group1(Fig. 1) show, 31 of the

FIG. 4. **Microarray data.** Group 4 includes 19 genes that are 4-fold or greater down-regulated at either 1 or 6 h. Definitions of column data headings, highlighting of values, and illustrative graph are as stated in the legend to Fig. 1.



41 LPS-up-regulated genes in this group are modulated by OxLDL. 9 of these genes are further up-regulated at 6 h (*light gray*), including the pro-inflammatory cytokine TNF- α . 6 of these 9 genes exhibit diminished up-regulation at the 1 h time point, suggesting a possible time delay in the induced expression of these genes compared with that of the controls. Included among these are 4 chemokines, Mip-1 α , Mip-1 β , Mip-3 α , and IL-8, which signal for the recruitment of lymphocytes and monocytes (23–25). Also included are the pro-inflammatory cytokine IL-1 β and one gene of unknown function, PMA-induced protein 1. The AP1 component Jun is further up-regulated at 1 h. AP1 is known to be activated by LPS and, like NF κ B, plays an important role in the activation of pro-inflammatory genes. The transcription factor activating transcription factor 3 and the cytoskeleton and cell migration regulator RhoE are up-regulated by OxLDL before the addition of LPS (*dark gray*), hence the message level of these genes is already elevated before further LPS induction. In the experiments discussed below, the expression pattern of several chemokine and pro-inflammatory cytokine genes was studied over a longer time course using RT-PCR. We also investigated the involvement of the nuclear receptors PPAR γ , LXR, and RXR in mediating the effects of OxLDL on these LPS-activated genes.

The remaining OxLDL-modulated genes in group 1 show diminished LPS up-regulation (*medium gray*). Included among these 20 genes are several pro-inflammatory mediators, including IL-1 α , Mip-2 α , pre-B-cell colony-enhancing factor, and cyclooxygenase 2, as well as the NF κ B signaling regulators I-Traf and A20. The repression of LPS-induced cyclooxygenase 2 expression is particularly strong, consistent with a previous study (26). Other genes with diminished up-regulation include the cell cycle-implicated genes GOS2, Pac1, and MCLS1 and three related orphan nuclear receptors, Nurr1, TR3, and MINOR. Originally studied in development, Nurr1 has recently been implicated in inflammation (27), and TR3 has been shown to play a novel role in triggering apoptosis (28). As the array data show, OxLDL strongly represses the LPS induction of these orphan nuclear receptors (confirmed by RT-PCR; data not shown). Two molecules, DSCR1 and EGR3, which modulate or are modulated by the calcineurin-NFAT pathway, also exhibit strongly repressed LPS induction. NFAT proteins play a critical role in T-cell activation, thus this pathway may have a role in macrophage inflammatory response. Interestingly, DSCR1 is both an OxLDL- and LPS-up-regulated gene. However, pretreatment with OxLDL precludes further induction by

LPS (confirmed by RT-PCR; data not shown). Two molecules that encode AP1 components, FosB, and Fos2A, also exhibit strongly repressed LPS induction, as do urokinase-type plasminogen activator and the G-protein-coupled receptor EB12. The reduction in FosB and Fos2A contrast with the OxLDL-mediated increased induction in c-Jun levels, which is also an AP1 component. Interestingly, RGS-1, like DSCR1, is both an OxLDL-induced and LPS-induced gene, but pretreatment with OxLDL precludes further induction by LPS (confirmed by RT-PCR; data not shown). RGS proteins have been shown to modulate chemotaxis through G-protein-coupled chemokine receptors (29, 30). The lack of LPS modulation of RGS-1 expression levels in the OxLDL-pretreated macrophages may impact control of chemotaxis in these cells. The nine LPS-induced genes in group 1 that show no OxLDL modulation are a mix of regulatory proteins (several of which are GTP-binding proteins), a chemokine, and genes of unknown function. Thus, in group 1, the majority of LPS-induced genes were altered in expression due to OxLDL pretreatment, including six of seven chemokines, eight of nine transcription factors, and three of three cytokines.

In group 2 (Fig. 2), none of the LPS-activated genes are further up-regulated by OxLDL pretreatment. Rather, 8 of the 14 LPS-up-regulated genes exhibit diminished up-regulation (*medium gray*). At 1 h, there are six genes showing diminished up-regulation. These include p105, the precursor of the p50 subunit of NF κ B, and the free radical-scavenging superoxide dismutase. NF κ B is a master regulator of inflammatory programs, and a reduction in this activity could help explain the general suppression of LPS-induced gene responses from OxLDL pretreatment. A functional drop in inflammatory activity would reduce the number of intracellular free radicals generated, thus reducing the need for high levels of superoxide dismutase. Other genes showing diminished up-regulation are the putative cell proliferation gene MRS1, the antiapoptotic gene GRS, and TNF- α -induced protein 6, which is implicated in adhesion and inflammation. Two other genes, connective tissue growth factor and the cell adhesion protein thrombospondin, are significantly reduced at both 1 and 6 h. LPS-activated genes in this group that show no OxLDL modulation are a mix of inflammatory markers and mediators and several regulatory proteins.

In Group 3 (Fig. 3), only 14 of the 53 LPS-up-regulated genes experience OxLDL modulation. Thus, for this group of late LPS-up-regulated genes, the effects of OxLDL pretreatment

appear to affect only a minority of genes. UBH1, a deubiquitinating enzyme, is further up-regulated at 6 h. Increased activity of this protein could affect the degradation rates of numerous proteins. The transcription factor ABF-1 is up-regulated by OxLDL before the addition of LPS. To date, this transcription factor has only been implicated in B-cell development. Of the 12 genes with diminished up-regulation (*medium gray*), 10 are affected at 6 h. Among these are five chemokine genes (SCYB11, BLC, IP-10, GCP-2, and MCP-2) coding for proteins chemotactic for B cells, granulocytes, activated T cells, and various other leukocytes (23–25). Thus, these later transcribed chemokines have suppressed LPS induction, whereas several early transcribed chemokines in group 1 are further up-regulated by the combination of OxLDL and LPS treatment. Dysregulation of chemokine expression and secretion would likely have a significant impact on macrophage function within the atherosclerotic lesion because such signaling determines the extent of further leukocyte infiltration and the severity of local tissue damage. The expression and regulation of these chemokine genes are investigated further below.

Other genes in group 3 that exhibit diminished LPS induction with OxLDL pretreatment include the IL-10 and CCR7 receptors. IL-10 functions to inhibit activation of macrophages (31) and ligands for CCR7 influence macrophage trafficking during inflammatory conditions (32). Loss of LPS inducibility for the IL-10 receptor is pronounced in the OxLDL-pretreated cells (confirmed by RT-PCR; data not shown). The effects of reduced sensitivity to IL-10 would likely result in a more sustained or chronic activation after inflammatory stimulus. Other genes that exhibit diminished LPS induction with OxLDL pretreatment include five genes known to be regulated by insulin or the interferons. The 39 remaining members in this group of late LPS-induced genes are unaffected by pretreatment with OxLDL. They include a mix of inflammatory markers or mediators, regulatory proteins, and transcription factors. Thus, in group 3, whereas the effects of OxLDL pretreatment affect only a minority of these late LPS-induced genes, the diminished up-regulation of five of six chemokines and the reduced expression of the IL-10 receptor could significantly impact macrophage inflammatory potential.

In group 4 (Fig. 4), there are 19 genes that are down-regulated by LPS at 1 or 6 h. In general, genes that are down-regulated by LPS account for only a small minority of LPS-responsive genes observed in this study (~15%). In addition, with the exception of genes involved in redox homeostasis, no clear functional groupings of genes occur within group 4 that might give mechanistic insights into the role that such LPS down-regulated genes play in the inflammatory response. Furthermore, this group exhibits only marginal OxLDL effects on LPS response. Three of the genes (V28 chemokine receptor, the cell surface glycoprotein Embigin, and an expressed sequence tag) have increased down-regulation at one or more time points in the OxLDL-pretreated cells.

It is worth noting that only a minority of the OxLDL-responsive genes listed in groups 1–4 are regulated by OxLDL before LPS treatment (13 genes of a total of 57 genes; see +Ox, 0hr / -Ox, 0hr values). Thus, until the majority of these genes are activated or repressed by an inflammatory stimulus like LPS, the fact that they are OxLDL-modulated is not apparent. In carrying out these experiments, we suspected that macrophage-derived foam cells might exhibit dysregulation of inducible gene expression programs. As our data indicate, OxLDL loading significantly impacts inflammatory gene expression patterns in the LPS-activated macrophage.

Extended Time Course Analysis of Select LPS-inducible Genes Affected by OxLDL Pretreatment—To further explore the

effects of OxLDL loading on the LPS response of THP-1 cells, we repeated the cell culture experiments as described previously. However, RNA was isolated at 0, 1, 3, 6, and 9 h after LPS treatment to establish a longer and denser time course of gene expression. These RNA samples were used in real-time quantitative RT-PCR experiments to both verify and extend observations made from the microarray data. We particularly wanted to distinguish genes that exhibited real and continuous increases in expression from those that were merely shifted in time.

As described previously, four LPS-activated chemokine genes (Mip-3 α , IL-8, Mip-1 α , and Mip-1 β) and two pro-inflammatory cytokines (IL-1 β and TNF- α) listed in Fig. 1 are further up-regulated in cells pretreated with OxLDL. The RT-PCR data in Fig. 5a shows that OxLDL induces significant increases in the LPS activation of Mip-3 α , IL-8, Mip-1 α , IL-1 β , and TNF- α . In almost all cases, these message levels remain elevated above the control values during the time course of observation. The changes in this group of cytokine and chemokine genes are particularly striking because the majority of LPS-induced genes in THP-1 macrophages are either repressed or unaffected by OxLDL pretreatment. The further up-regulation of these genes, if they reflect *in vivo* results, could result in increased recruitment and activation of macrophages and lymphocytes, an outcome consistent with *in vivo* observations of atherosclerotic lesions (1, 5, 6). RT-PCR values for MIP-1 β did not correspond to the microarray values. These differences are likely to reflect errors in the microarray measurements, potentially due to cross-hybridization or local defects in the array.

Five late LPS-activated chemokine genes (SCYB11, BLC, IP-10, GCP2, and MCP-2) listed in Fig. 3 show diminished up-regulation in cells pretreated with OxLDL. The RT-PCR data in Fig. 5b corroborate the microarray data for all these genes. However, the OxLDL effects on the LPS induction of BLC, SCYB11, IP-10, and MCP-2 are more extreme when measured by RT-PCR. There appears to be almost total suppression of the LPS induction of these genes in the OxLDL-pretreated cells. These results contrast significantly with those for the LPS-induced chemokines shown in Fig. 5a. Yet, taken together, these findings provide strong evidence that OxLDL loading in THP-1 macrophages introduces a significant imbalance in chemokine transcription under inflammatory conditions. An additional observation for these chemokine genes concerns the neutrophil attractant and activator GCP-2. The extension of the time course measurements reveals that whereas GCP-2 is repressed by OxLDL for the first 6 h of LPS induction, consistent with the array data, at 9 h its message level begins to increase strongly, whereas the control value decreases from its peak. A longer time course will be required to determine whether this change in GCP-2 expression represents a time-shifted expression pattern or a late but continuous increase in GCP-2 expression levels due to OxLDL pretreatment. Given the low level of neutrophils present in atherosclerotic lesions (2), it is unclear what effects changes in GCP-2 levels might have on lesion progression.

Regulatory Connections: NF κ B Signaling—The data in the control (non-OxLDL-treated) arm of this study shows that LPS induces significant changes in macrophage gene expression patterns, consistent with previous studies of LPS-treated monocyte/macrophages (33, 34). Upon LPS treatment, members of many protein classes are quickly up-regulated. These include chemokines, cytokines and associated receptors, adhesion molecules, transcription factors and other cell signaling regulators, and cell cycle or survival factors. The majority of gene expression changes appear to be up-regulation events, in seeming accordance with the transition of an “unstimulated”

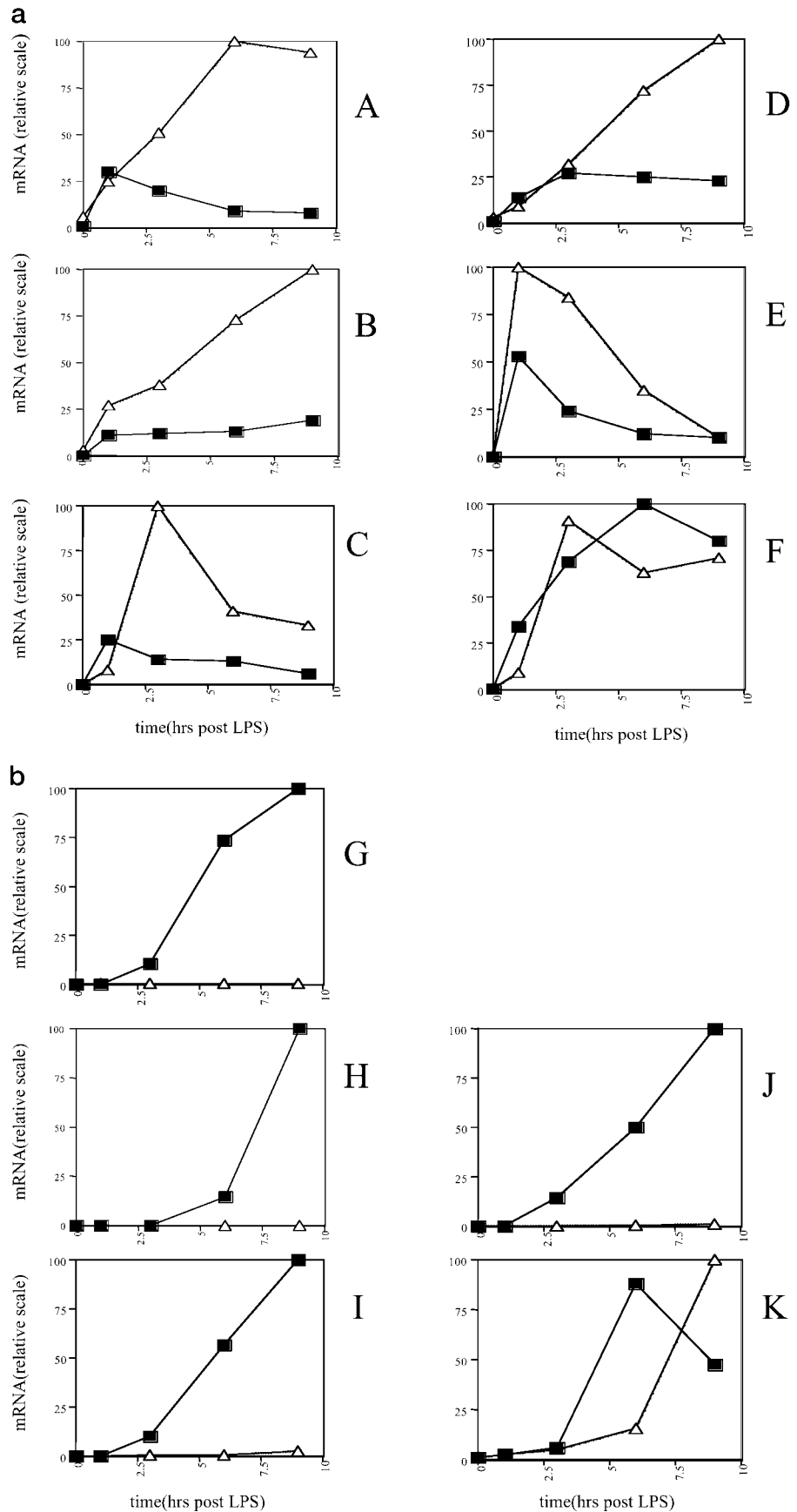


FIG. 5. *a* and *b*, RT-PCR data for selected chemokine and cytokine genes identified from the microarray results. RNA samples were collected at 0, 1, 3, 6, and 9 h after LPS treatment of \pm OxLDL-treated THP-1 cells. RNA levels for specific genes were then measured by quantitative RT-PCR. Genes in *a* are (A) Mip-3 α , (B) IL-8, (C) Mip-1 α , (D) IL-1 β , (E) TNF- α , and (F) Mip-1 β . Genes in *b* are (G) SCYB11, (H) BLC, (I) MCP-2, (J) IP-10, and (K) GCP-2. Δ , +OxLDL, +LPS; \blacksquare , -OxLDL, +LPS. The RT-PCR data were graphed by normalizing to the maximum LPS-induced expression value for each gene (on a scale of 1–100). The results shown in the figure are representative of this type of experiment. Cell culture, RNA sample preparation, cDNA synthesis, and RT-PCR conditions were as described under “Materials and Methods.”

macrophage to a “stimulated” or more active state.

NF κ B is an important transcription factor in LPS and inflammatory signaling (35, 36). The promoters of numerous

pro-inflammatory genes have been shown to contain NF κ B sites, and mice engineered to be deficient at different points in the NF κ B signaling pathway have deficient inflammatory re-

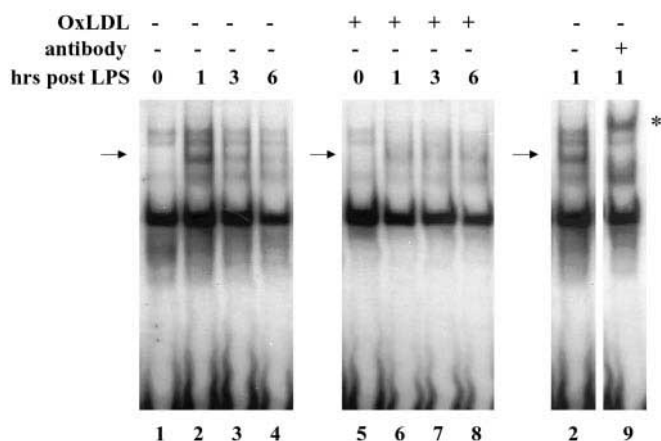


FIG. 6. Reduction in LPS-induced NF κ B DNA binding in cells pretreated with OxLDL. Nuclear extracts were prepared from THP-1 macrophages after LPS treatment at the times shown above each lane. The cells were first pretreated with OxLDL for 3 days (lanes 5–8) or left untreated (lanes 1–4 and 9). The arrow indicates the position of the NF κ B band. Extracts used in lanes 2 and 9 were identical, except that lane 9 includes NF κ B antibody. Right, lane 2 is shown again next to lane 9 to clearly indicate the supershifted NF κ B band (indicated by the asterisk). The results shown in the figure were observed in several repeats of the experiment. Preparation of nuclear extracts, DNA probes, and electrophoretic mobility shift analysis conditions were as described under “Materials and Methods.”

sponses (37–39). Previous studies have investigated the effects of OxLDL on NF κ B activation and found different outcomes depending on the cell types used. In smooth muscle and endothelial cells, reports have shown that OxLDL treatment activates NF κ B, often leading to increased apoptosis (40, 41). A study in LPS-treated monocyte/macrophages showed that OxLDL pretreatment down-regulates the early stages of NF κ B activation but possibly lengthens the time to full inactivation (42). We investigated the nuclear NF κ B signal in THP-1 macrophages pretreated with OxLDL for 3 days, followed by 0, 1, 3, and 6 h of LPS stimulation. We found that OxLDL significantly reduced the signal at 1 h compared with non-OxLDL-treated cells (Fig. 6), but the signal at later time points did not differ significantly between OxLDL-treated and untreated cells. In addition, the array data show that the LPS-induced increase of p105 (precursor to the p50 component of NF κ B) and I-Traf (an adaptor protein involved in NF κ B signaling) is diminished by OxLDL (Fig. 2). OxLDL diminishes the gene expression of six of nine transcription factors in group 1 (Fig. 1), whereas it has less effect on the expression of transcription factors up-regulated by LPS at a later time (group 3 genes). The reduction in the early stage of LPS-induced NF κ B activation, as well as the reduction in message levels of this and other transcription factors, may be responsible for many of the LPS-induced gene expression changes observed in the OxLDL-pretreated cells, particularly the reductions observed at 1 h after LPS treatment.

The majority of LPS-induced genes that are further up-regulated by OxLDL loading are observed at 6 h after LPS treatment. At this time, differences in NF κ B activation between control and OxLDL-pretreated cells appear negligible (Fig. 6). Whereas reductions in p105 and I-Traf message levels may conspire to lower NF κ B activity, increases in IL-1 β message and IL-1 β secretion, as measured by enzyme-linked immunosorbent assay (Fig. 7), may offset these effects to keep NF κ B activity levels near normal in the later stages of LPS activation. In addition, A20, a gene that encodes a powerful down-regulator of NF κ B-activated inflammatory responses (43), has message levels that are reduced in the OxLDL-pretreated cells. A reduction in A20 levels may also maintain

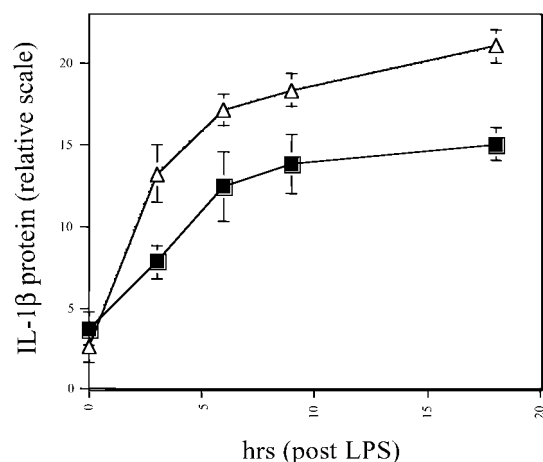


FIG. 7. Secreted levels of IL-1 β are increased in LPS-treated THP-1 cells pretreated with OxLDL. THP-1 cells were treated with OxLDL for 3 days as described previously. Culture media were harvested at the indicated times (in hours) after LPS treatment and assayed for IL-1 β levels by enzyme-linked immunosorbent assay using methods of the supplier (R&D Systems). Δ , +OxLDL, +LPS; \blacksquare , -OxLDL, +LPS. IL-1 β levels are expressed as the means \pm S.E. ($n = 3$) on a relative scale.

NF κ B activation levels near or even higher than normal at later time points. A recent study in endothelial cells shows that OxLDL treatment reduced A20 message and protein levels and that this correlated with persistent NF κ B activation in these cells (40).

Nuclear Receptor Involvement in OxLDL Modulation of LPS-responsive Genes—The mRNA levels of the nuclear receptors LXR α , RXR γ , and PPAR γ have been shown to be up-regulated by OxLDL treatment in THP-1 macrophages (9, 44). In addition, studies of OxLDL components have identified natural ligands for these receptors, and these receptors have been shown to be activated by OxLDL treatment as well (9, 45, 46). These receptors have been shown to regulate several macrophage genes involved in lipid metabolism, including apo E (47), CD36 (48), ABCA1 (49–51), and ABCG1 (52). PPAR γ and RXR have been shown to modulate inflammatory gene expression in both cell and animal models (53–58). In addition, PPAR γ and RXR have been shown to antagonize NF κ B activity, possibly through direct physical interaction (53, 58, 59). We therefore asked whether ligand activation of PPAR γ , RXR, and LXR, followed by LPS treatment, could recapitulate the effect of OxLDL on gene expression in LPS-treated THP-1 macrophages.

PMA-differentiated THP-1 cells were treated with either 15dPGJ2, 22RHC, or 9cisR for 2 days. These ligands have been shown to be selective binders of PPAR γ , LXR and RXR, respectively (21, 60, 61). Ligand treatment was then followed by stimulation with LPS. RNA samples were collected at 0, 1, 3, 6, and 9 h after LPS treatment and examined by real-time quantitative RT-PCR. Fig. 8a shows the effect of nuclear receptor ligand pretreatment on the LPS-induced chemokine and cytokine genes that were further up-regulated by OxLDL and LPS treatment (seen in Fig. 5a). Pretreatment with the PPAR γ ligand (15dPGJ2) further up-regulated the expression of both IL-8 and Mip-1 α at 6 and 9 h after the addition of LPS, whereas pretreatment with the RXR ligand (9cisR) further up-regulated Mip-3 α at all time points after the addition of LPS. Thus, for IL-8 and Mip-1 α , pretreatment of cells with 15dPGJ2 before LPS treatment recapitulates the results observed for OxLDL pretreatment. For Mip-3 α , 9cisR pretreatment recapitulates the OxLDL effects on this LPS-activated gene. The LXR ligand (22RHC) appears to have no effect on the LPS activation of these chemokine and cytokine genes, even though this concen-

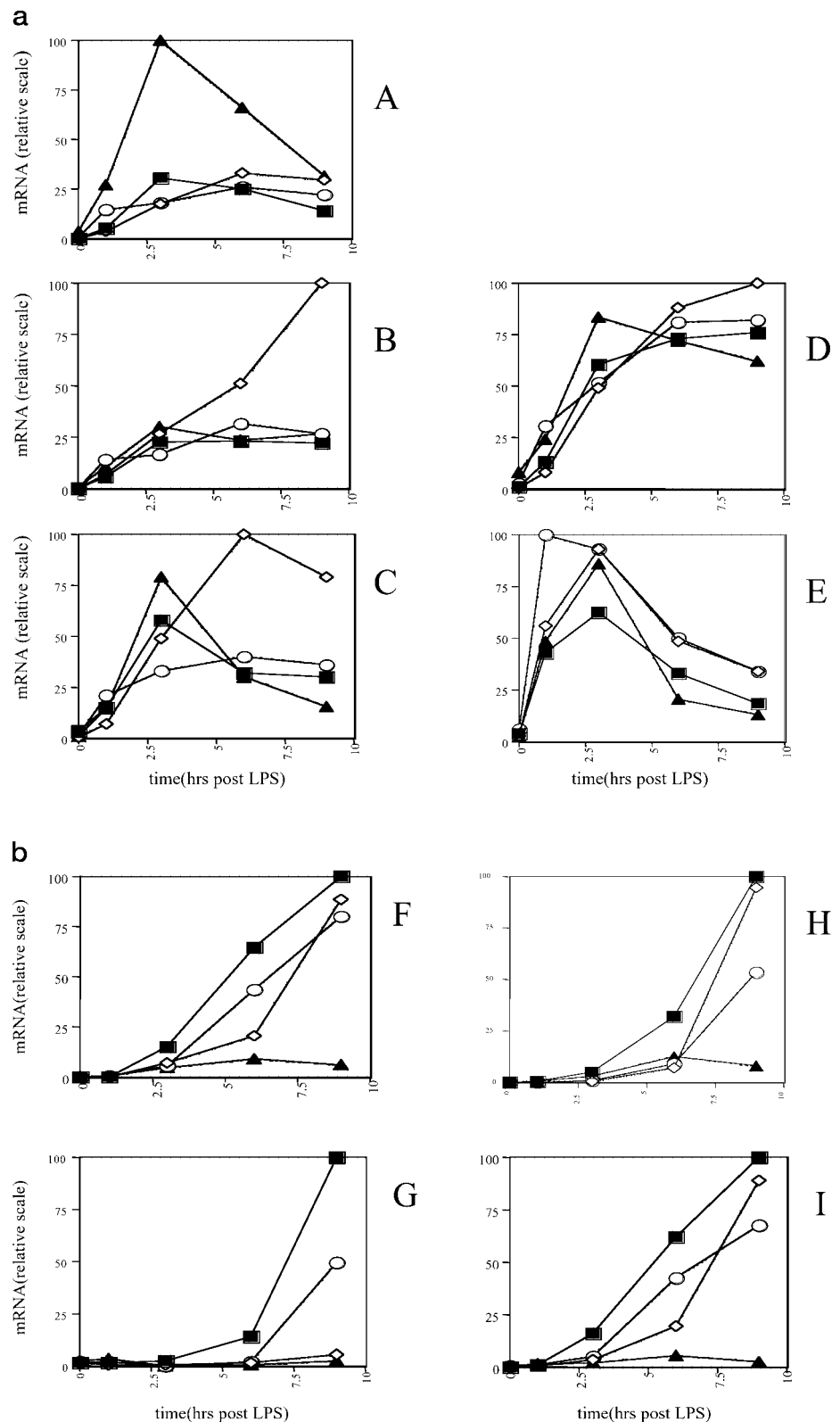


FIG. 8. *a* and *b*, pretreatment of THP-1 cells with specific nuclear receptor ligands before LPS treatment recapitulates OxLDL effects on LPS induction for several chemokine genes. PMA-differentiated THP-1 cells were treated with either 0.25 μ M 9cisR, 3 μ M 15dPGJ2, or 0.75 μ M 22 RHC or left untreated (control) for 2 days and then stimulated with LPS. RNA samples were collected at 0, 1, 3, 6, and 9 h after LPS treatment. mRNA levels for indicated chemokine genes were then measured by quantitative RT-PCR. Genes in *a* are (A) Mip-3 α , (B) IL-8, (C) Mip-1 α , (D) IL-1 β , and (E) TNF- α . Genes in *b* are (F) SCYB11, (G) BLC, (H) MCP-2, and (I) IP-10. ▲, +9cisR, +LPS; ◇, +15dPGJ2, +LPS; ○, +22RHC, +LPS; ■, no ligand, +LPS. The RT-PCR data were normalized and graphed as stated before. The results shown in this figure are representative of this type of experiment. Cell culture, RNA sample preparation, cDNA synthesis, and RT-PCR conditions were as described under "Materials and Methods."

tration of 22RHC was sufficient to induce the expression of the LXR target genes ABCA1 and ABCG1 severalfold (RT-PCR data not shown).

Fig. 8*b* shows the nuclear receptor ligand pretreatment effects for chemokine genes that exhibited suppressed LPS induction with OxLDL pretreatment (seen in Fig. 5*b*). As this figure shows, pretreatment with the PPAR γ ligand results in strong suppression of the LPS induction of the chemokine BLC.

Pretreatment with the RXR ligand results in strong suppression of the LPS induction of the chemokines SCYB11, BLC, IP-10, and MCP-2. For BLC, pretreatment with either 15dPGJ2 or 9cisR before LPS treatment recapitulates the OxLDL effects on this LPS-activated gene. For SCYB11, IP-10, and MCP-2, 9cisR pretreatment recapitulates the OxLDL effects on these LPS-activated genes. 22RHC has only weak effects on the early stages of SCYB11, BLC, and MCP-2 induc-

tion by LPS. These results suggest that in THP-1 macrophages, OxLDL-induced changes in LPS-activated chemokine expression occur through the involvement of the nuclear receptors RXR and PPAR γ .

Nuclear receptor involvement in modulating chemokine expression has previously been observed in several cell types. In endothelial cells, PPAR α ligands have been shown to up-regulate IL-8 and MCP-1 (62). In hepatic stellate cells, PPAR γ agonists have been shown to inhibit MCP-1 expression (63). In addition, PPAR γ agonists have been shown to inhibit interferon- γ -induced expression of IP-10 in endothelial cells (64). Our results further these observations by identifying several macrophage chemokines whose activation under inflammatory conditions appears to be modulated by the nuclear receptors RXR and PPAR γ . Given the known activation of these nuclear receptors by ligands present in OxLDL (9, 45, 46, 59), our results suggest that changes in LPS-induced chemokine expression in OxLDL-pretreated THP-1 macrophages may occur through activation of RXR and PPAR γ . To clearly show that OxLDL modulates the LPS induction of these chemokine genes through the activity of specific nuclear receptors, experiments involving cell lines deficient in the receptors will be required. In addition, more specific ligands will be needed because recent work has indicated that 15dPGJ2 exerts effects not limited to PPAR γ activity (48). Ligands with greater specificity for PPAR γ , LXR, and RXR have been developed or are currently being developed (49).

Conclusions—The results of this *in vitro* study with THP-1 macrophages show that significant changes in LPS-activated gene expression patterns occur if the cells are first pretreated with OxLDL. Included among these changes are genes that code for potent intracellular and extracellular signaling molecules such as NF κ B, A20, and numerous cytokines and chemokines. Changes in cytokine and chemokine gene expression, if found to correlate with secreted protein levels *in vivo*, would likely have profound effects on the recruitment and behavior of macrophages and lymphocytes within atherosclerotic lesions. Several studies have identified specific chemokine and cytokine increases at lesion sites, lending support to the observations made here in our *in vitro* study (1, 65). In addition, our data suggest that OxLDL modulation of macrophage inflammatory signaling is partially mediated through altered regulation of NF κ B signaling and activation of the nuclear receptors RXR and PPAR γ .

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