Oxidized Low Density Lipoprotein Blocks Lipopolysaccharide-induced Interferon β Synthesis in Human Macrophages by Interfering with IRF3 Activation*

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In response to lipopolysaccharide (LPS) exposure, macrophages activate the transcription of a large number of pro-inflammatory genes by way of signaling pathways downstream of the LPS receptor, Toll-Like Receptor 4. Many of these genes are expressed sequentially in time, with early synthesis events resulting in the secretion of soluble factors that drive the transcription of genes expressed later in the activation cycle. In this study we show that human blood-derived macrophages pretreated with oxidized low density lipoprotein (OxLDL) fail to transcribe and secrete interferon beta (IFNβ) immediately following LPS stimulation. As such, the normal downstream activation of Stat1 is blocked, and numerous IFNβ/Stat1-activated genes, including the chemokines IP10 and ITAC, are weakly expressed or not expressed at all in these cells. Inspection of the LPS-induced activation state of several transcription factors known to play a prominent role in IFNβ transcription reveals that, although NFκB, c-Jun, and ATF-2 activation appears normal, the LPS-induced activation of IFNβ regulatory factor 3 (IRF3), as measured by DNA-binding activity and association with the coactivator CBP, is inhibited in the OxLDL pre-treated cells. These IRF3 activities have been shown to be essential for the initiation of transcription of the IFNβ gene, and the loss of these activities presumably accounts for the lack of LPS-induced IFN β transcription seen in the OxLDL pre-treated cells.

Numerous studies support the concept that the oxidation of LDL is a pro-atherogenic process that contributes to a succession of localized arterial wall changes involving several cell types (1, 2). Oxidized LDL (OxLDL)¹ has been shown to compromise endothelial cell function by triggering the secretion of chemokines and increasing the expression of leukocyte adhesion proteins (3). This facilitates the entry of monocytes, and later, other leukocytes into the arterial wall (1, 4). Soon after entry, monocytes differentiate into macrophages, which scavenge the OxLDL that has accumulated in the sub-endothelial spaces of the affected artery. As professional scavenger cells, macrophages internalize OxLDL along with resident cell debris. However, the process of scavenging OxLDL, which harbors numerous biologically active molecules, initiates many changes in cell signaling, gene expression, cellular appearance, and function (1, 5–7). These changes are believed to differ from macrophase responses to scavenging of normal cell debris (1, 5, 8). Indeed, because the appearance of OxLDL-loaded macrophages is so altered, they are often referred to as foam cells (6).

Macrophage foam cells are not the only leukocyte present in atherosclerotic lesions, but from the earliest “fatty streak” to the late stage lesion, they are often the most abundant (1, 3). In addition, numerous animal studies have shown that specific gene expression changes associated with the foam cell phenotype are pro-atherogenic. Among these changes are the increased expression of the scavenger receptor CD36 (9, 10), the lipid-binding protein FABP4 (11), and the chemokine IL8 (12, 13). Because macrophages are inflammatory cells, as well as scavenger cells, it is believed that they contribute in important ways to vascular inflammation levels, a critical determinant of atherosclerotic lesion instability (3, 4, 14).

Activated macrophages are a major source of the soluble factors that coordinate the infiltration and local inflammatory response of numerous leukocytes in various tissue settings (15). Dysregulation or imbalances in these responses can lead to unresolved or chronic inflammatory states (16). Indeed, late stage atherosclerotic lesions often have features associated with sites of chronic inflammation, such as enrichment of inflammatory cells, persistent cell damage, local tissue remodeling, and fibrosis (1, 3). Furthermore, increases in circulating inflammatory factors like C-reactive protein and IL6 have been shown to be independent predictors of future cardiovascular disease (13,17). Other studies have implicated a role for infectious agents, like Chlamydia pneumoniae, in the pathogenesis of atherosclerosis (14, 18, 19). In addition, several receptors of the innate immune system are overexpressed in human lesions (20). A polymorphism in one of these receptors, the LPS receptor, Toll-like Receptor 4 (TLR4), has been shown to be associated with a reduced risk of atherosclerosis in humans (21), presumably because of the reduced inflammatory signaling associated with this receptor variant. Consequently, recent studies have begun to focus on ways in which the inflammatory response of arterial macrophages and macrophage foam cells may exacerbate the development of vascular disease and thus represent an area for therapeutic intervention (12, 22).

In an earlier report, we described the results of a DNA microarray study that characterized the altered gene expression responses of OxLDL pre-treated THP1 macrophages to LPS stimulation (23). This study revealed numerous early and

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1 The abbreviations used are: OxLDL, oxidized low density lipoprotein; ATF, activating transcription factor; CBP, CREM-responsive element-binding protein; EMSA, electrophoretic mobility shift assay; IFN, interferon; IRF, IFN regulatory factor; LPS, lipopolysaccharide; RT, reverse transcriptase; Stat, signal transducer and activator of transcription; IL, interleukin; TLR4, Toll-Like Receptor 4; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; JNK, c-Jun NH₂-terminal kinase; RANTES, regulated on activation normal T cell expressed and secreted.

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late changes in the LPS-induced inflammatory cascade, often involving inappropriate cytokine and chemokine transcription levels. In particular, a group of late expressed genes, which included the chemokines IP10 and ITAC, were no longer seen to be induced by LPS treatment in these OxLDL pre-treated cells. In the current study we show that human blood-derived macrophages pre-treated with OxLDL fail to transcribe and secrete interferon β (IFNβ) immediately following LPS stimulation. As such, the downstream activation of Stat1 is blocked, and as such IFNs/stat1-activated genes, like IP10 and ITAC, are weakly expressed or not expressed at all in these cells. OxLDL inhibits IFNβ transcription by apparently blocking the activation of the transcription factor IRF3, a step that has previously been shown to be essential for the initiation of transcription from the IFNβ promoter (24, 25).

MATERIALS AND METHODS

Cell Culture—Human monocyte-derived macrophages were isolated as described previously (26). Briefly,uffy coats from individual blood donors were adjusted to 75 ml with PBS containing 1 mM disodium EDTA and layered onto Ficoll-Paque (Amersham Biosciences). Following centrifugation at room temperature for 30 min at 400 g x g the layer of mononuclear cells was removed, diluted with three volumes of PBS/EDTA, and centrifuged at 800 g for 10 min at 4 °C. The cells were then washed three times with PBS/EDTA by centrifugation at 4 °C, and once with RPMI 1640 medium (Cellgro, Mediatech) without serum. The cells were then resuspended in RPMI 1640 medium and allowed to differentiate as described above. The cells were then treated with 0.5 g/ml human oxidized LDL (prepared by CuSO4 oxidation of LDL; Intracel Corp., Rockville, MD), and the cells were allowed to grow for 3 more days. The cells were then treated with 0.5 g/ml LPS to activate the macrophages. In some experiments, 3000 units/ml human IFNα (BIOSOURCE International; units are those of supplier) was used to treat the macrophages. OxLDL inhibits IFNα transcription by apparently blocking the activation of the transcription factor IRF3, a step that has previously been shown to be essential for the initiation of transcription from the IFNα promoter (24, 25).

RESULTS

Human blood-derived macrophages were isolated and allowed to differentiate in culture for 12 days, following standard procedures for the establishment of blood-derived macrophage cultures (26). On day 12, the cells were treated with OxLDL for an additional 3 days, to lipid load the cells and establish a foam cell-like phenotype based on increased levels of oil red-o staining of neutral lipids, and the increased expression of several marker genes, including ABCA1, ABCG1, Adipophilin, and FABP4 (as determined by RT-PCR, data not shown). Control cells were maintained in culture for the same length of time with no OxLDL treatment. The cells were then treated with LPS, and supernatants and RNA were collected at various times. Fig. 1 shows the results of ELISA measurements that were made to determine the amount of IP10 and ITAC being secreted into the media by these cells. As the data in this figure show, the LPS-inducible secretion of both IP10 (Fig. 1A) and ITAC (Fig. 1B) is dramatically reduced in the OxLDL pre-treated cells and that the inhibition occurs at the level of transcription (data not shown).

Results from other groups have shown that both of these genes can be induced in macrophages by a number of stimuli, including IFNγ (29, 30). Additional studies have shown that LPS treatment of macrophages immediately activates the transcription and secretion of IFNγ (29, 31). Accordingly, we investigated whether defects in LPS-induced IFNγ production could account for the lack of IP10 and ITAC synthesis in the OxLDL pre-treated cells. As the RT-PCR data in Fig. 2A show, LPS induces a rapid, but transient, increase in IFNγ transcription in the control cells. In contrast, the OxLDL pre-treated cells show an almost total absence of LPS-induced IFNγ transcription over the course of 9 h of observation. As the ELISA data in Fig. 2B show, LPS induces secretion of IFNγ in the control cells starting after 1 h. In contrast, very little IFNγ secretion is measured in the OxLDL pre-treated cells. These data show that LPS-induced IFNγ production is blocked in OxLDL pre-treated cells and that the inhibition occurs at the level of transcription. The RT-PCR data in Fig. 2C are included to show that the LPS-induced transcription of another immediate early transcription factor, C/EBP (28), is not affected by OxLDL treatment.
gene, TNFα, proceeds normally in the OxLDL pre-treated cells. Further support linking the early inhibition of IFNβ synthesis and secretion with the late inhibition of IP10 and ITAC synthesis was found in an additional set of experiments. Human blood-derived macrophages were isolated and grown in the presence or absence of OxLDL as described under “Materials and Methods.” The cells were then activated by treatment with LPS. Supernatants were collected at 0, 1, 3, 6, and 9 h post LPS stimulation. The amounts of IP10 and ITAC in the supernatants were determined by ELISA. A, IP10 ELISA; B, ITAC ELISA. Control/LPS data are plotted as squares; OxLDL/LPS data are plotted as triangles. The data are plotted as the mean of values from three independent experiments. Data from each independent experiment were normalized to the maximum LPS-induced expression value seen in the control cells of that experiment (on a scale of 1–100). Standard deviations are shown.

Fig. 1. OxLDL pre-treated macrophages fail to secrete IP10 and ITAC following LPS stimulation. Blood-derived macrophages were isolated, cultured, and grown in the presence or absence of OxLDL as described under “Materials and Methods.” The cells were then activated by treatment with LPS. Supernatants were collected at 0, 1, 3, 6, and 9 h post LPS stimulation. The amounts of IP10 and ITAC in the supernatants were determined by ELISA. A, IP10 ELISA; B, ITAC ELISA. Control/LPS data are plotted as squares; OxLDL/LPS data are plotted as triangles. The data are plotted as the mean of values from three independent experiments. Data from each independent experiment were normalized to the maximum LPS-induced expression value seen in the control cells of that experiment (on a scale of 1–100). Standard deviations are shown.

Fig. 2. OxLDL pre-treated macrophages fail to transcribe and secrete IFNβ following LPS stimulation. Macrophages were grown in the presence or absence of OxLDL as described under “Materials and Methods.” Cells were then treated with LPS to activate the cells, and RNA and supernatants were collected at 0, 1, 3, 6, and 9 h post LPS stimulation. Transcription levels of the IFNβ gene at 0, 1, 3, 6, and 9 h post LPS stimulation were determined by RT-PCR as shown in A. The amount of IFNβ secreted into the media by these cells was determined by ELISA and is shown in B. Transcription levels of the TNFα gene at 0, 1, 3, 6, and 9 h post LPS stimulation were determined by RT-PCR and are shown in C. Control/LPS data are plotted as squares; OxLDL/LPS data are plotted as triangles. The data are plotted as the mean of values from three independent experiments. Data from each independent experiment were normalized to the maximum LPS-induced expression value seen in the control cells of that experiment (on a scale of 1–100). Standard deviations are shown.
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Fig. 3. OxLDL pre-treated macrophages express normal levels of IP10 when treated with exogenously supplied IFNβ.

Blood-derived macrophages were grown in the presence or absence of OxLDL as described under “Materials and Methods.” Cells were then treated with IFNβ to activate the cells, and RNA was collected at 0, 3, 6, and 9 h post IFNβ stimulation. Transcription levels of IP10 were determined by RT-PCR. Controls/IFNβ data are plotted as squares; OxLDL/IFNβ data are plotted as triangles. The data are plotted as the mean of values from three independent experiments. Data from each independent experiment were normalized to the maximum IFNβ-induced IP10 expression value seen in the control cells of that experiment (on a scale of 1–100). Standard deviations are shown.

IFNβ production that is responsible for the lack of IP10 and ITAC expression seen in the OxLDL/LPS-treated cells.

Because IFNβ is known to act in an autocrine/paracrine fashion to activate a second wave of transcription, largely through the activation of the transcription factor Stat1 (29), we also checked the level of LPS-induced Stat1 activation in blood-derived macrophages. Cells were isolated, cultured, and treated with OxLDL and LPS as before. Cell lysates were prepared at various times following LPS treatment and analyzed on protein gels. As the data in Fig. 4 show, there is an equivalent level of Stat1 in both the control and OxLDL pre-treated cells, but only in the LPS-treated control cells does the active form of Stat1 appear. These results are thus consistent with the levels of IFNβ production seen in both the control and OxLDL pre-treated cells.

We also examined the expression levels of other IFNβ- and Stat1-activated genes identified from the literature. As before, macrophage cells were isolated, cultured, and treated with OxLDL for 3 days, followed by LPS treatment. Control cells were treated with LPS only. RNA was collected at various times and analyzed. As the RT-PCR data in Fig. 5 show, the LPS-induced expression levels of the chemokines, RANTES and MCP2; the tryptophan-catabolizing enzyme, indoleamine 2,3-dioxygenase; the antiviral protein, ISG54; and the IFN regulatory factors, IRF2 and IRF7b, were all found to be inhibited in the OxLDL pre-treated macrophages. Because these genes have all been shown to be either IFNβ- or Stat1-responsive (32–37), these data are consistent with the lack of IFNβ production and Stat 1 activation seen in the OxLDL/LPS-treated cells.

The next question we addressed was how OxLDL pre-treatment interferes with LPS-induced IFNβ transcription in human blood-derived macrophages. Previous work by others has identified the binding sites for numerous transcription factors within the IFNβ promoter (38, 39). Prominent among these factors are NFκB, c-Jun, ATF2, and IRF3 (40). We thus investigated the LPS-induced activation state of these four transcription factors in the control and OxLDL pre-treated cells. Cells were isolated, cultured, and treated with OxLDL and LPS as before. Nuclear extracts or whole cell lysates were prepared at various times following LPS stimulation. Transcription factor activity was assessed in these extracts and lysates as described below.

The EMSA result in Fig 6A shows the LPS-induced NFκB DNA binding signal in both the control and OxLDL pre-treated cell nuclear extracts. NFκB is similarly induced by LPS in both the control and OxLDL pre-treated cells. The NFκB activity induced consists of both p65 and p50 subunits, because antibodies against both NFκB forms supershift the LPS-inducible bands (data not shown). The stimulus-induced phosphorylation of NFκB at serine 536 is a modification known to augment NFκB transactivation potential (41), but here too we observed no difference in LPS-induced phosphorylation intensity between the control and OxLDL pre-treated cells (data not shown).

Fig. 6B shows a Western blot measuring the amount of phosphorylated c-Jun, the active form of the transcription factor, induced by LPS in both control and OxLDL pre-treated whole cell lysates. The active form of c-Jun is similarly induced by LPS in both control and OxLDL pre-treated cells, although there is some level of activation seen in the OxLDL pre-treated cells prior to LPS addition (lane 4). Finally, Fig. 6C shows a Western blot measuring the amount of phosphorylated ATF2, the active form of the transcription factor, induced by LPS in both control and OxLDL pre-treated whole cell lysates. The active form of ATF2 is similarly induced by LPS in both control and OxLDL pre-treated cells. The LPS-induced phosphorylation state of P38, an upstream activating kinase of ATF2, was also found to be comparable in the control and OxLDL pre-treated cells (data not shown). Thus, the LPS-induced activation of NFκB, c-Jun, and ATF2, three transcription factors known to be essential for the activation of IFNβ transcription (39, 40), were found to be comparable in both the control and OxLDL pre-treated cells. These figures were representative of data obtained from similarly treated macrophages isolated from several different blood donors.

We next measured the LPS-inducible DNA-binding activity of IRF3 in a standard pull-down assay. In this experiment, nuclear extracts are combined with a biotinylated DNA duplex that contains an IRF3 binding site. After a period of incubation, any DNA-protein complexes that form are pulled down with streptavidin-coated agarose beads via centrifugation. Bound protein is identified by SDS-PAGE, followed by immunoblotting with antibodies for IRF3. This assay, because of the large amount of nuclear extract that can be combined with the biotinylated DNA duplex, can often resolve DNA-protein complexes that are hard to detect in standard EMSA experiments. Fig. 7A shows the LPS-induced IRF3 DNA binding signal in both the control and OxLDL pre-treated cell nuclear extracts. As the
zero time point lanes in the figure show, IRF3 is not pulled down with the biotinylated DNA duplex in the absence of LPS stimulation. However, after 45 min of LPS stimulation, IRF3 is clearly pulled down in the control cell nuclear extracts. At 120 min of LPS stimulation, in these same cells, the amount of bound IRF3 detected was significantly diminished. In contrast, LPS treatment failed to induce the binding of IRF3 to the DNA probe in the OxLDL pre-treated cells, because IRF3 can not be detected at either of the post LPS time points. Fig. 7B shows that differences in IRF3 protein levels are unlikely to account for this difference in IRF3 DNA-binding activity, because comparable levels of IRF3 appear in whole cell lysates prepared from control and OxLDL pre-treated cells in standard Western blots.

Investigating further, we next measured the LPS-inducible interaction between IRF3 and CBP. The association of IRF3 and CBP/P300 has been shown to be essential for the activation of IFNβ transcription (24, 25). We therefore investigated whether OxLDL pre-treatment also interfered with the LPS-induced association of IRF3 with the coactivator CBP. In these experiments, whole cell lysates were first immunoprecipitated with antibody to IRF3, followed by probing of the precipitated material with antibodies to CBP. As the zero time point lanes in Fig. 7C show, CBP was not detected in the immunoprecipitate in the absence of LPS stimulation. However, after 45 min of LPS stimulation, CBP was clearly detected in the control cell immunoprecipitate. At 120 min of LPS stimulation, in these same cells, the association between IRF3 and CBP had weakened considerably, because very little CBP was then detected. However, LPS treatment failed to induce the association between IRF3 and CBP in the OxLDL pre-treated cells, because CBP could not be detected in the anti-IRF3 immunoprecipitate at either of the post LPS treatment time points. Fig. 7 (B and D) shows that differences in IRF3 and CBP protein levels were unlikely to account for this observed difference in association, because comparable levels of both proteins appeared in the control and OxLDL pre-treated cells in standard Western blots. Thus, OxLDL pre-treatment interferes in some way with the LPS-induced activation of IRF3, as measured in DNA binding assays with an IRF3-specific probe, and by measuring the LPS-inducible association of IRF3 with CBP in co-immunoprecipitations. Because these IRF3 activities have been shown to be essential for IFNβ transcription (24, 25), we conclude that the lack of LPS-induced IRF3 activation seen in the OxLDL pre-treated cells is responsible for the loss of LPS-induced IFNβ transcription seen in these same cells.

**DISCUSSION**

LPS activates the transcription of a large number of proinflammatory genes (42) by way of multiple signaling pathways downstream of TLR4 (43, 44). One major pathway involves the key adaptor protein MyD88 (44, 45). This protein facilitates the activation of the transcription factor NFκB, as well as the activation of the JNK and p38 kinases, which activate the transcription factors c-Jun and ATF2, respectively. Although the activation of genes like TNFα and IL1β are dependent on this pathway, the activation of IFNβ is not (43, 44). LPS-induced IFNβ transcription is activated via a MyD88-independent pathway that leads to the activation of IRF3 (43, 46). Recently it has been shown that the MyD88-independent pathway is also able to activate the transcription factor NFκB, but the mechanism is unclear and the kinetics of induction appears to be delayed (47, 48).

Previous studies have shown that NFκB, c-Jun, ATF2, and
IRF3 are all involved in the transcriptional activation of the IFNβ gene, although IRF3 appears to play a dominant role (38–40). Our studies show that LPS induced activation of NFκB, c-Jun, and ATF2 appear largely normal in OxLDL pre-treated blood-derived macrophages. However, the activation of IRF3, as measured by its LPS-inducible DNA-binding activity and association with CBP, is seen to be defective in the OxLDL pre-treated cells. Because these IRF3 activities have been shown to be essential for IRF3-mediated transcriptional activation, the absence of these activities would appear to explain the lack of IFNβ transcription that we observe in these cells. In turn, this lack of IFNβ synthesis and secretion accounts for the lack of Stat1 activation, because this transcription factor is known to be activated in macrophages by IFNβ, even when LPS is used as the initial treatment ligand (29, 31). Finally, the lack of Stat1 activation in the OxLDL/LPS-treated cells explains the extreme LPS hyporesponsiveness observed for genes like IP10 and ITAC and numerous other IFNβ-activated genes because of the prominent role Stat1 has been shown to play in their activation (29).

The cause and effect chain of events described above is further supported in our studies by the timing of our observations. In the control cells, NFκB, c-Jun, ATF2, and IRF3 activities exhibit peak activity at 45 min. This coincides with the peak activation time for IFNβ transcription. Stat1 activation peaks at 2 h, roughly corresponding with the peak in IFNβ secretion. In addition, the transcription of IFNβ/Stat1-activated genes, like IP10 and ITAC, peaks after 3 h. Thus, the lack of LPS-induced IRF3 activity in the OxLDL pre-treated cells not only blocks the activation of IFNβ but also a secondary wave of transcription and secretory events, dependent on IFNβ, that are part of the LPS-induced inflammatory cascade.

Our observation that the LPS-induced activation of IRF3 is blocked, while the LPS-induced activation of NFκB, c-Jun, and ATF2 activation appears largely unaffected, argues against direct antagonism between OxLDL and LPS at the point of binding to TLR4. Such antagonism would be expected to lead to reduced LPS activation of all signaling pathways downstream of TLR4, as well as reduced expression of all LPS-activated genes. This was not observed, because LPS induced the transcription of TNFα normally in the OxLDL pre-treated cells. Furthermore, we have examined the expression of numerous other genes induced by LPS at the same time as the IFNβ gene and find that many exhibit control levels of expression in the OxLDL pre-treated cells. Instead, our data support a mechanism whereby OxLDL/LPS-pre-treatment appears to block a step on the MyD88-independent pathway leading downstream from TLR4. Recent studies have shown that LPS-TLR4-mediated activation of IRF3 involves the upstream adaptor proteins Trif (49) and Tram (47, 48) and the midstream kinases TBK1 and IKKi (50). It is believed that one or both of these kinases may play a direct role in the phosphorylation of IRF3 monomers, which reside latent in the cytoplasm. Once IRF3 is phosphorylated, it homodimerizes (or heterodimerizes with the related protein, IRF7), and then translocates to the nucleus (46, 51). Inside the nucleus, IRF3 binds to cognate DNA sequences and to the coactivator protein CBP/p300. In the case of the IFNβ promoter, the DNA-bound IRF3-CBP/p300 complex collaborates with DNA-bound NFκB, c-Jun, and ATF2 to drive the transcription of the IFNβ gene (39, 40).

Although we have been able to observe a block in the MyD88-independent pathway occurring at the point of IRF3 DNA binding and IRF3-CBP association in the OxLDL/LPS-treated cells, we have not yet made observations further upstream on this pathway. Future studies will address whether OxLDL pre-treatment inhibits one or more of the kinases involved in IRF3 activation. Furthermore, OxLDL consists of numerous biologically active molecules, many of which have yet to be characterized. Recently, several lipid oxidation products associated with OxLDL have been reported to stimulate TLR4-mediated activation of IL8 transcription and to modulate the LPS activation of this gene as well (52–54). More work will be required to determine if these, or other OxLDL components, are responsible for the effects seen in our study.

Several nuclear receptors are reported to interact with CBP to facilitate the activation of transcription (55, 56). OxLDL treatment of macrophages is known to up-regulate, and to provide ligands for, several nuclear receptors, including liver receptor α, peroxisome proliferator-activated receptor γ, and retinoid receptor X (7, 57–59). It is possible that the increased

A. Marson and T. Mikita, unpublished data.
Macrophages were grown in the presence or absence of OxLDL as described under "Materials and Methods." Cells were then treated with LPS to activate the cells, and either nuclear extracts or whole cell lysates were prepared at the times indicated above the lanes in each of the figure panels. A, pull-down assay: nuclear extracts were incubated with a biotinylated DNA duplex containing an IRF3 binding site, followed by precipitation with streptavidin-coated agarose beads. The amount of DNA-bound IRF3, as shown in each lane, was determined by SDS-PAGE followed by immunoblotting with an IRF3 polyclonal antibody, as indicated under "Materials and Methods." B, in separate experiments, whole cell lysates from similarly treated cells were subjected to SDS-PAGE followed by immunoblotting with an IRF3 polyclonal antibody. C, co-immunoprecipitation: whole cell lysates were first incubated with IRF3 antibody and protein G-coupled beads. The beads were then pelleted by centrifugation and washed, and the immunoprecipitated protein was released into SDS buffer. The samples were then subjected to SDS-PAGE followed by immunoblotting with a CBP polyclonal antibody, as indicated under "Materials and Methods." The results of this co-immunoprecipitation are shown in the individually marked lanes. D, in separate experiments, the above lysates were subjected to SDS-PAGE followed by immunoblotting with a CBP polyclonal antibody. The data shown in each of the above panels were representative of results from at least three independent biological experiments.

In the current study we have shown that human blood-derived macrophages pre-treated with OxLDL fail to transcribe and secrete IFNβ immediately following LPS stimulation. As such, the downstream activation of Stat1 is blocked, and numerous IFNβ/Stat1-activated genes are weakly expressed or not expressed at all in these cells. Furthermore, we show that the inhibition of IFNβ transcription is likely due to a block in IRF3 activation. However, OxLDL, with its numerous oxidized lipid and protein molecules clearly has the potential for multiple and varied effects on a diverse set of macrophage responses. Although our current report has focused on the mechanism of how OxLDL inhibits LPS-activated IFNβ transcription, other signal transduction pathways lying downstream of TLR4 are also affected in ways that have yet to be characterized. For example, we have observed that the IL8 and IL1β genes are hyper-responsive to LPS stimulation in OxLDL pre-treated macrophages. Thus, OxLDL differentially affects the outcomes of TLR4 signaling in ways that are likely to involve several different downstream pathways. Further studies will be required to characterize these changes and to see if altered TLR4 signaling responses play a significant role in atherogenesis.

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A. Marson and T. Mikita, unpublished observations.
