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Oxidized LDL Immune Complexes and Oxidized LDL Differentially Affect the Expression of Genes Involved with Inflammation and Survival In Human U937 Monocytic Cells

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Abstract

Objective—To compare the global effects of oxidized LDL (oxLDL) and oxLDL-containing immune complexes (oxLDL-IC) on gene expression in human monocytic cells and to identify differentially expressed genes involved with inflammation and survival.

Methods and Results—U937 cells were treated with oxLDL-IC, oxLDL, Keyhole limpet hemocyanin immune complexes (KLH-IC), or vehicle for 4 h. Transcriptome profiling was performed using DNA microarrays. oxLDL-IC uniquely affected the expression of genes involved with pro-survival (*RAD54B, RUFY3, SNRPB2,* and *ZBTB24*). oxLDL-IC also regulated many genes in a manner similar to KLH-IC. Functional categorization of these genes revealed that 39% are involved with stress responses, including the unfolded protein response which impacts cell survival, 19% with regulation of transcription, 10% with endocytosis and intracellular transport of protein and lipid, and 16% with inflammatory responses including regulation of I-κB/NF-κB cascade and cytokine activity. One gene in particular, *HSP70 6*, greatly up-regulated by ox-LDL-IC, was found to be required for the process by which oxLDL-IC augments IL1-β secretion. The study also revealed genes uniquely up-regulated by oxLDL including genes involved with growth inhibition (*OKL38, NEK3*, and *FTH1*), oxidoreductase activity (*SPXN1* and *HMOX1*), and transport of amino acids and fatty acids (*SLC7A11* and *ADFP*).

Conclusions—These findings highlight early transcriptional responses elicited by oxLDL-IC that may underlie its cytoprotective and pro-inflammatory effects. Cross-linking of Fcγ receptors appears to be the trigger for most of the transcriptional responses to oxLDL-IC. The findings further strengthen the hypothesis that oxLDL and oxLDL-IC elicit disparate inflammatory responses and play distinct roles in the process of atherosclerosis.

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Introduction

Lipid-laden macrophages (foam cells) are the hallmark of the atherosclerotic process and main contributors to progression of cardiovascular disease. Therefore, understanding the processes by which foam cells are formed has been a major objective of atherosclerosis research. It is well established that oxidized LDL (oxLDL) particles are taken up by macrophages leading to accumulation of cholesteryl esters (CE) (1,2). On the other hand, oxLDL is immunogenic and elicits the production of antibodies, predominantly of the proinflammatory IgG1 and IgG3 isotypes (3,4). These antibodies form circulating immune complexes containing oxLDL (oxLDL-IC), and those immune complexes have pro-inflammatory properties (5–7) and are considerably more efficient than oxLDL in the induction of foam cell formation (8–10). While the uptake of both oxLDL and oxLDL-IC produce cells morphologically defined as foam cells, there is evidence for distinct molecular differences. In particular, foam cells formed through oxLDL exposure have reduced survival as compared to foam cells formed through oxLDL-IC exposure (11,12). The basis for this difference in not known. Macrophages exposed to oxLDL-IC have higher levels of CE and display an increased release of cytokines as compared to cells exposed to oxLDL (5,7). These findings have led to the hypothesis that although foam cells generated by exposure to oxLDL and oxLDL-IC appear morphologically similar, they differ in the profile of genes that regulate survival and inflammatory response. To test this hypothesis, we have used DNA microarray analysis and real time quantitative PCR (Q-PCR) to investigate the effects of oxLDL and oxLDL-IC on the transcriptome in human U937 monocytic cells. The results of these studies highlight specific transcriptional responses elicited by oxLDL-IC that may underlie its ability to promote prolonged activation of foam cells.

Materials and Methods

Cells

The human monocytic cell line U937 was obtained from the American Type Culture Collection (ATCC CRL-1593.2) (13). Cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 50μg/ml streptomycin at 37 C, 5% CO_2. Cells were seeded at 2×10^6 cells/2 ml in 6-well plates, and incubated in serum-free medium in the presence of IFN-γ (200 ng/ml) for 18 h prior to addition of experimental treatments. The rationale for IFN-γ treatment is that it is the major cytokine released by activated T cells in macrophage-containing atherosclerotic lesions (14). Thus, the response of IFN-γ primed macrophages to modified lipoproteins is likely to reproduce the conditions at the atheromatous plaque, where the same regions where macrophages and foam cells predominate are heavily infiltrated with CD4+ T cells releasing primarily IFN-γ (15).

Lipoprotein Isolation and Oxidation

LDL (d = 1.019 to 1.063 g/ml) was isolated from plasma of normal volunteers and oxidatively modified using Cu^{2+} as described previously (16,17). Under the conditions previously reported for copper oxidation of LDL (18) our oxLDL preparations have the following degree of modification: 4 to 7 mmol/mol lysine of malondialdehyde (MDA) (0.4 to 0.7% modification of lysine residues), 0.8 mmol/mol lysine of N^{ε}-(carboxymethyl) lysine (CML) (0.08% modification of lysine residues), and 0.25 mmol/ml lysine of N^{ε}(carboxyethyl)lysine (CEL) (0.025% modification of lysine residues). This degree of LDL modification is associated with formation of auto-antibodies in humans (17,18) and is optimally recognized by the antibody used to form oxLDL-IC (see next section). The endotoxin level in oxLDL preparations was measured using an endotoxin assay kit (Etoxate, Sigma), and found to be below the lower limit of detection (0.015 U/ml).

Preparation of Insoluble Immune Complexes

Soluble immune complexes stimulate macrophages only if carried by red blood cells or immobilized. Immobilization of oxLDL-IC by attachment to matrix proteins is likely to occur *in vivo*. We used insoluble oxLDL-IC as a form of immobilized immune complexes to stimulate macrophages. Insoluble oxLDL-IC were prepared with human oxLDL and human anti oxLDL antibodies. Anti oxLDL IgG was isolated from whole human serum using a combination of Protein G-Sepharose 4 Fast Flow chromatography (Amersham-Pharmacia Biotech) to isolate IgG, and affinity chromatography in Sepharose-linked human oxLDL to isolate oxLDL antibodies of the IgG isotype, as described previously (3,19). The amount of oxLDL combined with anti-oxLDL IgG to prepare insoluble oxLDL-IC was determined empirically by performing a precipitin curve, constructed by incubating aliquots of the IgG with varying amounts of oxLDL and determining the amount of oxLDL that induced the highest degree of insoluble oxLDL-IC formation as determined by nephelometry. The empirical measurements indicated that a ratio of 150 μg oxLDL protein/500 μg IgG produced peak precipitation. Human Keyhole limpet hemocyanin (KLH) immune complexes (KLH-IC) were prepared with the IgG fraction from anti-KLH human serum isolated using Protein G-Sepharose 4 Fast Flow chromatography (5). The optimal proportion of KLH (endotoxin-free, Calbiochem) to anti KLH IgG for preparation of insoluble human KLH-IC was 200μg/250μg (w/w). All immune complexes were prepared under sterile conditions, washed and re-suspended in PBS.

Measurement of Interleukin 1 beta (IL1-β)

IL1-β levels in conditioned medium were analyzed using two types of ELISA, SearchLight Human Cytokine Array, a multiplex sandwich ELISA (Pierce) and an IL1-β enzyme immunoassay kit from R&D Systems (DLB50; Minneapolis, MN).

Total RNA Preparation and DNA Microarray Hybridization

IFN-γ-treated U937 cells were exposed to oxLDL-IC, KLH-IC, oxLDL (150 μg/ml), or PBS vehicle for 4 h. KLH-IC was used as a control immune complex because KLH has a molecular weight comparable to LDL and because it can engage Fcγ receptors like oxLDL-IC but does not contain lipoproteins. Cells were pelleted by centrifugation at 400×*g* for 5 min. Total RNA was isolated using Trizol extraction (Invitrogen) and purified using RNeasy Mini kit (Qiagen). RNA quality was assured by using Agilent Bioanalyzer and RNA 6000 nano chip. Total RNA (8 μg) was converted into double-stranded cDNA with a T7-(dT) 24 primer (Genset) and a cDNA synthesis kit (Custom SuperScript; Invitrogen). Biotin-labeled cRNA was synthesized from cDNA by in vitro transcription (Enzo BioArray HighYield RNA Transcript Labeling Kit; Enzo Life Sciences). After purification (RNeasy kit; Qiagen), labeled cRNA was fragmented as recommended by Affymetrix. Hybridization of cRNA samples to Affymetrix HG-U133 plus2 GeneChips, post-hybridization washing, fluorescence staining and scanning were performed at the MUSC DNA Microarray and Bioinformatics Facility. DNA microarray data (raw and normalized) generated by this project are available online through the MUSC DNA Microarray Database (http://proteogenomics.musc.edu/ma/musc_madb.php?page=home&act=manage) and the NCBI Geo (http://www.ncbi.nlm.nih.gov/geo/).

Gene Array Analysis

Hybridization intensity data were normalized using the GCRMA algorithm (20). Identification of differentially expressed genes and hierarchical clustering were performed using dChip software (21). Genes differentially affected by the separate treatments were identified using ANOVA ($p<0.001$); hierarchical clustering was performed on the resulting genes using the distance metric of 1-Correlation and the Average linkage method. Genes presented here as uniquely affected by either oxLDL-IC or oxLDL were obtained from the resulting heat map.

Genes regulated similarly by oxLDL-IC and KLH-IC were identified using the following criteria: 1) FC>2 and $p<0.05$ (Student's unpaired t-test) for oxLDL-IC *vs* PBS and for oxLDL-IC *vs* oxLDL treatments; 2) FC>2 and $p<0.05$ (Student's unpaired t-test) for KLH *vs* PBS and for KLH *vs* oxLDL treatments. False discovery rate (FDR) approximated 0.0% as estimated by 50 iterations of randomized sample comparisons. Genes regulated similarly by oxLDL-IC and oxLDL were examined using the same criteria used to identify genes regulated similarly by oxLDL-IC and KLH-IC.

Real Time Quantitative PCR (Q-PCR)

PCR primers were designed using the Beacon Designer 5 software (Primer Biosoft Int., Palo Alto, CA). The forward and reverse primer sequences for the genes examined are shown in Table 1. PCR primers were synthesized by Integrated DNA Technologies, Inc. (Coraville, IA). IFN-γ-treated U937 cells were exposed to oxLDL-IC, oxLDL (150 μg/ml), or the PBS vehicle for 4 h. The RNAeasy mini kit was used to isolate mRNA (Qiagen), and complementary DNA (cDNA) was synthesized using iScript™ cDNA synthesis kit (Bio-Rad). Q-PCR was performed using the iCycler™ real-time detection system (Bio-Rad) with a two-step method using iQ™ SYBR Green Supermix (Bio-Rad). Amplification of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was performed to standardize the amount of sample RNA. Quantification was performed using the cycle threshold of receptor cDNA relative to that of GAPDH cDNA in the same sample.

siRNA Knockdown

U937 cells were transfected with Non-Targeting (control) or HSPA6 (HSP70 6) ON-TARGETplus SMARTpool siRNAs (Dharmacon RNA Technologies, Lafayette, CO) using the Nucleofector™ Device (Amaxa Inc., Gaithersburg, MD) according to manufacturer's instructions. After 48 h in serum-containing medium, IFN-γ was added and the cells incubated for an additional 18 h. The medium was then replaced with serum-free medium and the cells cultured for 2 h to eliminate all traces of lipoproteins in the serum-supplemented media before addition of the ligand. Longer serum starvation could independently activate the transfected cells and increase the release of cytokines in the media. oxLDL-IC (150μg/ml) was added and the cells incubated for up to 12 h. The conditioned culture medium was removed for IL-1β analysis and RNA extracted for Q-PCR. The extent of siRNA-mediated knockdown of HSP70 6 mRNA levels was confirmed using HSP70 6 and β-actin primers shown in Table I.

Results and Discussion

In this study, DNA microarray transciptomic profiling was performed on RNAs isolated from U937 cells treated with oxLDL-IC, oxLDL, KLH-IC, or PBS vehicle for 4 h. A rationale for choosing 4 h of treatment was based on the finding that between 2 and 6 h post treatment of U937 cells with oxLDL-IC there was an increase in levels of IL-1β released into the conditioned culture medium (Fig. 1). oxLDL did not elicit a similar response (Fig. 1). In light of these findings, changes in gene expression after 4 h of oxLDL-IC exposure could be due to secondary signaling effects of gene products such as IL-1β whose expression was augmented early by oxLDL-IC. Using ANOVA and pair-wise analyses of the resulting microarray hybridization data we sought to determine: *1)* genes uniquely regulated by ox-LDL-IC, *2)* genes similarly regulated by ox-LDL-IC and KLH-IC but not by oxLDL, 3) genes similarly regulated by ox-LDL-IC and oxLDL, and 4) genes uniquely regulated by ox-LDL.

Genes uniquely regulated by ox-LDL-IC

ANOVA analysis revealed that oxLDL-IC uniquely (as compared to the KLH-IC, oxLDL, and PBS treatments) affected the expression of 40 genes (Fig. 2A). From this set of genes, those having a fold change (FC) >2 (4 genes) were subjected to functional categorization. This

analysis showed that all four genes (*RAD54B, RUFY3, SNRPB2,* and *ZBTB24*) are involved with pro-survival functions (Table 2). These findings concur with previous evidence that oxLDL-IC treatment of U937 cells elicits a survival response (11,12).

Genes similarly regulated by ox-LDL-IC and KLH-IC

Differential expression analysis was also conducted to determine whether the regulatory activity of oxLDL-IC is similar to that of KLH-IC, and as such, likely to be mediated via engagement of Fcγ receptors. As a result, 43 genes were identified as being similarly regulated by ox-LDL-IC and KLH-IC (Fig. 2B). Functional categorization of the 31 up-regulated genes from this group (Table 3) revealed that 39% are involved with stress responses, including the unfolded protein response which impacts cell survival. Nineteen percent of the up-regulated genes are involved with regulation of transcription, 10% are involved with endocytosis and intracellular transport of protein and lipid, and 16% affect inflammatory responses including regulation of the I-κB/NF-κB cascade and cytokine activity (Table 3). Genes encoding the cytokines tumor necrosis factor (TNF) (superfamily member 9) and IL-1β were up-regulated 7 fold and 3 fold, respectively. These findings are consistent with evidence showing that human macrophages release significantly higher levels of pro-inflammatory cytokines after incubation with oxLDL-IC as compared to incubation with oxLDL, and support the hypothesis that the mechanisms of activation by oxLDL-IC are similar to those responsible for macrophage activation by KLH-IC, most likely via Fcγ receptor engagement (5).

It has been recently shown that immune-complexes activate monocytes/macrophages through the interaction with Fcγ receptors, triggering the secretion of inflammatory cytokines and inhibiting apoptosis (22). The apoptosis protection effects were shown to involve PI3K/Akt, MAPK, and NF-κB pathways (22). Earlier work by our group (11) and by others (12) has shown that oxLDL-IC also promoted survival of monocytes by cross-linking Fcγ receptors. Mechanistically, the pro-survival effects of oxLDL-IC have been shown to involve Akt signaling (12), as well as release of sphingosine kinase 1 (SK1), the enzyme responsible for generating the pro-survival signaling molecule sphingosine-1-phosphate (S1P) (11). Although analysis of the DNA microarray data did not show a statistically significant difference in SK1 expression in response to either oxLDL-IC or KLH-IC as compared to other treatments, Q-PCR analysis showed that SK1 mRNA levels were up-regulated in response to both oxLDL-IC and KLH-IC compared to oxLDL alone (2.3- and 2.2-fold, respectively) (Fig. 3A). Measurement of S1P levels together with SK1 gene expression are necessary since minor increases in the bioactive molecule S1P could be sufficient to transduce signals necessary for cell survival.

Of those genes similarly regulated by oxLDL-IC and KLH-IC, *Superoxide dismutase 2* (*SOD2*) was up-regulated 71 fold in response to oxLDL-IC and 20 fold in response to KLH-IC both as compared to PBS (Table 3). SOD2, a principal scavenger enzyme in the mitochondrial matrix, protects cells from oxidative stress by detoxifying superoxide generated in mitochondrial respiration by dismutation. High SOD2 expression can enhance fibrosarcoma cell survival in response to apoptotic stimuli via a mechanism that involves regulation of steady state levels of H_2O_2 (23). Therefore, the augmentation of *SOD2* expression that we observed in response to oxLDL-IC could account for the oxLDL-IC-induced cell survival reported previously (11).

Oxysterol binding protein (OSBP), a major sterol-binding protein implicated in lipid metabolism, vesicle transport, and signal transduction was found to be up-regulated in response to oxLDL-IC (4 fold) and KLH-IC (6 fold) compared to PBS (Table 3). Recently, OSBP was found to function as a cholesterol-binding scaffolding protein coordinating the activity of certain phosphatases to control the extracellular signal-regulated kinase (ERK) signaling pathway (24). Furthermore, Perry and Ridgway (25) have shown that when changes in sterol

metabolism (oxysterol) induce the translocation of OSBP to the *trans*-Golgi network, the ceramide transporter (CERT) also translocates to Golgi membranes, transporting more ceramide to sphingomyelin synthase at that site for sphingomyelin synthesis. Thus, up-regulation of OSBP in response to oxLDL-IC could lead to enhanced conversion of ceramide to sphingomyelin. It has been established that cellular accumulations of ceramide, which occur as a result of oxidative stress or in response to inflammatory cytokines, are associated with apoptotic responses (26). We have recently found that indeed short chain ceramides decreased in response to oxLDL-IC compared to controls (data not shown). Therefore, an OSBP-mediated decrease in ceramide levels in response to oxLDL-IC could account for the oxLDL-IC-induced cell survival reported previously (11).

The expression of *regulator of G-protein signaling 2* (RGS2) was up-regulated in response to oxLDL-IC (72 fold) and KLH-IC (98 fold) compared to PBS (Table 3). RGS2 has emerged as a multifunctional regulator of G-protein signaling (27). RGS proteins enhance the rate at which certain heterotrimeric G-protein α-subunits hydrolyze GTP to GDP limiting the duration that α-subunits activate downstream effectors (*i.e.*, negatively regulating G protein-coupled receptor signaling) (27). Targeted mutation of RGS2 in mice leads to reduced T cell proliferation and IL-2 production (28). The consequence of RGS2 up-regulation in response to cross-linking of Fcγ receptor on signaling pathways downstream to G protein-coupled receptors in human monocytes/macrophages should now be evaluated.

A member of the heat shock 70 kDa protein (HSP70) family, HSP70 6, displayed the greatest magnitude increase in expression (oxLDL-IC and KLH-IC were 1924-fold and 3516-fold greater, respectively, as compared to PBS) (Table 3). Augmented HSP70 6 expression in response to oxLDL-IC and KLH-IC was also shown by Q-PCR (Fig. 3B). At the present time nothing is known as to the function of HSP70 6. We sought to determine whether the augmented expression of HSP70 6 mRNA could account for oxLDL-IC-induced cytokine release by U937 cells. To address this question we used siRNAs to suppress HSP70 6 expression (Fig. 4A). As shown in Figure 4B, oxLDL-IC-induced secretion of IL1-β was completely inhibited when the expression of HSP70 6 was suppressed by siRNA. In cells treated with vehicle alone, the low level of IL1-β secretion was also decreased by HSP70 6 siRNA (Fig. 4B). The findings indicate that HSP70 6 expression is required in the process by which oxLDL-IC augments the secretion of IL1-β. Using Q-PCR analysis we found that IL1-β mRNA expression was not affected by HSP70 6 siRNA knockdown (data not shown).

Genes similarly regulated by ox-LDL-IC and oxLDL

Differential expression analysis of the array data was also conducted to determine whether the regulatory activity of oxLDL-IC is mediated by the oxLDL moiety of the complex. Using the same statistical criteria used to identify genes regulated similarly by oxLDL-IC and KLH-IC, the analysis revealed no genes similarly regulated by oxLDL-IC and oxLDL.

Genes uniquely regulated by ox-LDL

Differential expression analysis was also conducted to determine the transcriptional response of U937 cells to oxLDL. Genes regulated uniquely by oxLDL (31 genes) are shown in the heat map in Figure 2C. From this set of genes, those having a FC > (± 2) were subjected to functional categorization (Table 4). This analysis showed that up-regulated genes are involved with growth inhibition (*OSGIN1* and *FTH1*), oxidoreductase activity (*SPXN1* and *HMOX1*), and transport of amino acids and fatty acids (*SLC7A11* and *ADFP*, respectively) (Table 4).

The gene with the greatest level of increase in response to oxLDL was *heme oxygenase 1* (*HMOX1*) (Table 4). Several lines of evidence suggest that *HMOX1* expression can be increased several fold by stimuli that induce cellular oxidative stress, including oxidized LDL

(29,30). *HMOX1* induction is known to lead to an increase in catalytic free iron release. Importantly, *HMOX1* also mitigates the cytotoxic effects of iron by mediating the enhancement of intracellular ferritin (31). Consistent with this response, we see that oxLDL augments expression of *ferritin, heavy polypeptide 1* (*H-ferritin*) (Table 4). This is also consistent with other findings showing that oxLDL augments levels of L-ferritin (light-chain ferritin) in THP-1 cells (32,33).

The microarray results also show that the gene encoding *adipose differentiation-related protein* (*ADFP*) was uniquely up-regulated in response to oxLDL (Table 4). *ADFP* is expressed in many cell types and is associated with intracellular neutral lipid droplets (34). The precise relationship between ADFP and lipid droplet biology is still obscure (35,36).

Regulation of receptors involved in modified lipoprotein uptake and foam cell formation

We also examined the effects of oxLDL compared to oxLDL-IC on the expression of several receptors involved in lipoprotein uptake and foam cell formation. LDL receptor (LDLR) expression is characteristically down regulated in differentiated human macrophages (37). However, our group has previously shown that after stimulation with LDL-containing immune complexes, LDLR is up-regulated (38). LDL-containing immune complexes induce both transcriptional and post-transcriptional activation of the LDL-R gene in differentiated monocytes and this induction is independent of the free cholesterol pool of these cells (38). In this study, microarray analysis revealed that LDLR gene expression was induced 2.1 fold ($p<0.05$) in response to oxLDL-IC compared to oxLDL at 4 h post treatment. The level of expression of genes encoding the scavenger receptors *SRB-1 and CD36* were also increased in response to oxLDL-IC treatment (1.6 and 1.7 fold, respectively; $p<0.05$) as compared to oxLDL. Changes in *CD36* and *LDLR* receptor expression as revealed by DNA microarray analysis were verified using Q-PCR (Fig. 5).

The physiological and pathological significance of LDLR and CD36 upregulation in response to oxLDL-IC remains to be determined. CD36 is an integral membrane protein expressed on monocytes and macrophages and functions as a scavenger receptor for oxidized LDL. It has been shown that minimally oxidized LDL (MM-LDL) pretreatment increases both mRNA levels and protein levels of CD36, whereas “fully” oxLDL pretreatment does not increase expression of CD36 (39). This suggested that MM-LDL primes the macrophage for more effective clearance of oxLDL and foam cell formation (39). Our current data show that CD36 expression was down-regulated in response to oxLDL probably because of “full” oxidation of LDL and/or short incubation time (4 h). oxLDL-IC however induced CD36 upregulation, which could indicate more efficient uptake of existing oxLDL in the plaque.

Furthermore, a number of cytokines and inflammatory agents have been identified as possible mediators involved in regulating expression of scavenger receptors in human monocytes (40). It has been shown that expression of CD36 is upregulated by interleukin-4, monocyte colony-stimulating factor, and phorbol myristate acetate, while lipopolysaccharide and dexamethasone strongly downregulates CD36 mRNA (40); IFN-γ had no effect on CD36 mRNA (40). Thus, it is likely that CD36 upregulation in our study is secondary to the autocrine effect of secreted cytokines induced by oxLDL-IC.

We are currently investigating the possible role of scavenger receptors (class A and class B) and/or lipoprotein receptors in the uptake of oxLDL-IC and whether or not cross-linking of two receptors trigger distinct signaling pathways required to elicit an enhanced macrophage response.

Conclusion

We investigated whether oxidized LDL immune complexes and oxidized LDL differentially affect the expression of genes involved in activation and survival of IFN-γ-treated human monocytes. The findings highlight transcriptional responses elicited by oxLDL-IC that may underlie its reported cytoprotective and pro-inflammatory effects compared to oxLDL. Cross-linking of Fcγ receptor appears to be the principle trigger for macrophage responses to oxLDL-IC. Intriguingly, although oxLDL and oxLDL-IC share the same modified lipid moiety, the two do not regulate genes in common. The findings further strengthen the hypothesis that oxLDL and oxLDL-IC elicit disparate inflammatory responses and thereby play distinct roles in the process of atherosclerosis.

Recent reported findings indicate that there is an important immunological and inflammatory aspect of atherosclerosis. Several autoimmune diseases are associated with accelerated atherosclerosis, increased plasma levels of circulating oxLDL, anti oxLDL antibodies, dyslipidemia, and enhanced inflammation (41,42). Some groups have proposed that the immune response to oxidized LDL may be protective with regard to the development of atherosclerosis (43–45). This postulate is based on experiments carried out with animal models, which have limited relevance to human atherosclerosis (46) and on *in vitro* data obtained either with Fab fragments (47), which form non-inflammatory complexes due to the lack of the antibody Fc region (48), or with IgM oxLDL antibodies, which are unable to interact with phagocytic cells but represent a minority of the antibody population in humans (4), and have not been shown to confer protection in clinical studies (49). In contrast, human oxLDL-IC are not only pro-inflammatory *in vitro* (5–7), but are associated with diabetic nephropathy and with increased internal carotid intima-media thickness (50–53).

The striking difference in the cellular effects of oxLDL IC and uncomplexed oxLDL may result from a divergence in the processing of oxLDL and oxLDL-IC, perhaps due to differences in receptor binding, uptake and delivery to lysosomes and/or to lysosomal and post-lysosomal processing. Cross-linking of Fcγ receptor in response to oxLDL-IC is an extremely potent activation signal for human macrophages and is likely to trigger signal transduction pathways that modulate different macrophage functions (54), not likely to be induced as a consequence of scavenger receptor ligation by oxLDL. Our findings may advance efforts to reveal specific targets in the signaling pathway that can have therapeutic implications for blocking cytokine release and to prevent formation of vulnerable plaques.

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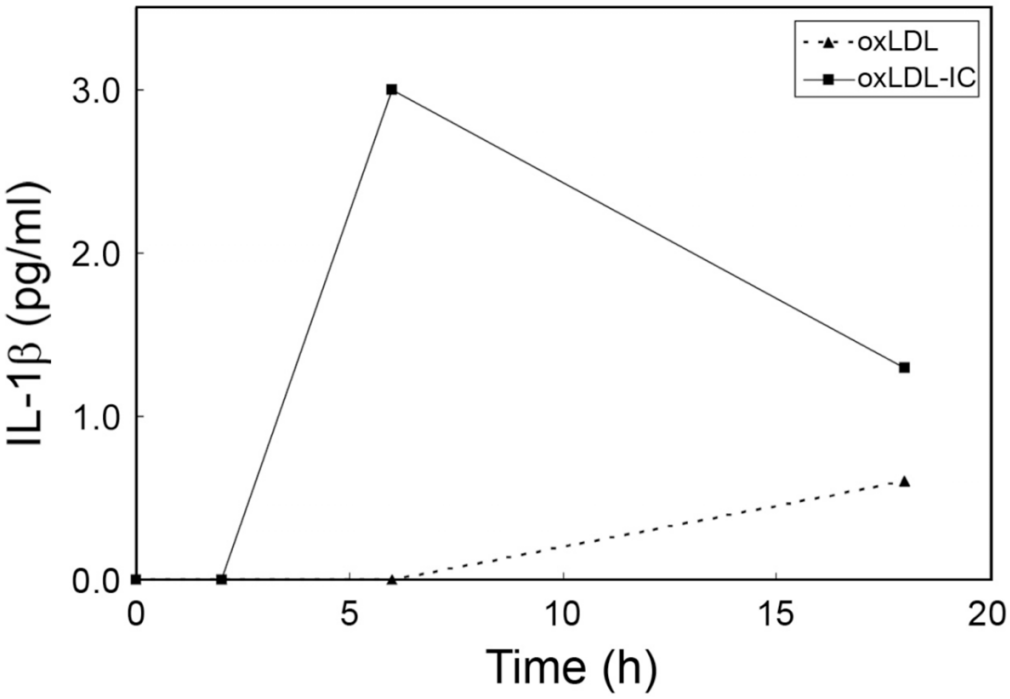

Figure 1.
Secretion of IL-1β is increased in response to oxLD-IC but not oxLDL. Shown are levels of IL-1β secreted into the conditioned culture medium of U937 cells treated with oxLDL-IC or oxLDL as measured using a multiplex sandwich ELISA. Data are representative of two independent experiments.

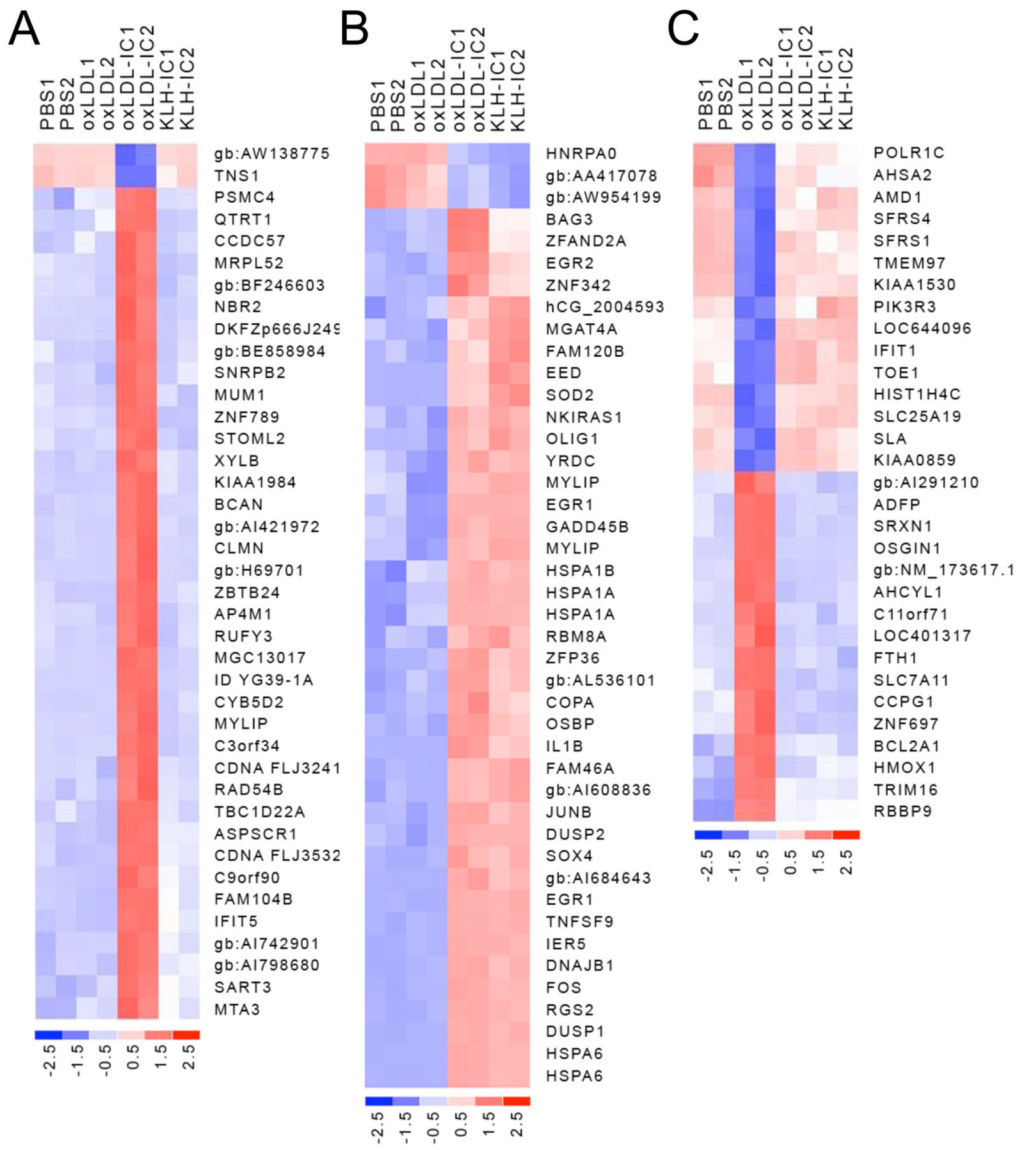

Figure 2.

Heat maps depicting expression profiles of regulated genes in U937 cells exposed to oxLDL-IC, KLH-IC, oxLDL, or PBS vehicle. U937 cells were exposed to oxLDL-IC, KLH-IC, oxLDL (150 μg/ml), or PBS vehicle for 4 h. DNA microarray experimentation was performed using Affymetrix U133 Plus 2.0 GeneChips (n = 2). ***A***, Heat map depicting expression profiles of genes uniquely affected by oxLDL-IC. ***B***, Heat map depicting expression profiles of genes regulated similarly by ox-LDL-IC and KLH-IC. ***C***, Heat map depicting expression profiles of genes uniquely affected by oxLDL. Statistical analysis was performed as described under Gene Array Analysis. Colorimetric scaling (Z-standardization) is indicated at the bottom in units of standardization deviation from the mean.

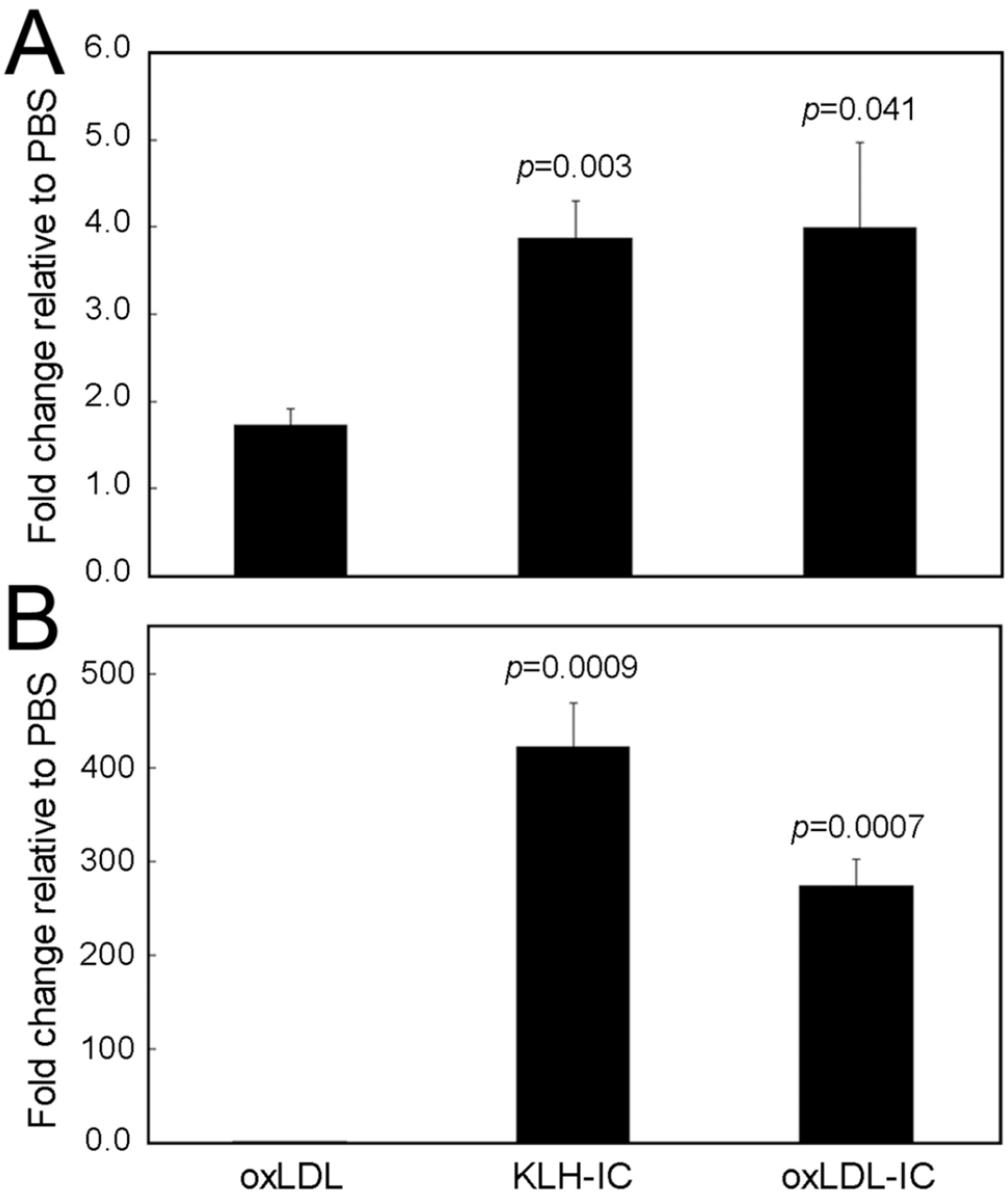

Figure 3.

Q-PCR analysis of sphingosine kinase 1 (SK1) and HSP70 6, two genes up-regulated in response to oxLDLIC and KLH-IC. U937 cells were exposed to oxLDL-IC, KLH-IC, oxLDL (150 μg/ml), or PBS vehicle for 4 h. ***A***, Q-PCR analysis of SK1 mRNA levels; and ***B***, Q-PCR analysis of HSP70 6 mRNA levels. Quantification of RNA was performed using the cycle threshold of SK1 and HSP70 6 cDNA relative to that of GAPDH. Values presented in ***A*** and ***B*** are means of triplicate determinations ± SE; data are representative of two experiments. Indicated *p* values were derived from a Student's unpaired t-test of data compared with oxLDL data.

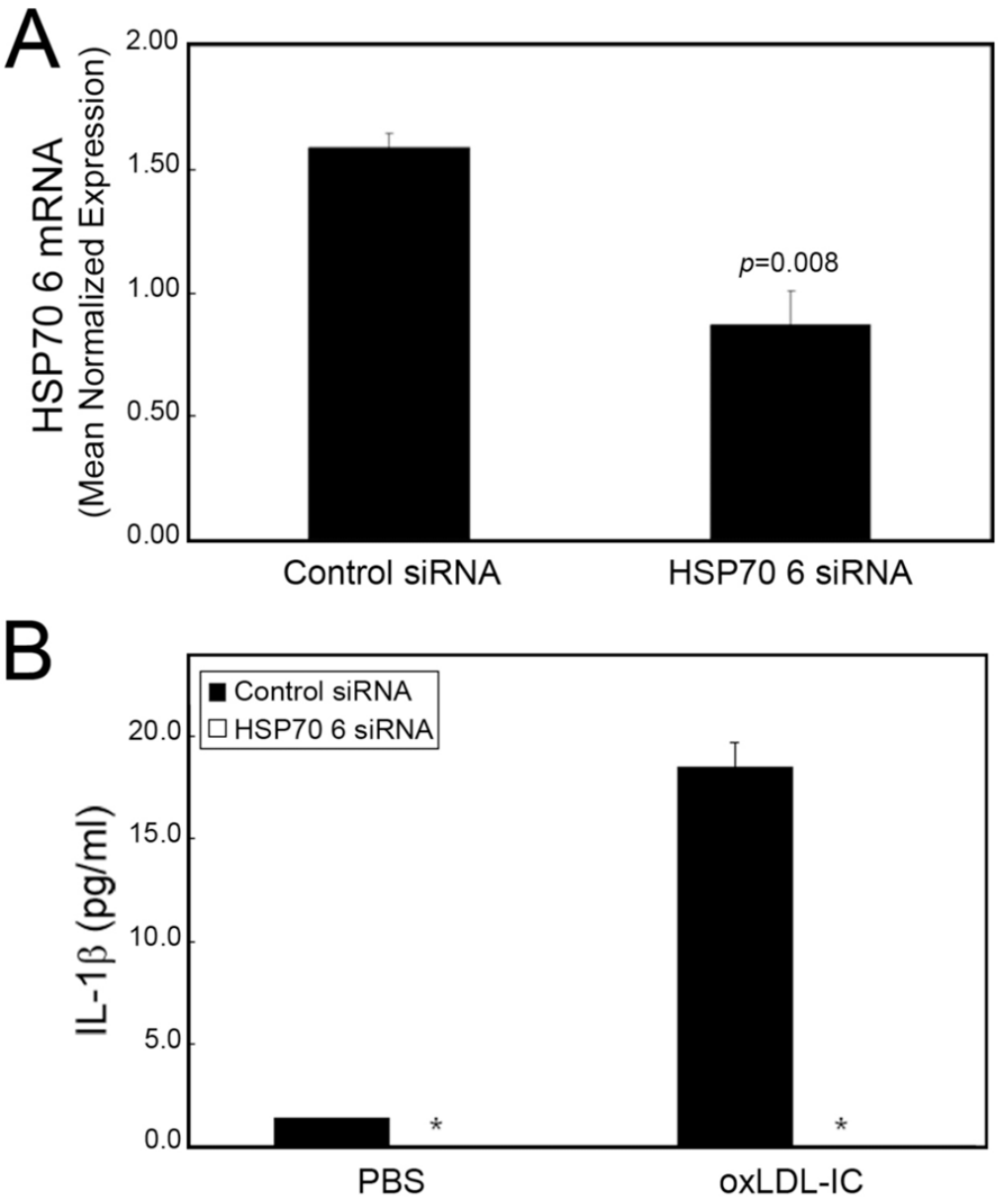

Figure 4.

siRNA-mediated suppression of HSP70 6 abrogates oxLDL-IC-induced IL-1β secretion. U937 cells were transfected with HSP70 6 siRNAs and treated with oxLDL-IC as described in Methods. ***A***, The effect on knockdown of HSP70 6 mRNA levels was measured by Q-PCR; quantification of RNA was performed using the cycle threshold of HSP70 6 cDNA relative to that of β-actin. Values presented in ***A*** are means of triplicate determinations ± SE; data are representative of three experiments. Indicated *p* values were derived from a Student's unpaired t-test of data compared with control siRNA. ***B***, The level of IL1-β secreted into the conditioned culture medium was measured by an IL-1β enzyme immunoassay. Asterisk indicated the undetectable levels of IL-1β in the conditioned culture medium of cells treated with HSP70 6 siRNA. Values presented in ***B*** are means of duplicate determinations ± SE; data are representative of two independent experiments.

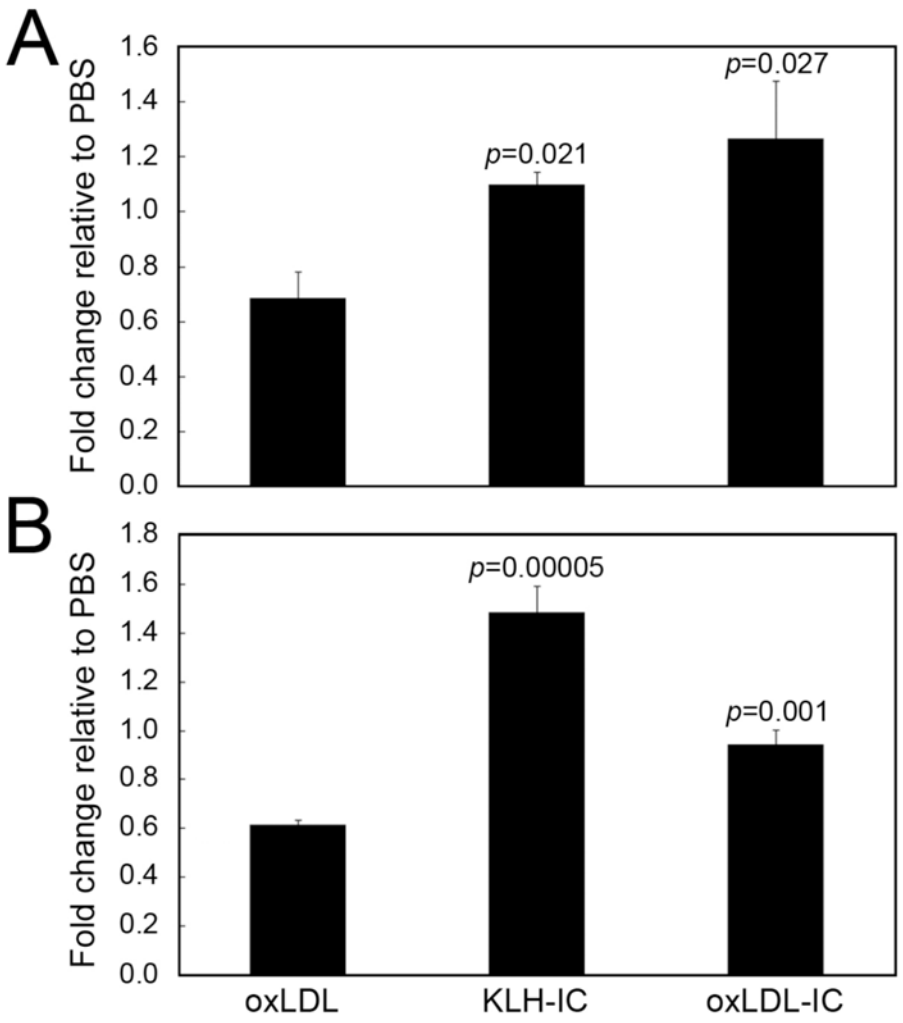

Figure 5.

Q-PCR analysis of the lipoprotein receptors CD36 ***(A)*** and LDLR ***(B)***. U937 cells were exposed to oxLDL-IC, KLH-IC, oxLDL (150 μg/ml), or PBS vehicle for 4 h. Quantification of RNA was performed using the cycle threshold of receptor cDNA relative to that of GAPDH. Values presented are means of triplicate determinations ± SE; data are representative of two experiments. Indicated *p* values were derived from a Student's unpaired t-test of data compared with oxLDL data.

Table 1

PCR primers

Gene	Accession No.	Symbol	Sequences of primers (5′ to 3′)
Sphingosine kinase 1	NM_021972	SPHK1	Forward: CTGGCAGCTTCCTTGAACCAT Reverse: TGTGCAGAGACAGCAGGTTCA
Heat shock 70kDa protein 6	NM_002155	HSPA6	Forward: CCCTAAGGCTTTCCTCTTGC Reverse: CATGAAGCCGAGCAGTACAA
CD36	NM_000072	CD36	Forward: AAGCAAAGAGGTCCTTATACG Reverse: TCTGTTCCAACTGATAGTGAAG
Low density lipoprotein receptor	NM_000527	LDLR	Forward: GCAAGGACAAATCTGACGAG Reverse: TAACGCAGCCAACTTCATC
GAPDH	NM_002046	GAPDH	Forward: CTGAGTACGTCGTGGAGTC Reverse: AAATGAGCCCCAGCCTTC
β-Actin	NM_001101	ACTB	Forward: TCTAAGAGAATGGCCCAGTC Reverse: GGCACGAAGGCTCATCATTC

Table 2

Genes uniquely up-regulated by oxLDL-IC

Gene	Symbol	Accession No.	Gene ID[1]	Function[2]	FC[3] *vs* PBS	FC *vs* oxLDL	FC *vs* KLH-IC
Similar to Fibrinogen silencer-binding protein	RAD54B	NM_006550	25788	DNA repair and mitotic activity	2.10	2.06	1.93
RUN and FYVE domain containing 3	RUFY3	NM_014961	22902	cell division; targets proteins to membrane lipids via interaction with PI_3P	2.12	2.18	2.17
Small nuclear ribonucleoprotein polypeptide B″	SNRPB2	BE466925	6629	RNA splicing, SLE[4] autoantigen	2.71	2.95	2.65
Zinc finger and BTB domain containing 24	ZBTB24	BC036731	9841	regulation of transcription	3.35	3.53	3.14

[1] NCBI Entrez Gene ID number

[2] based on information from GO biological process annotations and PubMed.

[3] FC, fold change

[4] systemic lupus erythematosus

Table 3

Genes similarly up-regulated by oxLDL-IC and KLH-IC

Gene	Symbol	Accession No.	Gene ID[1]	Function[2]
Heat shock 70kDa protein 1A	HSPA1A	NM_005345	3303	stabilizes proteins against aggregation, protein folding, ubiquitin-proteasome pathway
Heat shock 70kDa protein 1B	HSPA1B	NM_005346	3304	anti-apoptosis, response to unfolded protein, protein folding
Heat shock 70kDa protein 6 (HSP70B′)	HSPA6	NM_002155	3310	response to unfolded protein
DnaJ (Hsp40) homolog, subfamily B, member 1	DNAJB1	NM_006145	3337	response to unfolded protein, protein folding, HSP binding
BCL2-associated athanogene 3	BAG3	NM_004281	9531	anti-apoptosis, protein folding, modulates the activity of HSP70 class
Jun B proto-oncogene	JUNB	NM_002229	3726	anti-apoptosis
Growth arrest and DNA-damage-inducible, beta	GADD45B	NM_015675	4616	regulation of growth and apoptosis in response to stress
Immediate early response 5	IER5	NM_016545	51278	mediates cellular response to mitogenic signals
SRY (sex determining region Y)-box 4	SOX4	BG528420	6659	determination of the cell fate (death/survival)
Superoxide dismutase 2, mitochondrial	SOD2	AL050388	6648	response to oxidative stress
Dual specificity phosphatase 1	DUSP1	AA530892	1843	response to oxidative stress
Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isoenzyme A	MGAT4A	BC031487	11320	carbohydrate metabolic process, cell differentiation
Coatomer protein complex, subunit alpha	COPA	BC037941	1314	protein transport between endoplasmic reticulum and Golgi
yrdC domain containing	YRDC	NM_024640	79693	regulates the activity of a variety of transporters
Oxysterol binding protein	OSBP	BC017975	5007	intracellular lipid transport
Tumor necrosis factor (ligand) superfamily, member 9	TNFSF9	NM_003811	8744	inflammatory response, cytokine activity
Interleukin 1, beta	IL1B	NM_000576	3553	inflammatory response
v-fos FBJ murine osteosarcoma viral oncogene homolog	FOS	BC004490	2353	inflammatory response, regulator of cell proliferation, differentiation, and transformation.
NFKB inhibitor interacting Ras-like 1	NKIRAS1	AI970120	28512	inflammatory response, regulates the degradation of IkappaB
Ribosomal protein L17	hCG_2004593	AA522618	6139	inflammatory response, signal transducer activity, regulation of I-kappaB kinase/NF-kappaB cascade
RNA binding motif protein 8A	RBM8A	BC017770	9939	RNA splicing
Early growth response 1	EGR1	AI459194	1958	regulation of transcription
Early growth response 2	EGR2	NM_000399	1959	regulation of transcription
Oligodendrocyte transcription factor 1	OLIG1	AL355743	116448	regulation of transcription

Gene	Symbol	Accession No.	Gene ID[1]	Function[2]
Embryonic ectoderm development	EED	AF099032	8726	repression of gene activity, regulator of integrin function
Zinc finger protein 342	ZNF342	AA761573	162979	regulation of transcription
Zinc finger protein 36, C3H type	ZFP36	NM_003407	7538	mRNA catabolism
Zinc finger, AN1-type domain 2A	ZFAND2A	AI984061	90637	binding to metal ions
Dual specificity phosphatase 2	DUSP2	NM_004418	1844	MAPK signaling pathway
Regulator of G-protein signalling 2, 24kDa	RGS2	NM_002923	5997	cell cycle, regulation of G-protein coupled receptor protein
Myosin regulatory light chain interacting protein	MYLIP	NM_013262	29116	cell motility, protein ubiquitination

[1] NCBI Entrez Gene ID number

[2] based on information from GO biological process annotations and PubMed.

Table 4

Genes uniquely regulated by oxLDL

Gene	Symbol	Accession No.	Gene ID[1]	Function[2]	FC[3] *vs* PBS	FC *vs* oxLDL-IC	FC *vs* KLH-IC
Interferon-induced protein with tetratricopeptide repeats 1	IFIT1	NM_001548	3434	immune response	−2.58	−3.77	−3.25
Src-like-adaptor	SLA	U44403	6503	intracellular signaling cascade	−2.45	−2.72	−2.32
Target of EGR1, member 1 (nuclear)	TOE1	NM_025077	114034	growth inhibitory activity	−1.93	−2.45	−2.15
AHA1, activator of heat shock 90kDa protein ATPase	AHSA2	AI831431	130872	co-chaperone, response to unfolded protein	−3.20	−2.35	−1.87
Phosphoinositide-3-kinase, regulatory subunit 3 (p55, Gamma)	PIK3R3	BE622627	8503	modulates the activity of the enzyme phosphatidylinositol 3-kinase activity	−1.89	−1.76	−2.31
Zinc finger protein 697	ZNF697	AW003092	90874	regulation of transcription	1.85	2.09	2.08
Oxidative stress induced growth inhibitor 1	OSGIN1	NM_013370	29948	growth inhibition, cell death	2.54	2.57	2.58
Estrogen-responsive B box Protein	TRIM16	NM_006470	10626	enhancing the alternative secretion pathway of IL-1beta	4.26	2.62	2.76
Ferritin, heavy polypeptide 1	FTH1	AA083483	2495	inhibition of proliferation, ferric iron binding	3.23	3.40	3.92
Sulfiredoxin 1 homolog	SRXN1	AL121758	140809	oxidoreductase activity	3.26	3.49	3.56
Solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	SLC7A11	AB040875	23657	amino acid transport	3.15	4.04	3.84
Adipose differentiation-related Protein	ADFP	BC005127	123	long-chain fatty acid transport, sequestering of lipid	4.20	4.42	4.42
Heme oxygenase (decycling) 1	HMOX1	NM_002133	3162	oxidoreductase activity, iron ion binding	95.67	58.49	25.90

[1] NCBI Entrez Gene ID number

[2] based on information from GO biological process annotations and PubMed.

[3] FC, fold change