Current Pharmaceutical Design, 2018, *24*, 3143-3151

RESEARCH ARTICLE

Modified LDL Particles Activate Inflammatory Pathways in Monocyte-derived Macrophages: Transcriptome Analysis

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ARTICLE HISTORY

Received: July 27, 2018
Accepted: September 4, 2018

DOI:
10.2174/1381612824666180911120039

Abstract: ***Background*:** A hallmark of atherosclerosis is its complex pathogenesis, which is dependent on altered cholesterol metabolism and inflammation. Both arms of pathogenesis involve myeloid cells. Monocytes migrating into the arterial walls interact with modified low-density lipoprotein (LDL) particles, accumulate cholesterol and convert into foam cells, which promote plaque formation and also contribute to inflammation by producing pro-inflammatory cytokines. A number of studies characterized transcriptomics of macrophages following interaction with modified LDL, and revealed alteration of the expression of genes responsible for inflammatory response and cholesterol metabolism. However, it is still unclear how these two processes are related to each other to contribute to atherosclerotic lesion formation.

***Methods*:** We attempted to identify the main mater regulator genes in macrophages treated with atherogenic modified LDL using a bioinformatics approach.

***Results*:** We found that most of the identified genes were involved in inflammation, and none of them was implicated in cholesterol metabolism. Among the key identified genes were interleukin (IL)-7, IL-7 receptor, IL-15 and CXCL8.

***Conclusion*:** Our results indicate that activation of the inflammatory pathway is the primary response of the immune cells to modified LDL, while the lipid metabolism genes may be a secondary response triggered by inflammatory signalling.

Keywords: Atherosclerosis, inflammation, macrophages, LDL, modified LDL, pro-inflammatory signaling.

1. INTRODUCTION

Accumulation of lipid-laden macrophages (foam cells) in the arterial wall is an important early step in the development of atherosclerotic plaques and pathogenesis of atherosclerosis. The main source of accumulating cholesterol in foam is modified low-density lipoprotein (LDL), which is ingested by macrophages in an unregulated fashion via scavenger and pattern-recognition receptors, mostly by scavenger receptor A (SRA) and CD36 [1]. Native LDL can also cause accumulation of cholesterol, but high concentrations are required for this effect [2]. Foam cells exhibit diminished capacity to migrate while producing increased levels of pro-inflammatory cytokines, thus promoting inflammation and plaque progression [3]. Despite the central role of foam cells in atherogenesis, our knowledge about transcriptomic changes occurring during macrophages conversion into foam cells is surprisingly limited. In 2000, Shiffman with co-authors performed large scale gene expression experiment in a THP-1 macrophage model studying the response to oxidized LDL (Ox-LDL) [4]. They reported several clusters of genes up- or down-regulated in a timely fashion. A prominent group of genes upregulated early after Ox-LDL exposure involved genes regulating cholesterol metabolism, including scavenger receptors SCA and CD36, nuclear receptors PPARγ, LXRα and RXRγ, and cholesterol efflux protein ABCA1. A more recent study analyzed the transcriptome of bone-marrow-derived murine macrophages incubated with acetylated LDL (AcLDL) and confirmed activation of LXR and LXR-dependent cholesterol metabolism regulating *Abca1, Abcg1* and *Stac2* genes [5].

Another large group of genes that underwent changes of activity was related to inflammatory responses. Surprisingly, contrary to *in vivo* evidence, macrophage-derived foam cells in progressive atherosclerotic plaques were characterized by higher expression of genes involved in inflammation than cells from regressing plaques [6]. Shiffman with co-authors reported up-regulation of genes with anti-inflammatory activities, such as IL1-RA, DSCR1, annexin 1, and the Burton's tyrosine kinase repressor SH3 protein, and down-regulation of a number of pro-inflammatory genes, including leu-

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kotriene A4 hydrolase, cathepsin G, elastase 2, RNase A family 2 and 3 proteins, cytochrome*b*-245, and CD64 [4].

In this work, we used a transcriptomic analysis to identify genes responsible for the accumulation of cholesterol in human monocyte-derived macrophages exposed to modified LDL. Experiments generating "-omics" datasets are used to monitor the expression of proteins, lipids and RNAs in various physiological and pathological cellular conditions. A common challenge is to reveal the causal molecular mechanisms of diseases at the level of cellular regulatory networks. Due to specific pathological epigenetic changes in the genomes, cellular regulatory networks are often rewired in disease conditions as compared to the normal state. Reconstruction of disease-specific regulatory networks and identification of possible master regulators of these networks, i.e. transcription factors and genes that may regulate the activity of the whole network, can provide a clue to potential specific molecular targets for blocking the pathological regulatory cascades and suppressing the disease development.

There are several databases that collect transcriptomic data, such as ArrayExpress [7] and Gene Expression Omnibus (GEO) [8]. Specialized databases such as the Expression Atlas [9] and Mouse Expression Database (GXD) [10] contain identified sets of differentially expressed genes (DEG), also known as expression signatures. Expression signatures allow identifying potential drug targets by looking at statistical significance of the expression changes. Another approach, which can give more detailed results, is mapping the DEG sets to Gene Ontology (GO) categories or to KEGG pathways, for instance by GSEA (gene set enrichment analysis) [11, 12].

Unfortunately, the abovementioned methods provide only limited information on the causative relationships in gene expression changes. In a strategy introduced by our group over a decade ago, the "upstream analysis" approach for causal interpretation of the expression changes, has been designed to identify upstream master regulators [13-17]. This strategy has three major steps: (1) analysis of promoters and enhancers of identified DEGs to identify transcription factors (TFs) involved in the process under study; (2) reconstruction of signaling pathways that activate these TFs; and (3) identification of master-regulators of these pathways [18]. Using this approach, we revealed unexpectedly that regulation of some inflammation-associated genes was responsible for the accumulation of cholesterol in monocyte-derived macrophages exposed to modified LDL.

2. MATERIALS AND METHODS

2.1. Lipoproteins

Native human LDL, oxidized LDL, acetylated LDL, and high-density lipoprotein (HDL) were from Kalen Biomedical (Montgomery Village, MD, USA). LDL was desialylated by treatment of native LDL (2 mg of protein/ml) with agarose-bound neuraminidase 40 μU/ml for 2 hours at 37°C. This treatment results in a loss of up to 70% of sialic acid [19]. After desialylation, LDL was centrifuged for 10 minutes at 2,500 rpm to remove agarose particles and dialyzed against PBS.

2.2. Monocyte-derived Macrophages

Monocytes were prepared from peripheral blood of healthy individuals by plastic adhesion. The experiment was approved by the institutional Ethics committee, and all volunteers have agreed to participate in the experiment. Cells were incubated at 37°C in cell culture incubator with 5% CO_2 for 2 h, and rinsed three times with RPMI-1640 to remove non-adherent cells. The remaining adherent cells were mechanically detached and plated in 24-wells (1 x 10^6 cells per well) or 6-wells (3 x 10^6 cells per well) Primaria culture plates (Corning, USA) for intracellular cholesterol measurement. Cells were cultured in RPMI supplemented with 10% of human serum, 50 ng/ml human M-CSF (PeproTech, USA), and 25 ng/ml IL-10 (PeproTech, USA). Medium was refreshed on day 3 and replaced with serum-free X-VIVO medium (Lonza Group Ltd, Switzerland) on day 6. The resulting monocyte-derived macrophages were used for experiments on day 7. On day 7, X-VIVO medium was changed again and LDL (50 μg/ml) or HDL (30 μg/ml) was added to cell cultures for 24 hours. Then intracellular cholesterol measurement was performed, and total RNA was isolated from the cells by RNeasy Plus Mini kit (Qiagen).

2.3. Intracellular Cholesterol

To measure intracellular cholesterol, cells were rinsed three times in Dulbecco's phosphate-buffered saline containing Ca^{2+} and Mg^{2+} (DPBS), lysed with 1 ml/well of ultrapure water, and collected with a cell scraper. Lipids were isolated using the Folch method [20], and cholesterol was quantified as previously described [21]. Protein was measured in 40 μl aliquots of cell lysate using Lowry method [22] with bovine serum albumin solution as a standard. All measurements were performed in duplicate.

2.4. RNA Sequence

RNA-seq libraries were prepared using a NEBNext Ultra RNA library prep kit for Illumina according to the manufacturer's instructions. Libraries were PCR-amplified for 12-15 cycles, and sequenced on a HiSeq 1500 (Illumina).

2.5. Limma

In this study, we used Limma (Linear Models for Microarray Data) method to define fold changes of genes and to identify the statistically significantly expressed genes using a Benjamini-Hochberg adjusted p-value cutoff (≤0.05) [23]. The details of this method have been published previously [18]. The raw RNA-seq data were normalized and background corrected beforehand and converted into the RPKM values (Reads Per Kilobase of transcript per Million mapped reads) that were used as input values into the Limma analysis.

2.6. RankProd

Identification of differentially expressed genes that were up- or down- regulated, was based on the estimated percentage of false predictions (PFP), using the non-parametric RankProd method [24]. This method allows for meta-analysis of combined data sets from different origins to increase the power of the identification. The algorithm of RankProd works as follows: The rank of the genes is calculated as a sum of its ranks in all six Limma comparisons. The resulting ranks for all genes are then ordered by their values separately for up- and down-regulated genes. Using the RankProd algorithm the statistical analysis of gene changes among all six groups of comparisons was obtained. The threshold value of PFP = 0.15 (this threshold was recommended in ref. [24]) was then used for selection of the differentially expressed up- and down-regulated genes.

2.7. Upstream Analysis Pipeline in geneXplain Platform

The first step of "upstream analysis" strategy, identification of TFs of DEGs, was performed with the use of the TRANSFAC® database [11] and site identification algorithms, Match [25] and CMA [26]. The second step, reconstruction of signaling pathways that activate these TFs and identification of master-regulators, was carried out with the help of the TRANSPATH database [27], and graph search algorithms implemented in the geneXplain platform [16-18].

2.8. Analysis of Enriched Transcription Factor Binding Sites

TF binding sites in promoters of differentially expressed genes were analyzed using known DNA-binding motifs described in the TRANSFAC® library, release 2014.4 (geneXplain, Wolfenbüttel,

Germany) (http://genexplain.com/transfac). The motifs were specified using position weight matrices (PWMs) that give weights to each nucleotide in each position of the DNA binding motif for a TF or a group of TFs. TF binding sites (TFBS) enriched in the studied promoters compared to a background sequence set were identified using geneXplain tools. The study and background sets were marked as "Yes" and "No" sets. F-Match, the algorithm for TFBS enrichment analysis, has been described previously [15, 16]. TFBSs that achieved a Yes/No ratio >1 and a P-value < 0.01 were selected for further analysis. We considered promoter sequences of a standard length of 1,100 bp (−1000 bp to +100 bp from transcription start). The error rate in this part of the pipeline is controlled by estimating the adjusted p-value (using Benjamini-Hochberg procedure [28]) in comparison to TFBS frequency found in randomly selected regions of the human genome (adjusted p-value < 0.01).

2.9. Search for Composite Modules Using CMA Algorithm

We applied the CMA (Composite Module Analyst) algorithm for searching composite modules [26] in the promoters of up- and down-regulated genes. We searched for composite module consisting of a cluster of 10 TFs (represented by TF PWMs from TRANSFAC®) in a sliding window of 200-300 bp that statistically significantly separates sequences in the "Yes" and "No" sets (Wilcoxon p-value = 5.41E-24).

2.10. Finding Master Regulators in Networks

Master regulators upstream of the identified TFs were identified using geneXplain platform tools and the TRANSPATH® database (geneXplain) [27]. The main algorithm of master regulator search has been described earlier [15]. We run the algorithm with the maximum radius of 10 steps upstream of each TF in the input set. Control of the error rate of this algorithm was done by applying it 10,000 times to randomly generated sets of input transcription factors of the same size of the sets. Z-score and false discovery rate (FDR) value of ranks was calculated then for each potential master regulator node on based on such random runs (see detailed description in [27]). The error rate by the FDR threshold was 0.05.

3. RESULTS

3.1. Sample Preparation

Monocyte-derived macrophages were subjected to 5 different treatments in triplicate wells (details are in the Methods section): native LDL, oxidized LDL, acetylated LDL, desialylated LDL, and HDL, or were left untreated (no addition). After 24 hours, total cellular cholesterol and cholesteryl esters were determined (Table **1**). We confirmed that low concentrations of native LDL and HDL did not affect the total or esterified cholesterol content in cultured macrophages. All studied modified LDL samples caused a significant increase in total cholesterol and cholesteryl esters.

At the end of the experiment, RNA was isolated from the cells and mRNAs were analyzed by high-throughput sequencing on HiSeq 1500. In total, 16 samples from 6 groups indicated in Table 1 were obtained and analyzed (MDM from 3 different donors per each group, except the desialylated LDL, which was done with cells from a single donor). RNA sequence data were analyzed using "upstream analysis" [17, 18]. Our aim was to identify the so-called master regulators in gene regulatory networks, in our case looking for TFs that control the foam cell formation and potentially may be considered as drug targets. As explained in the Introduction, upstream analysis consists of three consequent steps: identification of DEGs, analysis of promoters and TFs of DEGs, and search for potential master-regulators.

3.2. Detection of Differentially Expressed Genes (DEGs)

To detect DEGs, we compared samples with cholesterol accumulation (cells incubated with modified LDL) to samples without cholesterol accumulation (HDL, native LDL and no addition). First, we applied Limma to all possible pair comparisons of the samples, except the cells treated with desialylated LDL (desLDL) which had only one replicate. This was followed by the RankProd analysis of the pairwise comparison results. Finally, we performed the full-Limma analysis with the use of all available samples gathered altogether (without group separation). As a result, using the RankProd cut-off PFP = 0.15 (recommended in the RankProd method description [24]), we obtained 4,573 upregulated and 4,072 downregulated genes (Supplementary Table **1**). With the chosen PFP cut-off, the method revealed a statistically significant difference in the expression of the selected genes averaged between all comparisons, whereas in each comparison pair the statistically significant difference was not guaranteed. No gene was found to be differentially expressed in all pairs at statistically significant levels. We also applied the full-Limma analysis by comparing all samples incubated with modified LDLs (including desLDL) versus all samples without cholesterol accumulation (HDL, native LDL and no addition). Among them, changes in 93 up-regulated genes and 54 down-regulated genes remained significant after adjusting the p-value following the full-Limma analysis (full logFC (logarithm of fold change) > 0 and adjusted p-value < 0.05) (Supplementary Table 1, genes marked by colored background).

Table 1. Cholesterol content in cultured MDM.

Treatment	Total Cholesterol (nmol/mg protein)		Cholesteryl Esters (nmol/mg protein)	
	Mean±SEM	P-value (vs control)	Mean±SEM	P-value (vs control)
No addition	53±3	-	1±2	-
Native LDL	62±2	NS	6±1	NS
Oxidized LDL	93±14	0.0361	35±14	0.0427
Acetylated LDL	106±4	0.0007	40±2	0.0001
Desialylated LDL	83±8	0.0498	24±3	0.0257
HDL	56±3	NS	0±1	NS

NS = Not significant.

Table 2. Atherosclerosis linked genes (based on HumanPSD database information) found as significantly up- or downregulated.

	Gene Symbol	Gene Description	logFC full*	logFC acLDL vs. Control	logFC acLDL vs. HDL	logFC acLDL vs. native LDL	logFC oxLDL vs. Control	logFC oxLDL vs. HDL	logFC oxLDL vs. native LDL
1	ABCA1	ATP binding cassette subfamily A member 1	1.22	1.56	2.68	0.83	1.08	2.21	0.36
2	CXCL8	chemokine (C-X-C motif) ligand 8	0.99	1.62	0.15	0.15	2.06	0.59	0.59
3	CXCL9	chemokine (C-X-C motif) ligand 9	0.61	-0.14	0.87	0.30	-0.25	0.75	0.19
4	IL15	interleukin 15	0.54	0.16	0.91	0.56	0.20	0.95	0.61
5	MMP1	matrix metallopeptidase 1	0.34	0.78	0.05	0.32	0.74	0.00	0.27
6	SOAT1	sterol O-acyltransferase 1	0.25	0.63	0.58	0.45	0.04	-0.01	-0.13
7	TNFSF14	tumor necrosis factor superfamily member 14	0.24	-0.18	-0.35	-0.01	0.69	0.52	0.86
8	CCL1	chemokine (C-C motif) ligand 1	0.22	0.67	-0.86	0.74	0.63	-0.89	0.71
9	CD1B	CD1b molecule	0.12	1.07	-0.23	0.94	0.28	-1.01	0.16
10	HBEGF	heparin-binding EGF-like growth factor	0.06	1.29	0.04	0.65	0.03	-1.22	-0.61
11	EGR1	early growth response 1	0.05	1.18	-0.22	1.48	-0.30	-1.70	0.00
12	FOS	FBJ murine osteosarcoma viral oncogene homolog	0.01	1.22	0.04	1.47	-0.42	-1.60	-0.17
13	CD1C	CD1c molecule	-0.01	0.48	0.01	0.74	-0.33	-0.80	-0.07
14	MMP7	matrix metallopeptidase 7	-0.02	-0.43	0.66	-0.28	-0.34	0.75	-0.19
15	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	-0.06	-0.44	-0.53	-0.31	0.00	-0.09	0.12
16	IL1B	interleukin 1 beta	-0.07	0.98	-0.89	1.02	-0.01	-1.87	0.03
17	CD1A	CD1a molecule	-0.10	0.31	-0.23	0.52	-0.28	-0.81	-0.07
18	IL10	interleukin 10	-0.16	-0.54	1.05	-0.15	-0.93	0.66	-0.54
19	TNFSF15	tumor necrosis factor superfamily member 15	-0.23	-0.22	-0.87	0.47	-0.12	-0.77	0.57
20	CD9	CD9 molecule	-0.31	-0.26	0.05	0.01	-0.78	-0.47	-0.51
21	CXCL10	chemokine (C-X-C motif) ligand 10	-0.36	-0.40	-0.30	0.35	-1.07	-0.97	-0.32
22	F3	coagulation factor III (thromboplastin, tissue factor)	-0.70	-0.15	-1.65	0.31	-0.54	-2.03	-0.07
23	TGFB1	transforming growth factor beta 1	-0.74	-1.02	-1.89	-1.28	0.17	-0.70	-0.09
24	TNFSF13	tumor necrosis factor superfamily member 13	-0.82	-1.14	-1.64	-0.90	-0.58	-1.08	-0.34
25	CSF1	colony stimulating factor 1 (macrophage)	-0.84	-0.57	-2.18	0.11	-0.61	-2.21	0.08

logFC full * show comparison of acLDL + oxLDL + desLDL *vs* Control+ HDL + native LDL

Table 3. Transcription factors selected for analysis.

ID	Title	Gene Description	Gene Symbol
MO000018136	c-Ets-1A(h)	v-ets avian erythroblastosis virus E26 oncogene homolog 1	ETS1
MO000019545	c-Ets-2(h)	v-ets avian erythroblastosis virus E26 oncogene homolog 2	ETS2
MO000019546	SAP-1a(h)	ELK4, ETS-domain protein (SRF accessory protein 1)	ELK4
MO000019614	GR-alpha(h)	nuclear receptor subfamily 3 group C member 1	NR3C1
MO000023577	hdac2(h)	histone deacetylase 2	HDAC2
MO000025650	GABP-beta1(h)	GA binding protein transcription factor, beta subunit 1	GABPB1
MO000046075	TAFII250(h)	TATA-box binding protein associated factor 1	TAF1
MO000056591	Kaiso(h)	zinc finger and BTB domain containing 33	ZBTB33
MO000057927	Elk1(h)	ELK1, member of ETS oncogene family	ELK1
MO000058770	Sp1(h)	Sp1 transcription factor	SP1
MO000079982	TEL1(h)	ets variant 6	ETV6
MO000080982	brca1(h)	breast cancer 1	BRCA1
MO000082496	Fli-1(h)	Fli-1 proto-oncogene, ETS transcription factor	FLI1
MO000083592	E2F-1(h)	E2F transcription factor 1	E2F1
MO000088374	GABP-alpha(h)	GA binding protein transcription factor alpha subunit	GABPA
MO000088705	Sp3(h)	Sp3 transcription factor	SP3
MO000088889	Egr-1(h)	early growth response 1	EGR1
MO000095458	KLF8(h)	Kruppel-like factor 8	KLF8
MO000095624	sp4(h)	Sp4 transcription factor	SP4
MO000102906	NRF-1(h)	nuclear respiratory factor 1	NRF1
MO000104451	FKLF(h)	Kruppel-like factor 11	KLF11
MO000114727	E2F-6(h)	E2F transcription factor 6	E2F6
MO000115949	CNOT3(h)	CCR4-NOT transcription complex subunit 3	CNOT3
MO000117843	TIEG-1(h)	Kruppel-like factor 10	KLF10
MO000138365	Net(h)	ELK3, ETS-domain protein (SRF accessory protein 2)	ELK3
MO000140876	znf580(h)	zinc finger protein 580	ZNF580
MO000176427	DEAF1(h)	DEAF1, transcription factor	DEAF1

ID is the unique identifier of corresponding transcription factor in the TRANSPATH® database.

The enrichment of genes for various categories of diseases can be performed by using the Gene Set Enrichment Analysis (GSEA) method [12]. We applied GSEA to the full list of the 4573 upregulated and 4072 downregulated genes obtained by PathProd analysis. Among up- and down-regulated genes, there was significant enrichment of the "Atherosclerosis" disease category (from the HumanPSD database found at www.genexplain.com/HumanPSD). Table **2** lists 25 genes, which are known markers of atherosclerosis (atherosclerosis linked genes in the HumanPSD database). The upregulated genes are numbered 1-12, and thirteen of the downregulated genes are under numbers 13-25.

Further, the analysis of the enrichment of the selected genes by signaling pathways was performed. For this, the GSEA algorithm was used with the TRANSPATH® database [27]. This analysis revealed a relationship to such important pathways as the TGFβ pathway, the p53 pathway, the E2F network, the EGF pathway, the HIF-1alpha pathway, and also the more specific pathways, such as IL-8 and IL-1 (Supplementary Table **2**). In total, we identified 480 upregulated and 380 downregulated genes that were mapped to several signaling pathways in TRANSPATH®.

3.3. Promoter Analysis

For identification of the mechanism of activation of the revealed genes and their regulation in the cells, for further analysis we have chosen the genes involved in cells' signaling pathways (based on application of GSEA method described above). Our focus on genes encoding components of signal transduction pathways (such as receptors, adaptors, intracellular kinases and phosphatases,

transcription factors, etc.) provided an opportunity to understand the mechanism of self-regulation of the regulatory machinery of the cells. For these genes, we searched their promoters for the binding sites of transcription factors. After filtration by a threshold value of statistical significance (Yes/No ratio >1 and a P-value < 0.01), we selected 27 transcription factors (listed in Table **3**) that are potentially responsible for the changes in gene expression after treatment of cells with modified LDLs. Among these transcription factors were c-Ets, GR-alpha, BRCA1, E2F-1, E2F-6 and EGR-1.

3.4. Search for Master-regulator Molecules Upstream of TFs

We used the discovered 27 transcription factors as an input for the algorithm of search for master regulators – master-genes and master-proteins that are responsible for regulation of large cascades of DEGs found in the performed experiments. From that analysis, we identified 148 potential master regulators (Supplementary Table **3**). Among the entire detected list of master-regulators, we performed additional selection for genes encoding the master-regulator proteins and fulfilling more stringent requirements: we required that the genes are characterized by RankProd score higher than 0.15 and full-LogFC higher than 0.7 (see above); and that the change of expression of these genes should be always in the same direction in all pairwise comparisons considered above. Applying these requirements, we selected the following genes as the most reliable master-regulators: IL7R, TIGIT, CXCL8, F2RL1, EIF2AK3, IL7, TSPYL2, ANXA1, DUSP1 and IL15. Expression of these genes in cells with the cholesterol accumulation was different compared to the cells without cholesterol accumulation in all samples in our experiments, and this difference was in the same direction in all samples. A diagram of signal flow from the revealed master-regulators to the identified transcription factors is presented in the Supplementary Fig. **1**.

4. DISCUSSION

Transcriptomic analysis is widely used to characterize gene expression in atherosclerosis [19, 29, 30]. This method is also used to study the interaction of vascular cells with atherogenic LDL [31, 32]. Previous studies produced large data sets of genes activated or down-regulated in macrophages exposed to modified LDLs. An important part of these sets are genes belonging to cholesterol metabolism and inflammation pathways. Such large sets of data hide a lot of information on gene interaction, which can be revealed only by applying dedicated bioinformatics approaches. However, no attempts have been made so far to determine the relationship between genes regulating cholesterol metabolism and inflammation following exposure of macrophages to modified LDLs. In particular, it remains unknown whether these two pathways are independently regulated by modified LDL, or regulation of one pathway drives changes in the other. Available literature presents evidence that these two pathways do interact [33], but it remains uncertain regarding the leader in this interaction, and examples of both possible scenarios have been described. Indeed, changes in cholesterol metabolism can affect inflammatory responses of macrophages [34, 35], likely via activation of the LXR nuclear receptor [36, 37], and changes in expression of inflammatory genes affect cholesterol metabolism [38-43]. The use of innovative bioinformatics methods in this study allowed us to identify 148 master genes responsible for the accumulation of intracellular cholesterol caused by atherogenic modified LDL (Supplemental Table **3**). The top ten genes (described below) may be the key regulators of foam cell formation. Surprisingly, seven of these top ten genes belong to the inflammatory pathway, and none was from the cholesterol metabolism pathway.

Interleukin 7 receptor (IL7R, ENSG00000168685) is best known for its role in V(D)J recombination during lymphocyte development [44], and defects in this gene may be associated with severe combined immunodeficiency (SCID). The role of this gene in atherosclerosis was first revealed in gene set enrichment analysis utilizing data from microarray experiments with obese white adipose tissue and atherosclerotic aortae in a combined insulin resistance-atherosclerosis mouse model. This study demonstrated a vast overlap in gene expression alterations in obese adipose tissue and atherosclerosis, with IL7R as one of the highest ranked genes for the inflammatory response pathway [45].

TIGIT (T-cell immunoreceptor with Ig and ITIM (immunoreceptor tyrosine-based inhibitory motif) domains, ENSG00000181847) encodes a member of the PVR (poliovirus receptor) family of immunoglobin proteins. The product of this gene is expressed on several classes of T cells including T regulatory cells (Tregs) [46]. Tregs plasticity and functionality in apoE$^{-/-}$ mice has an effect on the pathology of atherosclerosis, producing a unique transcriptional phenotype characterized by co-expression of Treg and Th1 lineage genes and a downregulation of Treg-related genes, including TIGIT [47].

CXCL8 (ENSG00000169429) is a member of the CXC chemokine family and is a major mediator of the inflammatory response. CXCL8 is expressed predominantly by neutrophils, but its upregulation was observed in human monocytes differentiated into macrophages in the presence of M-CSF and subjected to the M1-polarizing factors [48]. When macrophages were further converted into cholesterol-loaded foam cells by incubation with acetylated LDL, they showed a weaker response to the M1-polarizing factors, as indicated by reduced upregulation of the pro-inflammatory genes, including CXCL8.

F2RL1 (coagulation factor II receptor-like 1, ENSG00000164251) encodes a member of the G-protein coupled receptor 1 family of proteins. Mice lacking F2RL1 or its cytoplasmic domain were protected from weight gain and insulin resistance induced by a high-fat diet. Genetic ablation of tissue factor-F2RL1 signaling reduced adipose tissue macrophage inflammation, and specific pharmacological inhibition of macrophage tissue factor signaling rapidly ameliorated insulin resistance [49].

EIF2AK3 (eukaryotic translation initiation factor 2 alpha kinase 3, ENSG00000172071) phosphorylates and inactivates the alpha subunit of eukaryotic translation-initiation factor 2, resulting in global protein synthesis repression. The PERK/eIF2α/CHOP endoplasmic reticulum stress pathway largely mediates oxidized LDL-induced apoptosis in vascular endothelial cells [50].

IL-7 (ENSG00000104432) is a cytokine produced by a variety of cells, including follicular dendritic cells [51]. It is known to be important for B and T cell development, but its role in atherosclerosis has not been established.

TSPYL2 (testis-specific protein Y-encoded-like protein 2, ENSG00000184205) encodes a member of the testis-specific protein Y-encoded, TSPY-like/SET/nucleosome assembly protein-1 superfamily. The mRNA levels of TSPYL2 were elevated in the aorta of diabetic ApoE$^{-/-}$ mice, accompanied by increased levels of TGF-beta and extracellular matrix accumulation; knockdown of TSPYL2 blocked the pro-fibrotic effect of TGF-beta in vascular smooth muscle cells [52].

ANXA1 (annexin A1, ENSG00000135046) encodes a membrane-localized protein that binds phospholipids. ANXA1 and its derived peptides affect leukocytes as well as endothelial cells and tissue resident cells, like macrophages and mast cells; it also has a key role in limiting leukocyte recruitment and modulation of leukocyte adhesion cascade [53].

DUSP1 (dual specificity phosphatase 1, ENSG00000120129) is a phosphatase with dual specificity for tyrosine and threonine. The encoded protein can dephosphorylate MAP kinase MAPK1/ERK2 and plays an important role in the human cellular response to environmental stress as well as in the negative regulation of cellular proliferation [54]. DUSP1 is also known as mitogen-activated protein kinase phosphatase-1 (MKP-1), which is expressed in the athe-

rosclerotic lesions of mice, and inhibition of MKP-1 reduced atherosclerotic lesions in mouse models [55], whereas depletion of MKP-1 protected ApoE-null mice against atherosclerosis [56].

IL-15 (ENSG00000164136) is a pro-inflammatory cytokine that is constitutively expressed by a large number of cell types and tissues, including monocytes, macrophages and dendritic cells [57]. It plays an important role in innate and adaptive immunity by regulating T and natural killer cell activation and proliferation [58]. IL-15 is expressed in atherosclerotic plaques and is now considered a key component of atherosclerosis [59]. Genetic variants of IL-15 gene and IL-15 levels are associated with coronary heart disease and influence the risk of disease [60], and anti-IL-15 DNA vaccination strategy in mice markedly reduced atherosclerotic lesion size [61].

Therefore, nine of the top 10 master genes or the molecules encoded by them are known to be associated with the molecular and cellular mechanisms of atherosclerosis or are biomarkers of atherosclerotic diseases. In most cases (7 of the top 10 genes), the functions of the molecules encoded by the identified genes are related to the immune response and inflammation. Several studies evaluating the innate immune responses to modified LDL revealed an increase in the expression and production of cytokines resulting from the interaction of cells with modified lipoproteins. Oxidized LDL and lipid-derived species existing in oxidized LDL stimulated the release of IL-1β, TNFα, MCP-1 and MMP-2 [62-64]; minimally oxidized LDL induced secretion of pro-inflammatory cytokines MIP-2, MCP-1, TNFα, and IL-6 [65] and increased transcription of pro-inflammatory chemokines CXCL2 (MIP-2), CCL3 (MIP-1α), and CCL4 (MIP-1β) [66]; oxidized LDL upregulated the expression of pro-inflammatory IL-1β and induced its secretion [67]; and acetylated or oxidized LDL increased mRNA expression and secretion of pro-inflammatory cytokines (IL-6, IL-18, IL-8, TNFα) during the foam cell formation [68]. Thus, activation of inflammatory and immune responses following incubation of macrophages with modified LDL has been documented before. A surprising finding of this study is that none of the identified top 10 master-regulator genes are directly related to the intracellular metabolism of cholesterol. The currently accepted model assumes the primary role of lipid accumulation in functional changes associated with conversion of macrophages to foam cells [33]. Changes in lipid content then induce the pro-inflammatory responses.

CONCLUSION

In this work, we have demonstrated that treatment of macrophages with atherogenic LDL resulted in up-regulation of genes involved in the inflammation and immune responses, without affecting the activity of known cholesterol metabolism-regulating genes. Our results suggest a possibility that it is not cholesterol accumulation that causes an innate immunity response, but rather the immune response is a consequence of a cellular reaction to modified LDL. Such reassessment of the transcriptomic data suggests that targeting the inflammatory component of atherosclerosis may be the most effective way of slowing the development of this disease.

FUNDING BODY

This work was supported by Russian Science Foundation (Grant # 14-15-00112); the experiments on cell culture were supported by the intramural research program of the National Heart, Lung, and Blood Institute, NIH; by NIH grants R01 HL093818, R01 HL101274; and by the District of Columbia Center for AIDS Research (DC CFAR), an NIH-funded program (P30 AI055019).

AUTHORS' CONTRIBUTION

ANO, YO and HSK designed the experiments, XJ, NGN, AVZ, LD, KF and YO conducted the experiments, IAS, AK, DS and VJM analyzed the data, ANO, HSK, MB, IAS and AK wrote the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The experiment was approved by the Ethics committee Institute of General Pathology and Pathophysiology, Moscow, Russia.

HUMAN AND ANIMAL RIGHTS

No animal were used in this study. Reported experiments on humans were in accordance with the ethical standards of the committee responsible for human experimentation (institutional national), and with the *Helsinki Declaration* of 1975, as revised in 2008 (http://www.wma.net/en/20activities/10ethics/10helsinki/).

CONSENT FOR PUBLICATION

All volunteers have agreed to participate in the experiment.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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