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

Alexander N. Orekhov12*, Yumiko Oishi 3, Nikita G. Nikiforov1,4, Andrey V. Zhelankin5, Larisa Dubrovsky12, Igor A. Sobenin4, Alexander Kel6,7,8, Daria Stelmashenko6,7,8, Vsevolod J. Makeev9, Kathy Foxx10, Xueling Jin11, Howard S. Kruth11 and Michael Bukrinsky12

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 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, LXRA 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 IL-1 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-
kotriene A4 hydroxylase, cathepsin G, elastase 2, RNase A family 2 and 3 proteins, cytochrome-β-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 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% CO2 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 10⁶ cells per well) or 6-wells (3 x 10⁶ 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 Ca²⁺ and Mg²⁺ (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 Benjamin-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).
Table 1. Cholesterol content in cultured MDM.

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

<sup>*</sup> 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 | hda2c(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 | brcal(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 | FKL1(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 | zm580(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 up-regulated genes are numbered 1-12, and thirteen of the down-regulated 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) 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-
Roscelerotic 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.

REFERENCES

[1] Kunjathoor VV, Febbraio M, Podrez EA, et al. Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. J Biol Chem 2002; 277(51): 49982-8.
[2] Kruth HS. Receptor-independent fluid-phase pinocytosis mechanisms for induction of foam cell formation with native low-density lipoprotein particles. Curr Opin Lipidol 2011; 22(5): 386-93.
[3] Moore KJ, Sheedy FJ, Fisher EA. Macrophages in atherosclerosis: A dynamic balance. Nat Rev Immunol 2013; 13(10): 709-21.
[4] Shifman D, Mikita T, Tai JT, et al. Large scale gene expression analysis of cholesterol-loaded macrophages. J Biol Chem 2000; 275(48): 37324-32.
[5] Berisha SZ, Hus J, Robinet P, Smith JD. Transcriptome analysis of genes regulated by cholesterol loading in two strains of mouse macrophages associates lysosome pathway and ER stress response with atherosclerosis susceptibility. PLoS One 2013; 8(5): e65003.
[6] Feig JE, Rong JX, Shamir R, et al. HDL promotes rapid atherosclerosis regression in mice and alters inflammatory properties of plaque monocyte-derived cells. Proc Natl Acad Sci USA 2011; 108(17): 7166-71.
[7] Kolesnikov N, Hastings E, Keays M, et al. ArrayExpress update—simplifying data submissions. Nucleic Acids Res 2015; 43(Database issue): D1113-6.
[8] Barrett T, Wilhite SE, Ledoux P, et al. NCBI GEO: Archive for functional genomics data sets--update. Nucleic Acids Res 2013; 41(Database issue): D991-5.
[9] Petryszak R, Burdett T, Fiorelli B, et al. Expression Atlas update--a database of gene and transcript expression from microarray- and sequencing-based functional genomics experiments. Nucleic Acids Res 2014; 42(Database issue): D926-32.
[10] Finger JH, Smith CM, Hayamizu TF, et al. The mouse Gene Expression Database (GXD): 2017 update. Nucleic Acids Res 2017; 45(D1): D730-6.
[11] Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: A knowledge-based approach for interpreting genomewide expression profiles. Proc Natl Acad Sci USA 2005; 102(43): 15545-50.
[12] Kanelis M, Goto S, Sato Y, Furumichi M, Tanabe K, KEGG for integration and interpretation of large-scale molecular data sets. Nucleic Acids Res 2012; 40(Database issue): D109-14.
[13] Kel A, Voss N, Jauregui R, Kel-Margoulis O, Wingender E. Beyond microarrays: find key transcription factors controlling signal
transduction pathways. BMC Bioinformatics 2006; 7(Suppl. 2): S13.

[14] Michael H, Hogan J, Kel A, et al. Building a knowledge base for systems pathology. Brief Bioinform 2008; 9(6): 518-31.

[15] Stegmaier P, Voss N, Meier T, Kel A, Wingender E, Borlak J. Advanced computational biology methods identify molecular switches for malignancy in an EGF mouse model of liver cancer. PLoS One 2011; 6(3): e17738.

[16] Koschmann J, Bhar A, Stegmaier P, Kel AE, Wingender E. “Upstream Analysis”: An integrated promoter-pathway analysis approach to causal interpretation of microarray data. Microarrays (Basel) 2015; 4(2): 270-86.

[17] Kel A. Data on master regulators and transcription factor binding sites found by upstream analysis of multi-omics data on methotrexate resistance of colon cancer. Data Brief 2016; 10: 499-504.

[18] Kel AE, Stegmaier P, Valeev T, et al. Multi-omics “upstream analysis” of regulatory genomic regions helps identifying targets against methotrexate resistance of colon cancer. EuPA Open Proteome 2016; 13: 1-13.

[19] Shalhub J, Sikkel MB, Davies KJ, Vorkas PA, Want EJ, Davies AH. Systems biology of human atherosclerosis. Vasc Endovascular Surg 2014; 48(1): 5-17.

[20] Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957; 226(1): 497-509.

[21] Gamble W, Vaughan M, Kruth HS, Avigan J. Procedure for determination of free and total cholesterol in micro- or nanogram amounts suitable for studies with cultured cells. J Lipid Res 1978; 19(9): 1068-70.

[22] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193(1): 265-75.

[23] Smyth GK. 2005. Linear models for microarray data. Limma

[24] Hong F, Breitling R, McEntee CW, Wittner BS, Nemhauser JL, Chory J. RankProd: A bioconductor package for detecting differentially expressed genes in meta-analysis. Bioinformatics 2006; 22(22): 2825-7.

[25] Kel AE, Gösslöing E, Reuter I, Cheremushkin E, Kel-Margoulis V, Wingender E. MATCH: A tool for searching transcription factor binding sites in DNA sequences. Nucleic Acids Res 2003; 31(13): 3576-9.

[26] Waleev T, Shtokalo D, Konovalova T, et al. Composite Module Analyst: Identification of transcription factor binding site combinations using a scoring algorithm. Nucleic Acids Res 2006; 34(Web Server issue): W541-5.

[27] Krull M, Pistor S, Voss N, et al. TRANSPATH: An information resource for storing and visualizing signaling pathways and their pathological aberrations. Nucleic Acids Res 2006; 34(Database issue): D546-51.

[28] Ferreira JA. The Benjamini-Hochberg method in the case of discrete test statistics. Int J Biostat 2007; 3(1): 11.

[29] Anbazhagan K, Duroux-Richard J, Jorgensen C, Apparailly F. Transcription network support distinct roles of classical and non-classical monocytes in human. Int Rev Immunol 2014; 33(6): 470-89.

[30] Schultze JL. Transcriptional programming of human macrophages: on the way to systems immunology. J Mol Med (Berl) 2015; 93(6): 589-97.

[31] Diámán-Zamacona S, Toledo-Belles P, Ibarra-Abutidis MZ, et al. Early Transcriptomic Response to LDL and oxLDL in Human Vascular Smooth Muscle Cells. PLoS One 2016; 11(10): e0163924.

[32] Ho MM, Fraser DA. Transcriptome data and gene ontology analysis in human macrophages ingesting modified lipoproteins in the presence or absence of complement protein C1q. Data Brief 2016; 9: 362-7.

[33] Tall AR, Yvan-Charvet L. Cholesterol, inflammation and innate immunity. Nat Rev Immunol 2015; 15(2): 104-16.

[34] Dang EV, McDonald JG, Russell DW, Cyster JG. Oxysterol receptor mediated cholesterol synthesis prevents AIM2 inflammasome activation. Cell 2017; 171(5): 1057-1071.e11.

[35] Kelly B, O’Neill LA. Metabolic reprogramming in macrophages and dendritic cells in innate immunity. Cell Res 2015; 25(7): 771-84.

[36] Töröcsik D, Szanto A, Nagy L. Oxysterol signaling links cholesterol metabolism and inflammation via the liver X receptor in macrophages. Mol Aspects Med 2009; 30(3): 134-52.

[37] Im SS, Osborne TF. Liver x receptors in atherosclerosis and inflammation. Circ Res 2011; 108(8): 996-1001.

[38] Hajjar DP, Hajjar KA. (2016) Alterations of cholesterol metabolism in inflammation-induced atherogenesis. J Enzymol Metab 2016; 1: pii 104.

[39] Kovidhunkit W, Memon RA, Feingold KR, Grunfeld C. Infection and inflammation-induced proatherogenic changes of lipoproteins. J Infect Dis 2000; 181(Suppl. 3): S462-72.

[40] Ma AZ, Zhang Q, Song ZY, TNFa alter cholesterol metabolism in human macrophages via PFK-b-d-dependent pathway. BMC Biochem 2013; 14: 20.

[41] Kidani Y, Bensinger SJ. Reviewing the impact of lipid synthetic flux on Th17 function. Curr Op Immunol 2017; 46: 121-6.

[42] Ito A, Hong C, Oka K, et al. Cholesterol accumulation in CD11c+ immune cells is a causal and targetable factor in autoimmune disease. Immunity 2016; 45(6): 1311-26.

[43] Kidani Y, Bensinger SJ. Modulating cholesterol homeostasis to build a better T cell. Cell Metab 2016; 23(6): 963-4.

[44] Cândéias S, Muegge K, Durum SK. IL-7 receptor and VDJ recombination: tropic versus mechanistic actions. Immunity 1997; 6(5): 501-8.

[45] Moreno-Viedma V, Amor M, Sarabi A, et al. Common dysregulated pathways in obese adipose tissue and atherosclerosis. Cardiovasc Diabetol 2016; 15(1): 120.

[46] Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: Co-inhibitory receptors with specialized functions in immune regulation. Immunity 2016; 44(5): 989-1004.

[47] Butcher MJ, Filipowicz AR, Waseem TC, et al. Atherosclerosis-driven treg plasticity results in formation of a dysfunctional subset of plastic IFNγ+ Th1/Tregs. Circ Res 2016; 119(11): 1190-203.

[48] da Silva RF, Lappalainen J, Lace-Eueckert M, Kovanes PT. Conversion of human M-CSF macrophages into foam cells reduces their proinflammatory responses to classical M1-polarizing activation. Atherosclerosis 2016; 248: 170-8.

[49] Badeanlu I, Furlan-Freguia C, Yang G, Ruf W, Samad F. Tissue factor-protease-activated receptor 2 signaling promotes diet-induced obesity and adipose inflammation. Nat Med 2011; 17(11): 1490-7.

[50] Tso TK, Yu PL, Bai YP, Yan ST, Zhao SP, Zhang QG. Role of PERK/eIF2α/CHOP endoplasmic reticulum stress pathway in oxidized low-density lipoprotein mediated induction of endothelial apoptosis. Biomed Environ Sci 2016; 29(12): 868-76.

[51] Kröncke R, Loppnow H, Flad HD, Gerdes J. Human follicular dendritic cells and vascular cells produce interleukin-7: A potential role for interleukin-7 in the germinal center reaction. Eur J Immunol 1996; 26(10): 2541-4.

[52] Pham Y, Tu Y, Wu T, et al. Cell division autoantigen 1 plays a proflibitory role by modulating downstream signalling of TGF-beta in a murine diabetic model of atherosclerosis. Diabetologia 2010; 53(1): 170-9.

[53] de Jong R, Leoni G, Drechsler M, Soehnlein O. The advantageous role of annexin A1 in cardiovascular disease. Cell Adhes Migr 2012; 6(1): 24.

[54] Reddy ST, Nguyen JT, Grijalva V, et al. Potential role for mitogen-activated protein kinase phosphatase-1 in the development of atherosclerotic lesions in mouse models. Arterioscler Thromb Vase Biol 2004; 24(9): 1676-81.

[55] Shen J, Chandrasekharan UM, Ashraf MZ, et al. Lack of mitogen-activated protein kinase phosphatase-1 protects ApoE-null mice against atherosclerosis. Circ Res 2010; 106(5): 902-10.

[56] Grabstein KH, Eisenman J, Shanebeck K, et al. Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. Science 1994; 264(5161): 965-8.
Modified LDL Particles Activate Inflammatory Pathways in Monocyte-derived Current Pharmaceutical Design, 2018, Vol. 24, No. 26

[58] Lodolce JP, Burkett PR, Koka RM, Boone DL, Ma A. Regulation of lymphoid homeostasis by interleukin-15. Cytokine Growth Factor Rev 2002; 13(6): 429-39.

[59] Wuttge DM, Eriksson P, Sirsjö A, Hansson GK, Stemme S. Expression of interleukin-15 in mouse and human atherosclerotic lesions. Am J Pathol 2001; 159(2): 417-23.

[60] Gokkusu C, Aydin M, Ozkok E, et al. Influences of genetic variants in interleukin-15 gene and serum interleukin-15 levels on coronary heart disease. Cytokine 2010; 49(1): 58-63.

[61] van Es T, van Puijvelde GH, Michon IN, et al. IL-15 aggravates atherosclerotic lesion development in LDL receptor deficient mice. Vaccine 2011; 29(5): 976-83.

[62] Ku G, Thomas CE, Akeson AL, Jackson RL. Induction of interleukin 1 beta expression from human peripheral blood monocyte-derived macrophages by 9-hydroxyoctadecadienoic acid. J Biol Chem 1992; 267(20): 14183-8.

[63] Thomas CE, Jackson RL, Ohlweiler DF, Ku G. Multiple lipid oxidation products in low density lipoproteins induce interleukin-1 beta release from human blood mononuclear cells. J Lipid Res 1994; 35(3): 417-27.

[64] Yang K, Zhang XJ, Cao LJ, et al. Toll-like receptor 4 mediates inflammatory cytokine secretion in smooth muscle cells induced by oxidized low-density lipoprotein. PLoS One 2014; 9(4): e95935.

[65] Miller YI, Viriyakosol S, Worrall DS, Boullier A, Butler S, Witzum JL. Toll-like receptor 4-dependent and -independent cytokine secretion induced by minimally oxidized low-density lipoprotein in macrophages. Arterioscler Thromb Vasc Biol 2005; 25(6): 1213-9.

[66] Wiesner P, Choi SH, Almazan F, et al. Low doses of lipopolysaccharide and minimally oxidized low-density lipoprotein cooperatively activate macrophages via nuclear factor kappa B and activator protein-1: possible mechanism for acceleration of atherosclerosis by subclinical endotoxemia. Circ Res 2010; 107(1): 56-65.

[67] Jiang Y, Wang M, Huang K, et al. Oxidized low-density lipoprotein induces secretion of interleukin-1β by macrophages via reactive oxygen species-dependent NLRP3 inflammasome activation. Biochim Biophys Res Commun 2012; 425(2): 121-6.

[68] Bekkering S, Quintin J, Joosten LA, van der Meer JW, Netea MG, Riksen NP. Oxidized low-density lipoprotein induces long-term proinflammatory cytokine production and foam cell formation via epigenetic reprogramming of monocytes. Arterioscler Thromb Vasc Biol 2014; 34(8): 1731-8.