Induction of Dendritic Cell-like Phenotype in Macrophages during Foam Cell Formation

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Abstract

Foam cell formation from monocyte-derived macrophages is a hallmark of atherosclerotic lesions. Aspects of this process can be recapitulated in vitro by exposing MCSFinduced or platelet factor 4 (CXCL4)-induced macrophages to oxidized (ox) or minimally modified (mm) low density lipoprotein (LDL). We measured gene expression in peripheral blood mononuclear cells (PBMCs), monocytes and macrophages treated with CXCL1 (GRO- α) or CCL2 (MCP-1) as well as foam cells induced by native LDL, mmLDL or oxLDL using 22 Affymetrix gene chips. Using an advanced Bayesian errorpooling approach and a heterogeneous error model (HEM) with a false discovery rate (FDR) <0.05, we found 5,303 of 22,215 probe sets to be significantly regulated in at least one of the conditions. Among a subset of 917 candidate genes that were preselected for their known biological functions in macrophage foam-cell differentiation, we found that 290 genes met the above statistical criteria for significant differential expression patterns. While many expected genes were found to be upregulated by LDL and oxLDL, very few were induced by mmLDL. We also found induction of unexpected genes, most strikingly MHC-II and other dendritic cell markers such as CD11c. The gene expression patterns in response to oxLDL were similar in MCSF-induced and CXCL4-induced macrophages. Our findings suggest that LDL and oxLDL, but not mmLDL, induce a dendritic cell-like phenotype in macrophages, suggesting that these cells may be able to present antigens and support an immune response.

Introduction

Foam cell formation from monocyte-derived macrophages is a hallmark of atherosclerosis (4, 10, 14, 16). Atherosclerotic plaque contains foam cells that store cholesterol and cholesterol esters. In the later stages of the disease, foam cells undergo apoptosis and secondary necrosis, which leads to the formation of a lipid-rich, pro-coagulant core of the atherosclerotic lesion (34). When atherosclerotic lesions rupture, exposure of blood to this pro-coagulant material causes immediate and massive thrombosis, leading to serious or fatal cardiovascular events including myocardial infarctions (40) and ischemic strokes.

Macrophage-derived foam cells are of particular importance in the development and health consequences of atherosclerotic disease, because macrophages are found in the shoulder regions of "vulnerable" plaques, where they destabilize the plaque by expressing various pro-inflammatory chemokines, cytokines, matrix-degrading metalloproteases and other mediators that tend to promote plaque rupture (21, 42). Plaque macrophages are thought to be derived from blood monocytes, which are recruited into the arterial wall through adhesion molecules and chemokines. The adhesion molecule P-selectin transiently binds PSGL-1 to support rolling (26), and $\alpha_4\beta_1$ integrin binds to VCAM-1 to slow rolling cells and support adhesion (11). The chemokine CXCL1 (GRO-a/KC) is immobilized on the endothelial surface and binds its receptor CXCR2 on rolling monocytes to promote arrest (11). CCL5 (RANTES) also induces arrest by binding to its receptor CCR1 (11, 41), and CCL2 (MCP-1/JE) binds its receptor CCR2. Once monocytes enter the arterial wall, they differentiate to macrophages, probably under the influence of M-CSF, GM-CSF and their receptors. In the vessel wall, these cells continue to encounter an environment containing chemokines such as CXCL1, CCL2 and platelet factor 4 (CXCL4) as well as a variety of other differentiating factors. These factors modulate macrophage phenotype; in fact, CXCL4 alone is sufficient to induce macrophage differentiation even without M-CSF (29).

Plaque-resident foam cells are thought to form under the influence of modified forms of low density lipoproteins (LDL) (30). LDL may be modified by aggregation, oxidation, and other biochemical processes (17). Minimally modified (oxidized) LDL (mmLDL) can be produced in vitro by incubating native LDL with endothelial cells, smooth muscle cells, or cells transfected with 12/15 lipoxygenase (17). Chemical oxidation of LDL by exposure to copper salts (17) produces extensively oxidized LDL (oxLDL), a form of LDL that is known to have shared and unique biological effects on macrophages compared with mmLDL. Although not universally accepted, chemically unmodified LDL appears to also induce foam cell formation (14), perhaps after aggregation (24). Native LDL is taken up by a specific LDL receptor and perhaps by patocytosis (14), while oxLDL is bound and internalized through a large array of scavenger receptors including CD36 (9, 25), scavenger receptor that binds phosphatidylserine and oxidized lipoprotein SR-PSOX (32), and oxidized LDL receptor (LOX-1) (23).

Although much is known about the expression of adhesion molecules, chemokine receptors, nuclear receptors, scavenger receptors, lipoxygenases and other molecules in macrophages and foam cells, no comprehensive analysis of gene expression in these cells has been conducted. In two laser capture microdissection studies conducted in foam cells isolated from atherosclerotic lesions of apoE^{-/-} mice, the expression of nine genes was followed by qRT-PCR (36, 37). In a separate study, gene expression was analyzed in THP-1 cells in response to oxLDL and other interventions using Incyte microarrays (31).

In order to better understand macrophage differentiation and foam cell formation, the present study was undertaken using an unbiased, broad-based approach measuring the expression of 12,978 genes represented by 22,215 probe sets using the Affymetrix U133A gene chip on human blood mononuclear cells, isolated monocytes, macrophages, and foam cells with and without chemokine and LDL treatment.

Materials and Methods

Monocytes and Macrophages

Human blood was drawn from the antecubital veins of healthy blood donors and provided as buffy coats by the Virginia Blood Services (Richmond, VA). The mononuclear fractions were pooled from four unidentified donors to decrease individual variations in monocytes. Mixed peripheral blood mononuclear cells (PBMCs) were isolated by Histopaque 1.077 (Sigma Diagnostics, Inc., St. Louis, MO). Following centrifugation, the mononuclear layer was removed and washed with PBS containing 0.02% ethylenediaminetetraacetate (EDTA). The pellet was resuspended in 1X H-lyse Buffer (R&D Systems Inc., Minneapolis, MN), and washed with wash buffer. PBMCs contain mainly monocytes and lymphocytes as well as platelets that tend to be associated with blood monocytes (28). From these PBMCs, monocytes were isolated using a negative selection monocyte isolation kit and LS columns (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the isolated fraction was > 97% as estimated by flow cytometry using anti-CD14 (data not shown). Although these cells are often called "untouched" monocytes and thought to show little activation, the gene chip analysis conducted on these cells shows massive changes in gene expression compared to PBMCs (see below).

Monocytes were cultured in Macrophage Serum-Free Medium (MSFM, Invitrogen, Carlsbad, CA) in the presence of 1% media supplement nutridoma-HU (Roche Molecular Biochemicals, Indianapolis, IN) and 100 nM M-CSF for 6 days, after which the cells showed the expected morphological signs of macrophage differentiation. These macrophages were incubated either with 100 nM CCL2 or CXCL1 for 5 hours. CXCL1 was selected because we have previously found that it is an important arrest chemokine

for monocytes in vitro and in atherosclerotic arteries in vivo. CCL2 was chosen because mice lacking CCL2 (1) or its receptor CCR2 (3) are relatively resistant to atherosclerosis, suggesting a role in macrophage recruitment, differentiation and/or survival. Human monocyte-derived macrophages (MDM) were also incubated with native LDL, oxidized LDL (oxLDL) or minimally modified LDL (mmLDL) (each at a concentration of 100 µg/ml) for 2 days to induce foam cell formation. Foam cell formation was verified by oil red O staining (figure 2) and by determining their cholesterol and cholesterol ester content. OxLDL and mmLDL were prepared from the same native LDL for each experiment as described (18). Control experiments were conducted on macrophages cultured in M-CSF without LDL for an additional 2 days. Two separate sets of monocytes were incubated with CXCL4 (100 nM) for 6 days, another procedure known to induce macrophage differentiation (29), with and without oxLDL to induce foam cell formation. RNA was extracted from cells in all 11 conditions (table 1) and gene expression was measured in duplicates at the University of Virginia Gene Expression Core Facility using Affymetrix equipment.

Flow cytometry

Antibodies and appropriate isotype controls for protein expression studies were purchased from BD Biosciences (Franklin Lakes, NJ; CD11b, CD14, CD36, CD62L, CD68, CD206, DC-LAMP/CD208, PD-L2/CD273), BioLegend (San Diego, CA; CD64, CD83, CD86, CD163, CD205, CMKLR1), and R&D Systems (Minneapolis, MN; CCR7, DCIR, DC-SIGN). Macrophages and oxLDL treated macrophages were prepared as stated above. As a control, monocyte-derived dendritic cells were obtained by stimulation with

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IL-4 (10 ng/ml) and GM-CSF (50 ng/ml; both from Peprotech, Rocky Hill, NJ) for eight days. Cells were harvested by cell scraping, washed twice, incubated with Abs for 20 min at 4°C and again washed twice. Immunofluorescence was measured by flow cytometry (FACSCalibur), data were analyzed using FlowJO (Tree Star Inc.) software.

Data preprocessing, normalization, and analysis software

Signal intensity values were obtained from the Affymetrix MicroArray Suite software (MAS 5.0). Of 22,283 probe sets on the HG-U133A chip, 78 internal control probes were removed and 22,215 probe sets representing 12,978 gene products were analyzed. Microarray gene expression intensities were normalized in order to ensure that all 22 array chips have the same inter-quartile ranges (IQR). In addition, they were log-transformed with base 2, which allows transforms the right-skewed distribution closer to a normal distribution. While the log transformation enables a convenient interpretation of differential expression as fold changes, it is not a transformation that typically stabilizes variance. The variability of log-intensity measurements in oligonucleotide microarrays tends to decrease non-linearly with the increase in the mean expression intensity. This is in part due to common background noise at each spot of the microarray. At high intensity levels, this background noise is dominated by the expression intensity, while at low levels the background noise is a large component of the observed expression intensity.

The commonly used method of fold-change cutoff (for example, 2-fold) is not suitable for rigorous statistical analysis of gene expression, because at any given cutoff many genes with low levels of expression do not meet significance criteria, and other, highly expressed genes may miss the cutoff, although their change is really significant

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(13). For statistical analysis, an open source statistical software package **R** (www.rproject.org) was used, which includes the local pooled error (LPE) test for differential expression discovery under two conditions, the heterogeneous error model (HEM) for differential expression discovery under multiple conditions, hierarchical clustering & heatmap analysis, and self-organizing maps (SOM), especially the last two widely used in microarray data analysis (8, 35). The annotation information available from the Affymetrix website (www.affymetix.com) was used to identify the genes represented on the HG-U133A chip for the various classes of genes analyzed (see results). We eliminated non- expressed (within 2 SD from zero in all conditions) and housekeeping genes (not significantly regulated with false discovery rate FDR < 0.05) as described below. This eliminated 16,783 genes from analysis. We analyzed the regulated genes in two approaches; candidate gene analysis using LPE, HEM and heatmap analysis, and unbiased analysis of all regulated genes without prior knowledge such using hierarchical clustering analysis (figure 1).

LPE and HEM analyses

The LPE test (13) was used to investigate differential expression between two conditions because it is statistically powerful in identifying differentially expressed genes with low-replicated microarray data, e.g., duplicate or triplicate. LPE pools probe sets with similar expression levels provides a statistic for each probe set and the absolute value of the LPE-statistic is larger for more significantly differentially expressed probe set. HEM (6) is designed to investigate differential expression in microarray experiments comparing multiple conditions, taking advantages of the error pooling power of LPE. HEM captures

heterogeneous error variability of microarray data, so that it enabled us to reliably identify differentially expressed genes with a significantly higher statistical power from the macrophage microarray data with limited replication (duplicates). In addition, a false discovery rate (FDR) was calculated to discover probe sets differentially expressed with FDR < 0.05 (12).

Gene set enrichment analysis

Gene set enrichment analysis (33) was performed to demonstrate regulation of gene sets of interest. The analysis determines whether an a priori defined set of genes shows statistically significant differences between two conditions. To perform enrichment analysis, we used GSEA, which is software program freely distributed a at http://www.broad.mit.edu/gsea/. GSEA calculates an enrichment score reflecting the degree of overrepresentation of an a priori defined set of genes, and estimates its significance with adjusting for multiple hypothesis testing.

Results

After the IQR normalization of the microarray data, chip-by-chip correlations were calculated among the 22 arrays, which ranged from r=0.78 to 0.96 (supplemental table 1). We found that within-condition correlations were extremely high, average 0.95, which assures the specificity and quality of the array experiments. Between-condition correlations were lower (r averaged around 0.87), suggesting that some of the 11 conditions were significantly regulated, potentially with a large number of genes with differential expression patterns.

Of the 22,215 probe sets representing 12,978 genes, 129 probe sets showed no significant expression (NE) and 16,783 probe sets (supplemental table 2) showed no significant regulation across the 11 conditions tested and were therefore eliminated from further analysis. The remaining 5,303 probe sets were subjected to unbiased expression analysis using SOM and Venn diagrams to analyze monocyte differentiation to macrophages and foam cells (11 conditions, figure 3a). Since gene expression changes between monocytes and macrophages dominated many of the patterns, we analyzed macrophage and foam cell conditions (9 conditions, figure 3b) and chemokine conditions (CXCL1, CCL2 and CXCL4, data not shown) separately.

Inspection of SOM (figure 3 and supplemental table 3) reveals distinct patterns that formed the basis for further analysis. In the 11-condition analysis, 1511 probe sets (figure 3A, shaded, top left corner) showed increased expression by monocyte isolation (2 to 4-fold upregulation), followed by decreased expression upon differentiation to macrophages. This very large number of up-regulated genes was surprising, given that the method used to purify monocytes was the best method available and these cells are commonly called "untouched" monocytes (22). The reasons for the observed changes include the removal of contaminating lymphocytes, which tends to concentrate monocytespecific genes, and gene expression induced by the isolation procedure used.

A reasonably large number of genes (925) showed increased expression after monocyte isolation, followed by a further increase upon macrophage differentiation (figure 3A, bottom left). If monocyte isolation is considered an "activating" procedure, this set of probe sets may contain genes that show "increased activation". Four hundred thirtyseven probe sets showed no significant change between PBMCs and isolated monocytes, but were up-regulated by macrophage differentiation. These genes will be discussed in more detail below. A similar number of probe sets (477) showed no difference between PBMCs and monocytes, but was down-regulated upon macrophage differentiation.

The comparison of nine conditions revealed groups of genes upregulated by oxidized and native LDL (2108 probe sets, figure 3B, top boxes, oxLDL and LDL indicated by arrows) and a similar number that is down-regulated by oxidized and native LDL (1831 probe sets, figure 3B, bottom boxes, oxLDL and LDL indicated by arrows). In general, minimally modified LDL (mm-LDL) did not induce significant changes that penetrated the SOM maps. However, mmLDL induced foam cell formation at least as strongly as oxLDL as judged by oil red O incorporation (see figure 2) and total cholesterol (cholesterol ester) content, which was elevated from 96 (6) nmol/mg protein in untreated macrophages to 118 (21) nmol/mg protein in oxLDL-treated macrophages.

To focus on contrasts between biologically interesting conditions, we constructed Venn diagrams showing the number of genes that are significantly up- or down-regulated during macrophage differentiation from monocytes and compared them to genes that are up- or down-regulated by addition of LDL (figure 4A, see also supplemental table 4). Only small numbers of genes were found to be shared, but overall most genes that were regulated by LDL were also regulated by macrophage differentiation. A similar analysis was also conducted for oxLDL (figure 4B) and mmLDL (figure 4C). Lists of these genes are found in supplemental table 4. Interestingly, only six genes were uniquely and significantly up- and seven downregulated by mmLDL. The corresponding numbers are 14 and 6 for oxLDL and 99 and 52 for LDL.

When comparing gene expression in macrophages treated with the chemokines CXCL1 or CCL2, no genes were found that were up-regulated by CXCL1 and downregulated by CCL2 or vice versa (figure 4D). Most genes (322) were concomitantly up-regulated, or concomitantly down-regulated (115). A larger number of genes were uniquely regulated by CXCL1 (314 up, 274 down) than by CCL2 (98 up, 55 down).

Next, we compared the response to mmLDL with the response to unmodified LDL (figure 4E). A surprisingly small number of genes was regulated concomitantly between these two conditions (11 up, 10 down), and only seven genes were regulated in opposite directions. Similarly, we compared gene expression by oxLDL and LDL (figure 4F). To make the comparison more stringent, we only considered those genes that were up-regulated in both oxLDL conditions, whether the macrophages were differentiated with M-CSF or with CXCL4. This analysis yielded two empty sets: there were no genes that were up-regulated by oxLDL and down-regulated by LDL or vice versa. Almost all genes that were regulated by oxLDL were also regulated in the same direction by native LDL. In addition to the unbiased approach, 917 probe sets (see supplemental table 5 for list of genes) for candidate genes were analyzed separately. These candidate genes were grouped into genes involved in antigen presentation, cytokines and their receptors, heat shock proteins, chemokines and their receptors, cytochrome p450 CYPs, integrin alpha and beta subunits, genes associated with dendritic cell differentiation, phospholipases A, matrix metalloproteinases (MMPs), toll-like receptors (TLRs), immunoglobulin superfamily adhesion molecules, MAP kinases, selectins and their ligands, lipoxygenases, cyclooxygenases, connexins and channel proteins. Among these groups, the highest number of regulated genes was found in the genes regulating antigen presentation with 71 regulated genes, while at the other end of the scale none of 19 significantly expressed genes encoding for ion channels were regulated under the conditions tested (figure 5).

Altogether, 290 probe sets were significantly regulated and further analyzed by constructing heat maps (figure 6), where red indicates higher and green lower than average expression across all conditions, respectively, black indicates average expression, and blank (white) indicates fields where the duplicates were too different from each other to allow conclusions. For these heatmaps, probe sets and conditions were allowed to cluster freely in y and x direction, respectively. All duplicate conditions clustered together, indicating that the replicates were closer to each other in all cases than to any other condition and thus validating the quality of the data obtained. Gene expression in isolated monocytes (figure 6A, right) clustered far away from unfractionated PBMCs (left), indicating that many genes were heavily regulated by the procedures used to isolate monocytes and many genes were affected by removing lymphocytes and other contaminating cells from the mixed PBMCs. The chemokine-treated conditions (CXCL1, CXCL4 and CCL2) clus-

tered together, suggesting that these three chemokines induce similar sets of genes, but the number of genes that were changed from the untreated control was small.

The analysis for all 11 conditions showed six major clusters of overexpressed genes (figure 6A). Cluster **a** is highly expressed in PBMCs, but not in all other conditions except monocytes, where about half of cluster \mathbf{a} genes are highly expressed (marked by **b**) and the other half is underexpressed (supplemental table 6A). Many genes that are overexpressed in PBMCs reflect contaminating lymphocytes. Examples of genes found highly expressed in PBMCs but not in purified monocytes include TCR-β, CD8, CCR4, 7, 8 and CXCR3, IL-2 and IL-7 receptors and β_7 integrins, all of which likely result from lymphocyte contamination. Interestingly, no platelet or neutrophil genes are overexpressed. Genes in cluster **b** are overexpressed in purified monocytes, but not highly expressed elsewhere except in PBMCs. CXCR4 is overexpressed in both PBMCs and monocytes (multiple probe sets), which means that CXCR4 is lost during macrophage and foam cell differentiation. Some pro-inflammatory genes such as IL-8, CXCR2, CCR2 and COX-1 are also found in this cluster. Unexpectedly, β_2 microglobulin, a component of the MHC complex, is also overexpressed in **a** and **b**. Another unexpected finding is that IL-23 p19 is apparently constitutively expressed in PBMCs and monocytes, but downregulated during macrophage differentiation. Glucocorticoid receptor is also highly expressed in these two groups, but lost later in differentiation.

Cluster **c** represents genes that are highly expressed in monocytes only and not in PBMCs. Some of the genes are likely overexpressed secondary to the isolation procedure. This group of isolation-induced genes includes many MHC-II genes, including DM, DP, DQ and DR α , β_1 , β_4 and β_5 . $\alpha_x\beta_2$ integrin or CD11c was also found in this cluster, consis-

tent with dendritic cell-like differentiation. The tyrosine kinase Syk is involved in outside-in signaling through Mac-1 ($\alpha_M\beta_2$ integrin) and other adhesion molecules. Among toll-like receptors, TLR1 and 4 are also found overexpressed in this cluster. All these genes are also overexpressed in cluster **d** and thus in macrophages treated with LDL or oxidized LDL, but not mmLDL. This would therefore comprise a group of genes that is expressed in monocytes and in LDL- or oxLDL-differentiated foam cells.

A separate group of genes is induced in monocytes, but is not found in LDL or oxLDL-treated macrophages (**c** but not **d**). This group contains a number of chemokines like CCL2, 3, 4, 7 and 8, CXCL10 and the chemokine receptor CCR1. IL-1, IL-13 and interferon- γ receptors are also overexpressed in monocytes. Among adhesion molecules, ICAM-1 and β_2 integrins are overexpressed.

Cluster **e** represents a group of genes that are expressed in all macrophages, whether or not treated with chemokines. Most of these genes are suppressed in macrophages treated with LDL or oxidized LDL (**e** but not **f**). This group includes β_7 integrins, which are associated with gut-specific homing, and the NK-cell genes KIR2DS1, 2 and 5, and KIR3DL2. None of these genes were expected to be expressed in macrophages. In this group, only a few pro-inflammatory genes are found: VCAM-1, CCL1, CXCR6 and fucosyl transferase VII were significantly overexpressed in macrophages.

Genes found in clusters **e** and **f** (overexpressed across all forms of macrophages) include antigen presentation genes like MHC IIDQ α_1 and β_1 and MHC IG. Among chemokines, CXCL9, CCR5, 18 and 22 fall in this group, where the first two are proinflammatory. Another pro-inflammatory gene expressed in all macrophages is MMP-9. Some genes are expressed in LDL and oxLDL-treated macrophages, but less elsewhere (**f**

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but not **e**). This group includes 10 heat shock proteins, suggesting that LDL treatment causes significant stress to the macrophages. Surprisingly, only two bona-fide pro-inflammatory genes were induced in LDL- or oxLDL-treated macrophages; STAT-1 and MMP-12. PPAR- γ was overexpressed in these oxLDL-treated cells, a gene that is generally considered anti-inflammatory.

To allow closer analysis of gene expression in the macrophages and foam cells only, the clustering was repeated for nine conditions, leaving out PBMCs and monocytes (figure 6B). The general patterns of clustering were preserved (chemokines together, oxLDL together), but new clusters became apparent by removing the overbearing influence of the PBMC and monocytes conditions. The heatmap was bisected by vertical and horizontal borders, defining a large cluster of genes overexpressed in macrophages treated with oxLDL or LDL (a and supplemental table 6B), which is mirrored by the remaining genes that are more highly expressed in all other conditions (b). The expression was consistent whether foam cells were made by oxLDL from M-CSF-differentiated or CXCL4-differentiated macrophages. Almost none of these genes were induced by mmLDL. Cluster a includes 123 genes, 33 of which are involved in antigen presentation, including MHC class II DM, DP, DQ and DR as well as several accessory proteins such as TAP2, a molecule involved in peptide antigen processing. Twenty-eight heat shock proteins are overexpressed after LDL or oxLDL treatment. Among 18 cytokines and cytokine receptors, most probe sets for GM-CSF receptor showed overexpression, which would tend to support differentiation towards dendritic cells. Interferon gamma receptor and its signal transducing molecule STAT-1 as well as receptors for IL-1, 6, 7, 13 and 15 are also overexpressed. Seven probe sets for integrin subunits are induced by LDL or

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oxLDL treatment, including both subunits of $\alpha_5\beta_1$ integrin, β_3 , β_5 and α_x , which encodes the antigen CD11c, a dendritic cell marker. Other dendritic cell genes include three Sadenosylhomocysteine hydrolase probe sets. Among 13 genes encoding nuclear receptors, PPAR- γ , retinoic acid receptor and retinoid X receptor are all overexpressed. Overexpression of TLR1, 2 and 4, MMP14 and 19 are all consistent with a pro-inflammatory phenotype. Among pro-inflammatory chemokines, IL-8 and one of its two receptors, CXCR2, are significantly overexpressed, suggesting an autocrine pro-inflammatory loop. As expected, most of these probe sets overlap with clusters **d** and **f** in the 11-condition heatmap.

Clusters \mathbf{c} and \mathbf{d} are practically identical, although they do not cluster together because of the intervening LDL-treated macrophages. Clusters \mathbf{c} and \mathbf{d} represent genes induced by all modified LDLs, but not native LDL. The largest group of 9 genes concerns antigen presentation, mostly MHC class II, followed by seven chemokines and their receptors.

A unique group of genes formed cluster **e** of suppressed expression in CXCL4differentiated macrophages treated with oxLDL. Both probe sets of CCL5 are suppressed, as is CXCR6. These changes and suppression of IL-23 would have to be considered antiinflammatory. Among adhesion molecules, ICAM-2 and α_4 integrin are consistently suppressed (2 probes sets each), as are PSGL-1 and L-selectin.

A separate cluster of 63 genes is underexpressed in untreated macrophages (cluster **f**). This cluster is a subset of cluster a (overexpressed in LDL and oxLDL-treated macrophages). The three TLRs 1, 2 and 4 and many nuclear receptors as well as 21 of the 33 genes encoding antigen presentation molecules are significantly missing from un-

treated macrophages. Both probe sets for CCL18 are also underexpressed, as is GM-CSF receptor, interferon gamma receptor, IL-10 and IL-13 receptors and IL-15.

Given that DC genes appeared to be upregulated in response to oxLDL, we analyzed a set of DC genes (supplemental table 7) using gene set enrichment analysis (33). We performed the enrichment analysis for the set of DC genes by GSEA with the default options. We found that the set of DC genes was enriched significantly in macrophages treated with oxLDL, mmLDL, and CXCL4+oxLDL at a nominal p-value < 0.05, but not with LDL.

The biological validation of the findings obtained by the gene chip experiment was based on a three-tiered system. First, we compared our findings to those obtained by others in THP-1 cells (31). THP-1 cells were incubated with oxLDL for various times, including 2 days, which is a time point we investigated in the present study using primary cells. The THP-1 cell study (31) was conducted using a different hybridization platform (Incyte). We identified 238 genes that could be matched between their experiment and ours and expressed the relative expression as a ratio of gene expression in oxLDL-treated cells divided by control cells. The comparison analysis shows that there are statistically significant correlations between the two studies (r=0.39, p-value < 0.001), which is reasonable considering that the earlier study (31) was done with a cell line and our study was done with primary blood monocyte-derived macrophages and using a different assay platform (Affymetrix). Next, the medical literature was searched for each of the gene products represented by the 290 probe sets in the candidate gene approach. The information was categorized as confirmatory, conflicting, inconclusive or no information (supplemental table 8). In some particularly interesting cases, additional laboratory experiments were conducted at the protein level. Specifically, we used flow cytometry to analyze the expression of CD11b, CD14, CD36, CD62L, CD68, CD83, CD86, CD163, CD205, CD206, CD208, CD273, CMKLR1, CCR7, DCIR and DC-Sign in macrophages treated with and without oxidized LDL and compared these to bona fide dendritic cells. oxLDL caused an increase in CD206 and a decrease in CD62L, CD68 and CD83, all consistent with a dendritic cell-like phenotype (figure 7). One of seven genes upregulated at the mRNA level also showed increased surface expression of its product, six of ten unregulated genes were also unregulated on the cell surface, and three were found to be downregulated although their gene expression was not changed significantly. Five molecules could not be detected at the protein level.

Discussion

The comprehensive and unbiased analysis of gene expression during foam cell formation yielded the expected induction of inflammatory genes, but also many unexpected results. Among the unexpected results, the bias toward dendritic cell differentiation induced by foam cell-inducing agents stood out. Dendritic cells differentiation were previously postulated to be anti-atherosclerotic, whereas macrophage polarization was considered pro-atherosclerotic (27). This view is not supported by the current data. Out of 9 DC genes, 7 genes were significantly up-regulated by CXCL4 and oxLDL, compared to CXCL4-differentiated macrophages (condition 11 versus 10). Similar but less dramatic changes were also seen in foam cells induced by oxLDL, mmLDL, and LDL from M-CSF-differentiated macrophages (conditions 7, 8 and 9 versus 6; supplemental table 5).

In a microarray analysis of expression of 6805 human genes expressed in THP-1 cells (31), 268 were found to be upregulated at least two-fold. We identified 238 genes that could be compared with our study, and we determined a reasonable degree of correlation between the two studies, given that different platforms and different cells were used (supplemental figure 2 and supplemental table 9). In another study, 640 genes were significantly up- or down-regulated by oxidized LDL in endothelial cells (7). Of the 640 genes, 150 were also significantly regulated in CXCL4-induced macrophages exposed to oxidized LDL (present study). The top genes that were concomitantly regulated in both experiments were metallothionein 1G, H, F and 2H, heme oxygenase (decycling) 1, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble) and farnesyl-diphosphate farnesyltransferase. Interestingly, all metallothionins were strongly up-regulated by oxLDL in both endothelial cells and macrophages. Metallothioneins are known to have

protective and antioxidant functions and can inhibit peroxinitrite-induced DNA and lipoprotein damage (5), but were not previously known to be regulated by oxidized LDL. 3hydroxy-3-methylglutaryl-Coenzyme A synthase 1 is a key enzyme in cholesterol biosynthesis (2) and is strongly downregulated by oxLDL. This gene encodes a membraneassociated enzyme located at a branch point in the mevalonate pathway. Farnesyldiphosphate farnesyltransferase is the first specific enzyme in cholesterol biosynthesis, catalyzing the dimerization of two molecules of farnesyl diphosphate in a two-step reaction to form squalene (20). As expected, this gene is also strongly downregulated by oxLDL. The similarity of genes regulated by oxLDL in endothelial cells and macrophages further validates our observations.

Scavenger receptors induce uptake of modified LDLs and are therefore thought to be pro-atherogenic (9, 19). However, in our analysis, scavenger receptors were not uniformly induced by foam cell formation. In CXCL4-differentiated macrophages, oxidized LDL significantly induced 2 of 3 probe sets for CD36 and one probe set each for CD68, SCARA3, SCARB1, SCARB2 and CD14. All probe sets for LOX and LOXL-1 were unchanged. Similarly, the regulation of pro-inflammatory cytokines and chemokines was more complicated than expected. While some well-known inflammation genes are clearly upregulated in foam cells, others are downregulated or remain unchanged. Thus, the foam cell is not simply a more pro-inflammatory version of the macrophage.

Surprisingly, native LDL and oxidized LDL induced similar patterns of gene expression, while the set of genes induced or suppressed by mmLDL was clearly different. This challenges the prevailing view of a gradual progression from LDL to mmLDL to oxLDL as moieties with increasing pro-atherogenic potential. Among the genes induced

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by LDL or oxLDL treatment, antigen presentation genes were most prominent, including MHC-II. MHC-II overexpression is associated with macrophage activation, but also with differentiation towards dendritic cells. The dendritic cell marker CD11c as well as several other dendritic cell genes were found overexpressed after treatment with LDL or oxLDL. TLR1, 2, and 4 were also overexpressed and may suggest that LDL-treated macrophages respond more vigorously to their respective ligands. Some differences of gene expression may be the related to different degrees of cholesterol loading, but cholesterol loading was not systematically investigated in all conditions.

The effect of chemokines on macrophages has not been investigated previously. CCL2 and CXCL1 induced only a handful of significant gene changes, all in the same direction. Thirty-two probe sets were significantly induced by CCL2, 43 by CXCL1, while 10 and 19 were downregulated, respectively (supplemental table 10). We hoped to find survival genes induced by CCL2, but further research will be necessary to assess whether CCL2 is involved in macrophage survival and proliferation, because no clear evidence for this hypothesis was found in the present data set.

Previous studies analyzed gene expression in portions of atherosclerotic lesions obtained by laser capture microdissection (36, 38, 39). This method provides the most relevant samples, because the mRNA comes from the actual lesions. However, alterations in cellular composition are superimposed on the changes in gene expression induced during foam cell formation. For example, the reported increase in CD11a/CD18 (LFA-1) (39) is likely due to increased leukocyte content rather than upregulation of gene expression in individual cells. Trogan et al. (36) specifically dissected out macrophage-rich areas and analyzed expression of a housekeeping transcript, cyclophilin A, a smooth muscle cell marker, α -actin, a macrophage marker, CD68, and three inflammatory transcripts, VCAM-1, ICAM-1 and MCP-1 (CCL2). They demonstrate enrichment for CD68, suggesting that macrophages were indeed acquired from the lesions. VCAM-1, ICAM-1 and CCL2 were all upregulated by LPS, but a comparison of foam cells with bone marrowderived macrophages or blood monocytes was not reported. In a follow-op study, CCL2 and VCAM-1 were reported to decrease in regressing plaques, while SRB1, ABCA1 and LXR α were increased. Macrophages and dendritic cells are able to leave from regressing lesions as observed by transplanting aortic arches from *apoE*^{-/-} mice into wild-type recipients (15). Interestingly, CCR7 was also induced in these regressing lesions, allowing macrophages to leave the lesions and appear in draining lymph nodes. CCR7 is known to induce DC migration to lymph nodes (37), thus supporting our conclusion that a dendritic cell-like phenotype is induced in atherosclerosis.

Shiffman et al. (31) investigated gene expression in THP-1 cells at seven time points (0, 0.5, 2.5, 8 hours, 1, 2, and 4 days) using oxLDL treatment. They found that 268 genes were at least 2-fold regulated at one or more time points (no statistical analysis provided) and were classified into seven clusters of expression profiles. We found the probe sets in Affymetrix HG-U133A that correspond to those in the seven clusters, and compared the expression of oxLDL (2 days) relative to control (figure S2 and table S9). As seen in Figure S2, we observed a statistically significant correlation between our and Shiffman's fold changes although the correlation was not large.

In conclusion, while analysis of gene expression gives only a limited view of the process of foam cell formation, it reveals that it is more complex than previously appreciated. New findings include the induction of dendritic cell-like gene program by modified

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LDLs and the surprisingly large biological difference between the effects of mmLDL and oxLDL. It remains to be established how similar in vitro generated foam cells are to foam cells in atherosclerotic lesions. Better in situ techniques such as single cell analysis by cell sorting followed by single cell RT-PCR are needed to fully understand foam cell formation in atherosclerosis.

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Figure Legends

- Figure 1. Flow chart showing analysis strategy. Of 22,215 probe sets, only 129 were not expressed in any of the conditions (NE), but 16,783 were not significantly regulated and considered housekeeking genes (HK) for this experiment, leaving 5,303 probe sets for unbiased analysis by SOM for 11, 9 or 4 conditions (details see text). 917 probes sets were picked as candidate genes, of which 290 were significantly regulated and analyzed in detail.
- Figure 2. Macrophages differentiated with M-CSF for 6 days followed by oxLDL (top) or mmLDL (bottom) were stained with oil red O to demonstrate uptake of neutral lipids. Control macrophages did not stain for oil red O (data not shown).
- Figure 3. SOM of all regulated genes (see also supplemental table 4). A. all 11 conditions; sequence of conditions is PBMC, monocytes, M-CSF induced macrophages, CCL2, CXCL1, M-CSF macrophages differentiated for 8 days (control for LDLs), oxLDL, mmLDL, LDL, CXCL4-induced macrophages, CXCL4-induced macrophages plus oxLDL, two chips each; shaded boxes: up- (top left) or down-regulated (bottom right) by monocyte isolation. B. 9 macrophage and foam cell conditions; sequence of conditions is M-CSF induced macrophages, CCL2, CXCL1, M-CSF macrophages differentiated for 8 days (control for LDLs), oxLDL, mmLDL, LDL, CXCL4-induced macrophages, CCL2, CXCL1, M-CSF macrophages differentiated for 8 days (control for LDLs), oxLDL, mmLDL, LDL, CXCL4-induced macrophages, CXCL4-induced macrophages plus oxLDL, two chips each; arrows point to oxLDL and LDL conditions; n indicates the number of probe sets in each panel and vertical scale is change in expression (log₂).

- Figure 4. Venn diagrams for up- or down-regulation of probe sets by macrophage differentiation from A. monocytes (condition 2 vs. 3) and LDL (condition 6 vs. 9);
 B. oxLDL (condition 6 vs. 7); C. mmLDL (condition 6 vs. 8); D. up- or down-regulation by culture in the presence of CCL2 (condition 3 vs. 4) or CXCL1(condition 3 vs. 5); E. up- or down-regulation by mmLDL (condition 6 vs. 8) and LDL (condition 6 vs. 9); F. up- or down-regulation in CXCL4-differentiated macrophages by oxLDL (condition 10 vs. 11) and also in M-CSF-induced macrophages by oxLDL (condition 6 vs. 9).
- Figure 5. Numbers of regulated (black) and non-regulated (white) genes in the 23 groups of candidate genes. Among 917 candidate probe sets, 290 were found to be significantly regulated.
- Figure 6. Heatmap of the 290 regulated candidate probe sets shown in supplemental table 5. A. all 11 conditions with clusters outlined by yellow boxes. Both probe sets and conditions were free to self-organize; therefore, the sequence of conditions on the x axis is different from that listed in table 1. B. 9 macrophage and foam cell conditions with clusters outlines.
- Figure 7. Expression of similarly regulated surface markers as measured by flow cytometry on monocytes-derived macrophages, monocyte-derived macrophages after stimulation with oxLDL for two days, and monocyte-derived dendritic cells (all from the same donor). Open histograms show CD206, CD62L, CD68 and CD83; filled histograms represent isotype controls.

SUPPLEMENTS

SUPPLEMENTAL FIGURES

- Figure S1. Heatmap of 290 regulated candidate genes
- Figure S2. Comparison with previously published result of oxLDL-differentiated THP-1 cells (31)

SUPPLEMENTAL TABLES

- Table S1. Chip-by-chip correlations of 22 array chips and their specificity/sensitivity
- Table S2.HEM and LPE results for 22,215 genes
- Table S3. SOM results for 5,303 regulated genes
- Table S4. Regulation of genes used for Venn diagram (Figure 3)
- Table S5. Normalized expression data and HEM/LPE results for 290 regulated known genes
- Table S6. A Gene cluster within the 290 regulated candidate genes (11 conditions)B Gene cluster within the 290 regulated candidate genes (9 conditions)
- Table S7. A set of DC genes used for gene set enrichment analysis
- Table S8. Up- or downregulation of 290 regulated known genes under the chemokine conditions (CCL1, CXCL1 and 4)
- Table S9. Comparison with previously published result of oxLDL-differentiated THP-1 cells (31)
- Table S10. LPE results for the 290 regulated candidate genes under the chemokine conditions (CCL2, CXCL1 and 4)

#	Name	Preparation	Remarks
1	PBMC	Buffy coat prepared	Monocytes, lymphocytes, some platelets
		on histopaque 1077	
2	monocytes	Adhesion-purified	Monocytes with 5% lymphocyte and some
			platelet contamination
3	macrophages	M-CSF for 6 days	Adherent macrophages, visually pure and
			homogeneous cell population
4	CCL2	M-CSF for 6 days	Acute CCL2 effects on macrophages
		plus 5 hours CCL2	
5	CXCL1	M-CSF for 6 days	Acute CXCL1 effects on macrophages
		plus 5 hours CXCL1	
6	macrophages	M-CSF for 8 days	Timed control for foam cell differentiation
7	oxLDL	M-CSF for 8 days	Foam cell formation induced by classical
		and oxLDL	Cu-oxidized LDL
8	mmLDL	M-CSF for 8 days	Foam cell formation by mmLDL prepared
		and mmLDL	by incubation with 12/15-LO-transfectants
9	LDL	M-CSF for 8 days	Native LDL, possibly somewhat aggre-
		and native LDL	gated
10	CXCL4	Platelet factor 4 for	Macrophage differentiation induced by
		6 days	platelet factor 4
11	CXCL4+oxLDL	Platelet factor 4 for	Foam cell formation in CXCL4-
		6 days+oxLDL (2d)	macrophages

Table 1. Description of the 11 experimental conditions

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SOM (11 conditions)



Figure 3A



SOM (9 conditions)





■ regulated □ not regulated



Figure 6A



Figure 6B



Figure 7