Transcriptional Profiling of Foam Cells Reveals Induction of Guanylate-Binding Proteins Following Western Diet Acceleration of Atherosclerosis in the Absence of Global Changes in Inflammation

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Background—Foam cells are central to two major pathogenic processes in atherogenesis: cholesterol buildup in arteries and inflammation. The main underlying cause of cholesterol deposition in arteries is hypercholesterolemia. This study aimed to assess, in vivo, whether elevated plasma cholesterol also alters the inflammatory balance of foam cells.

Methods and Results—Apolipoprotein E–deficient mice were fed regular mouse chow through the study or were switched to a Western-type diet (WD) 2 or 14 weeks before death. Consecutive sections of the aortic sinus were used for lesion quantification or to isolate RNA from foam cells of mice under prolonged hypercholesterolemia or to microarray and quantitative polymerase chain reaction analyses. WD feeding for 2 or 14 weeks significantly increased plasma cholesterol, but the size of atherosclerotic lesions increased only in the 14-week WD group. Expression of more genes was affected in foam cells of mice under prolonged hypercholesterolemia than in mice fed WD for 2 weeks. However, most transcripts coding for inflammatory mediators remained unchanged in both WD groups. Among the main players in inflammatory or immune responses, chemokine (C–X–C motif) ligand 13 was induced in foam cells of mice under WD for 2 weeks. The interferon-inducible GTPases, guanylate-binding proteins (GBP)3 and GBP6, were induced in the 14-week WD group, and other GBP family members were moderately increased.

Conclusions—Our results indicate that acceleration of atherosclerosis by hypercholesterolemia is not linked to global changes in the inflammatory balance of foam cells. However, induction of GBPs uncovers a novel family of immune modulators with a potential role in atherogenesis. (J Am Heart Assoc. 2016;5:e002663 doi: 10.1161/JAHA.115.002663)

Key Words: atherosclerosis • cholesterol • gene expression • inflammation • macrophages

Lipid-laden macrophages, or foam cells, are chief cellular components of atherosclerotic lesions through all stages of development.\(^1\) Circulating monocytes are recruited to the arterial wall in response to inflammatory stimuli induced, among other factors, by modified low-density lipoprotein (mLDL) particles deposited in the subendothelial space. Monocytes differentiate into macrophages that take up mLDL in an unfettered fashion and become heavily loaded with lipoprotein-derived cholesterol, while they also orchestrate the development of a local inflammatory process.\(^2\)–\(^4\) Given that, presumably, the amount of mLDL deposited in arteries is related to the concentration of circulating LDL, it might be inferred that, by promoting monocyte immigration, hypercholesterolemia directly contributes to vascular inflammation.\(^3\) However, it could also be possible that exposure to different lipoprotein concentrations alters the inflammatory balance of foam cells. The main LDL modifications related to atherosclerosis development involve lipid peroxidation.\(^5\) Transcriptional response of macrophages to exposure to oxidized (ox) LDL has been tested in cultured macrophages of different sources, with diverse results that, depending on the study, suggested predominantly proinflammatory or anti-inflammatory effects.\(^6\)–\(^9\) In some cases, the outcome was significantly affected by changes in experimental variables, such as the cell type, form of LDL modification and presentation to cells, or time of exposure to the lipoproteins. For example, Brand et al. found that short-term exposure of THP-1 macrophages to oxLDL induced activation of nuclear factor kappa B (NF-κB), whereas long-term exposure to oxLDL not only did not activate NF-κB, but actually prevented NF-κB activation by...
lipopolysaccharide; Hammad et al. observed different effects on inflammatory gene expression depending on whether U937 monocytic cells were treated with oxLDL or with oxLDL immune complexes; and Shiffman et al. identified gene clusters in THP-1 macrophages with different temporal patterns of expression in response to treatment with oxLDL. An added challenge that might contribute to the variability of studies on macrophages is the remarkable plasticity of these cells, which allows them to change their phenotype depending on the surrounding environment. Thus, given that the complex atherosclerotic milieu is difficult to reproduce in vitro, cell-culture approaches to characterize macrophages may not represent the functional relevance of the variables being studied as well as studies performed on macrophages resident within actual atherosclerotic lesions.

The apolipoprotein E-deficient (apoE) mouse is a widely used mouse model of atherosclerosis. From a molecular point of view, gene expression patterns in mouse aortas that define different stages of atherosclerosis development also correlated with severity of human coronary lesions. Like in humans, atherosclerosis development in apoE mice is driven by hypercholesterolemia, and plasma cholesterol levels and the extent of lesion development are directly related to the cholesterol content in their diet. Thus, to study the effect of hypercholesterolemia on the transcriptional response of lesional foam cells, here we have fed apoE mice regular chow through the study or have switched their diet to a Western-type diet (WD) for a short (2 weeks) or for a longer (14 weeks) time period. We have assessed the effects of the WD on atherosclerosis development in cross-sections of the aortic sinus, and we have used sections consecutive to the used for lesion quantification to selectively isolate RNA by laser-capture microdissection (LCM) from lesional macrophages to perform a broad analysis of gene expression, with an emphasis on the expression of genes that regulate inflammatory and immune responses.

Methods

Experimental Design

At 8 weeks of age, 15 female apoE mice in C57BL6/J background were divided into 3 groups with similar cholesterol levels. One group was maintained on regular mouse chow (2020X Teklad Global Soy Protein-Free Extruded Rodent Diet; Harlan Laboratories, Indianapolis, IN) until death at 22 weeks. This is a cholesterol-free diet that provides 3.5 kcal/g, including 16% of calories from fat. From 8 weeks of age to the end of the study, the diet of a second group of 5 mice was switched from regular chow to a WD (TD.88137; Harlan Laboratories). This diet provides 4.5 kcal/g and contains 20% (wt/wt) milk fat (42% of total calories) and 0.15% (wt/wt) cholesterol. The third group of mice remained on regular chow until the age of 20 weeks, when mice were fed WD for the remaining 2 weeks of the study. The study design is summarized in Figure 1A. Cholesterol measurements and lipoprotein fractionation by fast-performance liquid chromatography were performed as we previously described. Plasma oxLDL levels were determined by ELISA (USCN Life Science Inc., Wuhan, China), following the manufacturer’s instructions. Additional mice were used to obtain aortic arches for RNA isolation from whole artery, isolate thioglycollate-elicited peritoneal macrophages, and assess the rate of increase of plasma cholesterol upon introduction of the WD. All animal experiments were conducted following protocols approved by the institutional animal care and use committees at Albany Medical College (Albany, NY) and Baylor College of Medicine (Houston, TX).

Lesion Analysis

After death, mouse hearts were perfused with sterile PBS, bisected, and the upper half immediately embedded in OCT (Sakura, Torrance, CA) and stored at −80°C. Approximately 30 consecutive 7-μm cryosections of each aortic sinus were sequentially mounted on 3 slides. The first and third slides, which were used for macrophage isolation by LCM, were immediately fixed, cell nuclei were stained with toluidine blue, and sections were dehydrated with the HistoGene LCM Frozen Section Staining Kit (Applied Biosystems, Foster City, CA). The second slide was stained for macrophages with anti-Lamp2/Mac3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and used as a template to identify macrophage-rich areas within the lesions. Images were acquired with a Zeiss AxioObserver.D1 microscope using an AxioCam MRc camera (Carl Zeiss, Jena, Germany), and analyzed with AxioVision software (Zeiss). Lesion area was measured by outlining the perimeter of the area between the vessel lumen and media layer of the arteries. The areas within lesions that stained positive for macrophages were determined using Image software (National Institutes of Health [NIH], Bethesda, MD), as we previously described.

LCM and RNA Amplification

LCM of macrophages and RNA processing were performed as we previously described. Briefly, ~2000 laser shots (power ~65 mV; pulse ~2500 μs) were performed with a Veritas Microdissection System (Applied Biosystems), and cells were collected on CapSure HS LCM Caps (Applied Biosystems). RNA was extracted with the PicoPure RNA Isolation Kit (Applied Biosystems). mRNA was submitted to 2 rounds of amplification with the RiboAmp HS RNA Amplification Kit (Applied Biosystems), each of which consisted of synthesis of
double-stranded cDNA using oligo(dT) primers tagged with the T7 promoter sequence, followed by in vitro transcription (IVT) with T7 polymerase.22 A260/A280 ratios and size distribution analysis of amplified cRNAs were assessed with an Agilent 2100 bioanalyzer (Agilent Technologies Inc., Santa Clara, CA).

**Gene Expression Analysis**

Fifteen micrograms of each cRNA were biotin-labeled with the TURBO Labeling Biotin kit (Applied Biosystems), fragmented, and hybridized to Mouse Genome 430 2.0 Arrays (Affymetrix, San Diego, CA), as we previously described.22 Data were filtered with dChip software to include probes with ≥50% presence call in the arrays and with expression levels of ≥25 in ≥50% samples. The filtered data were transferred to MeV software,23 log2 transformed, and differential gene expression in the 3 experimental groups was assessed by ANOVA. Welch’s t test (assuming unequal variances) was used for pairwise comparisons. Differences were considered significant when P≤0.01. To visualize the patterns of gene expression between data sets, volcano plots were generated by plotting significance versus fold change in the Y and X axes, respectively. Pathway Express was used to identify the most relevant pathways affected by the dietary manipulations.24 This software calculates P values based on the number of genes differentially expressed in each pathway in relationship to the number of genes expected to change by chance. It also produces a gamma P value that, in addition to classical statistics, takes into consideration parameters such as the fold change and the topology of genes within each given pathway.24 Thus, the gamma P value is influenced by biologically meaningful factors that are not usually captured by classic statistics.

Primers for quantitative real-time polymerase chain reaction (qPCR) were designed in the 3’-terminus region of mRNA, including the 3’ untranslated region (Table). Relative gene expression was determined from threshold cycle values normalized to cyclophilin A, as we previously described.22 For gene expression analyses in cultured primary macrophages, thioglycollate-elicited peritoneal macrophages were

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**Figure 1.** A, Study design. Blue lines represent time periods in which mice were fed regular chow; periods of WD feeding are represented in orange. Red arrows indicate times of blood sampling. B, Time course of plasma cholesterol elevation after introduction of WD. Mice were not fasted for these plasma measurements (n=5). C, D, and E, Fasting plasma cholesterol (C), triglyceride (D), and oxLDL (E) levels at time of death in mice used for gene expression studies (n=5). F, Cholesterol distribution in different lipoprotein fractions in pooled plasmas obtained at week 22 under overnight fasting. *P<0.005 and **P<0.001 with respect to CHOW; #P<0.05 with respect to 2-week WD. HDL indicates high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; oxLDL, oxidized low-density lipoprotein; VLDL, very low-density lipoprotein; WD, Western-type diet.
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Table. Primer Sequences

| Name       | Sequence                      |
|------------|-------------------------------|
| Cyclophilin A | Forward 5'-TGCTTTTGGAGAGTCTGAA -3'  |
| CD68       | Forward 5'-TTCACGCTGGCTCTCAT -3'  |
| CD14       | Forward 5'-GTCGGCTTGTCAAGGAATCT -3'  |
| SR-A1      | Forward 5'-ATCGGTTGCAATGTGGT -3'  |
| ABCA1      | Forward 5'-GGGCAGATCTGCCGGTGA -3'  |
| α-actin    | Forward 5'-GGACAGGACGGCTATGGA -3'  |
| SM22       | Forward 5'-GACAGAGGACGGCTATGGA -3'  |
| MYH11      | Forward 5'-CTGAGAGGACGGCTATGGA -3'  |
| VE-cadherin| Forward 5'-TGTATATGAAATGTTGGAC -3'  |
| IL-6       | Forward 5'-ATGACCAATATGAAATGTTGGAC -3'  |
| CCL2       | Forward 5'-TCCCTCTTGAGATGACCGA-3'  |
| CXCL13     | Forward 5'-CTGGGAGGAAACCTCCTAC -3'  |
| TNF-α      | Forward 5'-GTCTGGAGAGCCAGTGT-3'  |
| IL-18      | Forward 5'-GCAGCTGGATGTATCTGCTG-3'  |
| GBP3       | Forward 5'-ACATGGCCAATGAGGACACA-3'  |
| GBP6       | Forward 5'-TCATCGTTGGAAGGACAACA-3'  |
| GBP8       | Forward 5'-TGAGGTATTCATGAGAACA-3'  |
| GBP4       | Forward 5'-CTTCGTATATGAAATGTTGCA -3'  |
| GBP2       | Forward 5'-TGTCCTCTTGTCAGAGTACG-3'  |

ABCA1 indicates ATP binding cassette A1; CCL2 indicates chemokine (C-C motif) ligand 2; CXCL, chemokine (C-X-C motif) ligand 13; GBP, guanylate-binding protein; IL, interleukin; MYH11, myosin heavy chain 11; SM22, smooth muscle protein 22; SR-A1, scavenger receptor A1; TNF-α, tumor necrosis factor alpha.

cultured in DMEM/0.2% BSA containing oxLDL (1, 50 or 100 μg/mL; Alfa Aesar, Ward Hill, MA) for 4 or 24 hours. RNA was purified and digested with DNase using the Absolutely RNA miniprep kit (Agilent Technologies). Reverse transcription of 500 ng of total RNA was performed with SuperScript III (Invitrogen, Carlsbad, CA). The qPCR protocol was the same used for analysis of samples isolated by LCM.

Statistical Analysis

Statistical analysis of nonmicroarray data was carried out using parametric methods when the data followed a normal distribution and the samples had equal variances. Otherwise, nonparametric tests were used for the analysis. When parametric tests were used, multiple comparisons were analyzed by ANOVA, and post-hoc pair-wise comparisons were performed using the Holm–Sidak test. When nonparametric tests were used, multiple comparisons were performed with the Kruskal–Wallis test, followed by pair-wise comparisons with the Mann–Whitney U test. Statistical analysis involving comparisons between two groups were performed using a 2-tailed Student t test (parametric) or the Mann–Whitney U test (nonparametric). Differences were considered significant when P < 0.05. Values are presented as mean ± SEM.

Results

Effects of WD on Plasma Lipids and Atherosclerosis Development

To determine the adequate length of the study in which foam cells were exposed to hypercholesterolemia for a short time period, we performed a preliminary study to assess the rate of increase of plasma cholesterol in apoE−/− mice upon introduction of the WD. As seen in Figure 1B, plasma cholesterol was markedly higher at 24 hours of WD feeding, but levels continued to increase gradually to reach concentrations of ≈1000 mg/dL (under fed conditions) at day 11. Thus, in order to expose foam cells to plasma cholesterol levels similar to the achieved under prolonged WD, but for a period of time not long enough to affect atherosclerosis development, mice were fed WD for 2 weeks. Fasting plasma cholesterol levels measured at the time of death in mice used for gene expression studies were still moderately (≈10%) higher in mice fed WD for 10 weeks than in mice that were fed WD for only 2 weeks. Although these differences did not reach statistical significance. However, in both WD groups, cholesterol levels were significantly (≈2-fold) higher than in mice fed regular mouse chow (Figure 1C and 1F). As expected...
in apoE<sup>−/−</sup> mice, plasma triglycerides did not increase in response to WD (Figure 1D). Although oxidative modifications are believed to take place mainly on apoB-containing lipoproteins retained at the arterial wall, oxLDL is also present in the circulation.<sup>25,26</sup> Plasma oxLDL were very similar between mice fed chow through the study and mice fed WD for the last 2 weeks, whereas oxLDL levels were slightly reduced in mice fed WD for 14 weeks (Figure 1E). This observation is consistent with recent studies that showed that plasma oxLDL decreases concomitantly with increase in lesion size.<sup>26</sup> Indeed, the prolonged hypercholesterolemia in the 14-week WD group resulted in a very substantial (>3-fold) increase in atherosclerosis development at the aortic sinus: 696±32×10<sup>3</sup> µm<sup>2</sup> in mice fed WD for 14 weeks versus 221±13×10<sup>3</sup> µm<sup>2</sup> in mice fed regular chow through the study. However, the size of the lesions in the 2-week WD group was similar to that of mice fed regular chow: 263±37 µm<sup>2</sup> (Figure 2A and 2B). Next, we performed immunohistochemical analyses to quantify the areas of macrophages within lesions. The relative macrophage content was similar in mice fed chow through the study and in mice fed WD for 2 weeks (≈35–40%), but it was lower (≈25%) in mice fed WD for 14 weeks (Figure 2C). However, the total area of macrophages, calculated by multiplying the percentage of macrophages by the total lesion area, was higher in mice fed WD for 14 weeks (Figure 2D). Thus, WD feeding for either 2 or 14 weeks significantly increased plasma cholesterol levels. However, lesions in mice fed WD for 2 week were similar in size and contained a similar amount of macrophages than those of mice fed chow through the study, whereas the longer WD feeding protocol markedly increased the progression of atherosclerotic lesions.

**Gene Expression Analyses of Lesional Foam Cells**

RNA from lesional foam cells was isolated by LCM and amplified by IVT to obtain enough material for a broad gene expression analysis.<sup>22</sup> First, to control the quality of the isolation and amplification processes, we assessed whether the gene expression patterns in the cRNA amplified from cells captured by LCM were the expected in macrophage/foam cell populations. Using qPCR, we compared expression of several macrophage markers between these cRNA samples and cRNA amplified from lysates of aortic arches (whole artery) of apoE<sup>−/−</sup> mice. As seen in Figure 3A through 3D, levels of transcripts that are typically elevated in macrophage populations, including CD68, CD14, scavenger receptor A1 (SR-A1), and ATP binding cassette A1 (ABCA1), were enriched in LCM-cRNAs. Conversely, levels of transcripts coding for the smooth muscle cell (SMC) markers, α-actin, smooth muscle myosin heavy chain 11 (MYH11), and smooth muscle protein 22 (SM22), were significantly reduced in macrophages isolated by LCM (Figure 3E through 3G). Likewise, mRNA coding for the endothelial cell marker VE-cadherin, was also

![Figure 2. A, Representative images of sections of the aortic sinus in the 3 experimental groups stained with the macrophage marker, Mac-3 (Brown), and counterstained with hematoxylin. Bar=200 µm. B, Area of atherosclerosis involvement was determined by quantifying the area between the media layer and the vessel lumen. Dots represent individual values. Bars represent average±SEM. C, Percentage of lesion area occupied by macrophages and (D) absolute of macrophages at the aortic sinus. *P<0.05 and **P<0.001 with respect to CHOW. WD indicates Western-type diet.](image-url)
Reduced in LCM samples (Figure 3H). Importantly, both the enrichment in macrophage markers and the decrease in SMC markers and VE-cadherin were similar in LCM-cRNAs in the 3 experimental groups.

For broad gene expression profiling of foam cells, amplified cRNAs were biotinylated and hybridized to Affymetrix DNA chips. The microarray data have been deposited at the GEO (Gene Expression Omnibus) repository and can be accessed through the accession number GSE70619. Data was filtered to remove probes with low presence of call and/or low intensity across the board of arrays, and 15,433 targets satisfied the filtering criteria. Among them, ANOVA analysis at a P value of 0.01 followed by pair-wise comparisons against the “CHOW” group identified 52 targets significantly affected in the 2-week WD group and 366 targets in the 14-week WD group. Volcano plots summarizing these changes are shown in Figure 4A and 4B, and the genes affected by the dietary manipulations are listed in Table S1 (2-week WD vs CHOW) and Table S2 (14-week WD vs CHOW). To determine the main biological processes affected in response to hypercholesterolemia, the data were analyzed with Pathway Express. Two pathways, “antigen processing and presentation” and “ubiquitin-mediated proteolysis,” were commonly affected in both WD groups (Figure 4C). Changes in genes in the antigen processing and presentation pathway could be related to the increased uptake of modified lipoproteins by foam cells of mice under WD. The reasons for the over-representation of genes in the ubiquitin-mediated proteolysis pathway are not clear, though it was reported that protein ubiquitination was increased in atherosclerotic plaques, and ubiquitin-mediated proteolysis was the most over-represented pathway in plaques of diabetic patients.27,28 Whereas these two pathways were the only ones affected in the 2-week WD group, the higher number of genes differently expressed in the 14-week WD group was reflected in 11 additional pathways significantly affected in this group (Figure 4C). Given that lesions were more developed in response to prolonged WD, but the plasma lipid profiles were similar in both WD groups, it is likely that changes in the lesional microenvironment contributed to the more-robust changes in gene expression observed in the 14-week WD group. Indeed, the modest changes in gene expression observed in mice fed WD for only 2 weeks suggest that hypercholesterolemia is not a major determinant of transcriptional response of foam cells. However, whether the changes in gene expression were directly related to the plasma cholesterol concentrations, or were the result of other changes in the lesional milieu, neither WD feeding protocol affected the expression of the vast majority of genes in inflammatory and immune response categories. The most noteworthy exceptions were chemokine (C-X-C motif) ligand 13 (CXCL13), which was upregulated in the 2-week WD group by ~4.4-fold, and two members of the p65 guanylate-binding proteins (GBP) family of interferon-inducible GTPases, GBP3 and GBP6, which were induced in the 14-week WD group by ~3.2- and ~5.2-fold, respectively.

Analysis of Inflammatory Mediators

The inflammatory nature of atherosclerosis has been supported by numerous studies, including analysis of gene
expression in both clinical samples and animal models of atherosclerosis.\textsuperscript{2,4} Thus, to assess whether genes involved in inflammation could have been affected, but to an extent that did not meet the criteria of this analysis, we performed a similar analysis at a significance level of \( P < 0.05 \) instead of \( P < 0.01 \). As expected, the less-conservative analysis yielded a larger number of genes significantly affected by both dietary manipulations (78 targets significantly changed when comparing 2-week WD vs CHOW and 571 targets when comparing 14-week WD vs CHOW). However, the absence of immune and inflammatory mediators was still evident, and, accordingly, pathways related to the inflammatory and immune responses were not significantly affected (data not shown). Next, we focused the analysis on expression of transcripts coding for major cytokines and chemokines that had been related to progression of atherosclerosis or development of more-severe plaque phenotypes.\textsuperscript{16,28–31} As seen in Figure 5A, in both WD groups, the levels of most transcripts coding for interleukins or for CC or CXC cytokines were very close or within \( \approx 1 \)-fold change of the levels observed in the CHOW group. There were no obvious trends toward higher or lower ratios in any of the 2 WD groups that suggested an overall proinflammatory or anti-inflammatory effect. Among these players in inflammation, only CXCL13 (in the 2-week WD group) was significantly elevated. This result was confirmed by qPCR analysis (Figure 5B). Interleukin-6 (IL-6) was increased by \( \approx 5 \)-fold in the 14-week WD group, although the signal intensities for IL-6 were very variable and the differences did not reach statistical significance. Changes in IL-6 mRNA levels were not supported by qPCR analysis (Figure 5C). Other inflammatory mediators that displayed modest and nonstatistically significant increases in the microarray analyses, namely, chemokine (C-C motif) ligand 2 (CCL2) in the 2-week WD group or IL-18 in the 14-week WD group, also remained similar among groups.
by qPCR analysis (Figure 5D and 5E). Interestingly, whereas CXCL13 was induced by short-term hypercholesterolemia, it was not elevated in foam cells from chow-fed mice when compared to whole aortic arches. In contrast, levels of transcripts coding for IL-6, CCL2, and IL-18 were significantly higher in all 3 foam cell populations.

**Induction of GBPs by Hypercholesterolemia**

A remarkable observation in the microarray analyses was the induction of two p65-GBPs (GBP3 and GBP6) in foam cells of mice fed WD for 14 weeks. Several studies have shown that different GBPs are concomitantly induced.32 Thus, we asked whether in the 14-week WD group there would also be some degree of induction of other GBP family members. Interestingly, as seen in Figure 6A, levels of 5 of the 6 GBPs included in the microarray was higher in foam cells isolated from mice fed WD for 14 weeks than in mice fed regular chow. Intensity of GBP2, GBP7, and GBP8 was 2-fold higher, although these differences were not statistically significant. In general, GBPs were not elevated in mice fed WD for only 2 weeks, although GBP6 was relatively higher than in control CHOW-fed mice both in the microarray and qPCR analyses (Figure 6A and 6C).

Results of qPCR analyses were consistent with the microarray data (Figure 6B through 6F). Next, we asked whether the changes observed in vivo would also be observed in vitro in peritoneal macrophages treated with oxLDL. It was reported that oxLDL in human endarterectomy specimens was nearly 70 times higher than plasma oxLDL in the same patients.33 Thus, given that measurements of oxLDL concentrations within mouse atherosclerotic lesions are extremely challenging, we performed a dose-response study that included a low dose of oxLDL close to the circulating levels (1 \( \mu \)g/mL) and 2 higher doses of 50 and 100 \( \mu \)g/mL. Mouse peritoneal macrophages were exposed to these concentrations of oxLDL for 4 and 24 hours. None of the treatments altered the levels of the CXCL13 mRNA. However, as seen in Figure 7A, we observed significantly increased GBP3 and GBP6 mRNA in peritoneal macrophages cultured with 100 \( \mu \)g/mL of oxLDL. Overall, GBP6 seems to be the most responsive GBP family member both in vivo and in vitro. Next, we assessed whether expression of other GBP family members was also induced in response to oxLDL, and found that GBP2 and GBP7 were also increased upon treatment with oxLDL (100 \( \mu \)g/mL). Thus, whereas CXCL13 was not induced by oxLDL in vitro, several GBP family members were induced, suggesting that oxLDL
might be one of the factors involved in the induction of GBP in response to hypercholesterolemia.

Discussion

Hypercholesterolemia is a leading risk factor for development of atherosclerosis, an inflammatory disease of the arterial wall. There is compelling evidence supporting the notion that hypercholesterolemia enhances vascular inflammation, including gene expression profiles of aortic sinuses isolated from apoE−/− mice that showed increased expression of inflammatory mediators after the introduction of a Western diet. Upregulation of proinflammatory genes observed in these studies could be the logical consequence of the increased

![Figure 6](image)

**Figure 6.** A, Average signal intensity for GBPs present in the microarray. Bars represent the ratios (fold increase) with respect to LCM macrophages in the CHOW group (dashed line). B through F, qPCR analysis of expression of GBP3, GBP6, GBP2, GBP7, and GBP8 in aortas and LCM macrophages of the 3 experimental groups. *P<0.05 and **P<0.001 with respect to RNA isolated from aortic arches (Aorta); ^P<0.05 with respect to CHOW macrophages (MØ). GBP indicates guanylate-binding protein; LCM, laser-capture microdissection; qPCR, quantitative polymerase chain reaction; WD, Western-type diet.

![Figure 7](image)

**Figure 7.** A, qPCR analysis of expression of CXCL13, GBP3, and GBP6 in mouse peritoneal macrophages that remained untreated or were stimulated with oxLDL (1, 50, or 100 µg/mL) for 4 or 24 hours. B, Expression of other GBP family members in response to oxLDL (100 µg/mL for 4 or 24 hours; n=3). *P<0.05, **P<0.01 and ***P<0.001 with respect to untreated control. CXCL, chemokine (C-X-C motif) ligand; GBP, guanylate-binding protein; oxLDL, oxidized low-density lipoprotein; qPCR, quantitative polymerase chain reaction.
influx of inflammatory cells to the arterial wall during progression of the disease, but could also be related to other factors, such as the activation of lesional cells, in response to hypercholesterolemia. In this line, there is evidence that exposure to modified lipoproteins might regulate the inflammatory response of foam cells. For example, oxLDL was shown to activate NF-κB by binding to Toll-like receptors (TLRs) such as TLR2 and TLR4. Accumulation of cholesterol crystals in the cytoplasm of macrophages was also proposed to stimulate a proinflammatory cascade. Alternatively, lipoprotein-derived oxysterols are natural liver X receptor ligands, which can counter-regulate induction of inflammatory gene expression by NF-κB by recruiting corepressors to the promoters of inflammatory genes. Thus, we asked whether increases in plasma cholesterol that significantly accelerate atherosclerosis development would actually affect the inflammatory balance of lesional foam cells. Answering this question using complex heterogeneous samples, such as fragments of atheromatous plaques or diseased arteries, may be quite challenging, mainly because of the variable number of inflammatory cells that can be found in these specimens. To circumvent this challenge, here we have used LCM to specifically isolate and assess the inflammatory status of macrophages resident within atherosclerotic lesions. We fed apoE−/− mice for a period of time that was not long enough to affect the size of atherosclerotic lesions (2 weeks) or for a longer period that significantly enhanced the development of atherosclerosis (14 weeks). Whereas WD feeding resulted in a ∼2-fold elevation in plasma cholesterol, neither dietary manipulation affected expression of the vast majority of genes coding for inflammatory mediators. Thus, a first and foremost conclusion of this study is that accelerated development of atherosclerosis in response to hypercholesterolemia was not linked to major changes in the inflammatory balance of foam cells, as it would be expected if the activity of central regulators of inflammation, such as NF-κB, were affected.

Although we did not observe global changes in inflammation, we observed certain changes that might be relevant to the pathogenesis of the disease. Expression of the chemokine CXCL13, was elevated only in the short-term WD group. CXCL13 is a homeostatic chemokine that has been primarily linked to lymphocyte trafficking, but has also been shown to influence other key processes such as activation of T cells and macrophages. CXCL13 is produced by macrophages and is expressed in human atherosclerotic lesions. However, its role in atherogenesis remains poorly characterized, and it has actually been proposed to play a role both in plaque stabilization and plaque destabilization. It is noteworthy that the in vivo changes in CXCL13 expression were not recapitulated in peritoneal macrophages cultured with various doses of oxLDL. This may simply stress the importance of performing gene expression analyses on macrophages within actual atheroma, but could also indicate that CXCL13 expression is induced by other factors involved in the pathogenesis of atherosclerosis.

In the long-term WD group, we observed a significant induction of 2 GBPs, GBP3 and GBP6, as well as a more-moderate elevation of other GBP family members. The GBPs were among the first interferon (IFN)-inducible genes identified, and, like other interferon target genes, the function of GBPs has been primarily associated to protection against viral and bacterial infections. Although their mechanism of action is still under investigation, p65-GBPs have been shown to localize to vacuoles containing pathogens and play a role in transport of autophagic machinery, antimicrobial peptides, and NADPH oxidase (NOX) enzymes for assembly on phagosomal membranes. Interestingly, both the phagocytic clearance of apoptotic cells, known as efferocytosis, and the production of reactive oxygen species by NOX enzymes are processes associated with the development of atherosclerosis. Furthermore, similar to what happens during bacterial phagocytosis, efferocytosis was shown to induce an oxidative burst in macrophages in a NOX-dependent fashion. Importantly, in follow-up studies using cultured macrophages, we found that several GBPs were induced in vitro by oxLDL, which indicates that oxLDL may be one of the factors responsible for the induction observed in vivo. However, the reason for the specific upregulation of GBPs among the various players in inflammation and immunity is not clear. A possible explanation is that the genes coding for GBPs may be more sensitive to modest changes in inflammation that may take place in more-advanced lesions. Indeed, GBPs are known to be very strongly induced by IFN and other inflammatory stimuli, a fact that even facilitated the characterization of signaling pathways such as the Janus kinase/signal transducer and activator of transcription and the IFN-γ and IFN-α/β pathways. However, to our knowledge, this is the first report linking this family of IFN-induced GTPases with the pathogenesis of atherosclerosis. Thus, additional studies will be necessary to determine whether GBPs play a significant role in regulation of atherogenesis, whether it is through regulation of macrophage function during efferocytosis or by other mechanisms.

In conclusion, this study challenges the notion that acceleration of atherogenesis by hypercholesterolemia is linked to a global impact on the inflammatory balance of foam cells. Significant changes among inflammatory and immune mediators included induction of CXCL13 in response to short-term increases in plasma cholesterol, and induction of GBPs in foam cells resident within the more-advanced lesions that formed in response to prolonged hypercholesterolemia. Further research will be necessary to elucidate the role of these players in the development of atherosclerosis.
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Disclosures
None.

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**Table S1.** Genes differentially expressed in foam cells of mice fed WD for 2 weeks with respect to mice fed regular chow through the entire study.

| Affymetrix Probe ID | Fold Change | Gene Symbol | Gene Name |
|---------------------|-------------|-------------|-----------|
| 1448859_at          | 4.38        | Cxcl13      | chemokine (C-X-C motif) ligand 13 |
| 1439276_at          | 3.89        | Adar        | adenosine deaminase, RNA-specific |
| 1424436_at          | 3.60        | Gart        | phosphoribosylglycinamidase formyltransferase |
| 1431028_a_at        | 3.56        | Pank1       | pantothenate kinase 1 |
| 1431544_at          | 2.85        | 4930524B17Rik | |
| 1451381_at          | 2.76        | 1810020D17Rik | RIKEN cDNA 1810020D17 gene |
| 1421290_at          | 2.62        | Hspb7       | heat shock protein family, member 7 |
| 1444328_at          | 2.37        | Cita        | clathrin, light polypeptide |
| 1424713_at          | 2.06        | Calml4      | calmodulin-like 4 |
| 1424428_at          | 2.05        | Ino80e      | INO80 complex subunit E |
| 1453320_at          | 2.04        | 1700027A23Rik | RIKEN cDNA 1700027A23 gene |
| 1448104_at          | 1.69        | Aldh6a1     | aldehyde dehydrogenase family 6, subfamily A1 |
| 1420691_at          | 1.49        | Il2ra       | interleukin 2 receptor, alpha chain |
| 1442004_at          | 1.40        | Trim65      | tripartite motif-containing 65 |
| 1435091_at          | 1.39        | Zfp568      | zinc finger protein 568 |
| 1432511_at          | -1.32       | Cnot1       | CCR4-NOT transcription complex, subunit 1 |
| 1426446_at          | -1.39       | 6430548M08Rik | RIKEN cDNA 6430548M08 gene |
| 1431420_s_at        | -1.47       | Prelid1     | PRELI domain containing 1 |
| 1428760_at          | -1.54       | Snapc3      | small nuclear RNA activating complex, polypeptide 3 |
| 1433621_at          | -1.60       | Wdr41       | WD repeat domain 41 |
| 1417226_at          | -1.62       | Fbxw4       | F-box and WD-40 domain protein 4 |
| 1421888_x_at        | -1.76       | Aplp2       | amyloid beta (A4) precursor-like protein 2 |
| 1440168_x_at        | -1.82       | Kctd7       | potassium channel tetramerisation domain containing 7 |
| 1415911_at          | -1.85       | Impact      | imprinted and ancient |
| 1431716_at          | -1.92       | Herc4       | heat domain and RLD 4 |
| 1435867_at          | -1.92       | Jhdm1d      | jumonji C domain-containing histone demethylase 1 homolog D |
| 1418971_x_at        | -1.98       | Bcl10       | B-cell leukemia/lymphoma 10 |
| 1423899_at          | -1.99       | Trip12      | thyroid hormone receptor interactor 12 |
| 1434660_at          | -2.01       | Alkbh1      | alkB, alkylation repair homolog 1 |
| 1437361_at          | -2.01       | OTTMUSG00000002043 | predicted gene, OTTMUSG00000002043 |
| 1450905_at          | -2.06       | Plxnc1      | plexin C1 |
| 1423066_at          | -2.07       | Dnmt3a      | DNA methyltransferase 3A |
| 1452769_at          | -2.07       | Rnf145      | ring finger protein 145 |
| 1417622_at          | -2.08       | Slc12a2     | solute carrier family 12, member 2 |
| 1426227_s_at        | -2.17       | Vps37c      | vacuolar protein sorting 37C |
| 1449347_a_at        | -2.24       | Xlr4c       | X-linked lymphocyte-regulated 4B |
| Probe ID         | Value  | Description                                      |
|-----------------|--------|--------------------------------------------------|
| 1437087_at      | -2.29  | 2210408K08Rik major facilitator superfamily domain containing 1 |
| 1424129_at      | -2.32  | Mfsd1 cell division cycle and apoptosis regulator 1 |
| 1436157_at      | -2.34  | Ccar1 solute carrier family 20, member 1          |
| 1448568_a_at    | -2.49  | Slc20a1 glycine amidinotransferase               |
| 1423569_at      | -2.59  | Gatm potassium channel tetramerisation domain containing 20 |
| 1416324_s_at    | -2.85  | Kctd20 serine palmitoyltransferase, long chain base subunit 2 |
| 1460243_at      | -2.87  | Sptlc2 predicted gene, 100042616                 |
| 1439251_at      | -2.87  | 100042616 exonuclease domain containing 1        |
| 1416324_s_at    | -2.85  | Kctd20 potassium channel tetramerisation domain containing 20 |
| 1460243_at      | -2.87  | Sptlc2 serine palmitoyltransferase, long chain base subunit 2 |
| 1439251_at      | -2.87  | 100042616 predicted gene, 100042616             |
| 1435390_at      | -3.08  | Exod1 exonuclease domain containing 1            |
| 1426894_s_at    | -3.18  | Fam102a family with sequence similarity 102, member A |
| 1451798_at      | -3.35  | Il1m interleukin 1 receptor antagonist           |
| 1449363_at      | -3.38  | Atf3 activating transcription factor 3           |
| 1448883_at      | -3.40  | Lgmn legumain                                    |
| 1440342_at      | -4.27  | G530011O06Rik                                    |
| 1422650_a_at    | -4.90  | Riok3 RIO kinase 3 (yeast)                       |
| 1435539_at      | -6.28  | N/A N/A                                          |
**Table S2.** Genes differentially expressed in foam cells of mice fed WD for 14 weeks with respect to mice fed regular chow through the entire study.

| Affymetrix Probe ID | Fold Change | Gene Symbol | Gene Name |
|---------------------|-------------|-------------|-----------|
| 1457566_at          | 12.40       | Zfp677      | zinc finger protein 677 |
| 1428600_at          | 6.53        | Nin         | ninein    |
| 1435422_at          | 5.85        | 4933433P14Rik | RIKEN cDNA 4933433P14 gene |
| 1439363_at          | 5.63        | 1200014J11Rik | RIKEN cDNA 1200014J11 gene |
| 1438676_at          | 5.28        | Mpa2l       | macrophage activation 2 like |
| 1452833_at          | 4.67        | Rapgef2     | Rap guanine nucleotide exchange factor (GEF) 2 |
| 1422492_at          | 4.54        | Cpox        | coproporphyrinogen oxidase |
| 1438643_at          | 4.28        | Camk1d      | calcium/calmodulin-dependent protein kinase ID |
| 1439276_at          | 4.27        | Adar        | adenosine deaminase, RNA-specific |
| 1445702_x_at        | 4.07        | Ppapdc2     | phosphatidic acid phosphatase type 2 domain containing 2 |
| 1452960_at          | 4.07        | Scyl3       | SCY1-like 3 |
| 1452232_at          | 4.03        | Gaint7      | UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 7 |
| 1416866_at          | 3.99        | Bet1        | blocked early in transport 1 homolog |
| 1425327_at          | 3.98        | Fam76a      | family with sequence similarity 76, member A |
| 1427990_at          | 3.96        | Usp45       | ubiquitin specific petidase 45 |
| 1460389_at          | 3.88        | Cdk8        | cyclin-dependent kinase 8 |
| 1427938_at          | 3.85        | Mycbp       | c-myc binding protein |
| 1436736_x_at        | 3.84        | DHO4S114    | DNA segment, human D4S114 |
| 1423408_a_at        | 3.80        | 2500003M10Rik | RIKEN cDNA 2500003M10 gene |
| 1435164_s_at        | 3.80        | Uba3        | ubiquitin-like modifier activating enzyme 3 |
| 1456060_at          | 3.75        | Maf         | avian musculoaponeurotic fibrosarcoma (v-maf) AS42 oncogene homolog |
| 1460391_at          | 3.73        | Ola1        | Obg-like ATPase 1 |
| 1452593_a_at        | 3.72        | Tceb1       | transcription elongation factor B (SIII), polypeptide 1 |
| 1429519_at          | 3.68        | Fpgt        | fucose-1-phosphate guanylyltransferase |
| 1424436_at          | 3.68        | Gart        | phosphoribosylglycinamide formyltransferase |
| 1433730_at          | 3.68        | Elmod2      | ELMO domain containing 2 |
| 1437901_a_at        | 3.65        | Vps41       | vacuolar protein sorting 41 |
| 1451572_a_at        | 3.60        | Mff         | mitochondrial fission factor |
| 1456319_at          | 3.60        | N/A         | N/A       |
| 1436048_at          | 3.58        | Exoc8       | exocyst complex component 8 |
| 1424360_at          | 3.56        | BC019943    |         |
| 1435694_at          | 3.53        | Arhgap26    | Rho GTPase activating protein 26 |
| 1428453_at          | 3.47        | Nat12       | N-acetyltransferase 12 |
| 1426669_at          | 3.43        | Cpped1      | Vcalcineurin-like phosphoesterase domain containing 1 |
| 1452047_at          | 3.43        | Cacybp      | calcyclin binding protein |
| 1434853_x_at        | 3.38        | Mkrn1       | makorin, ring finger protein, 1 |
| 1424099_at          | 3.38        | Gpx8        | glutathione peroxidase 8 |
| Gene Symbol      | Log2 Fold Change |
|------------------|------------------|
| Kdelc2           | 3.35             |
| Lrrc58           | 3.35             |
| 4921524J17Rik    | 3.27             |
| Rpl15            | 3.24             |
| Ppp1cb           | 3.21             |
| Ildr2            | 3.21             |
| Ptch1            | 3.21             |
| Lrrc58           | 3.20             |
| Gbp3             | 3.20             |
| Top2a            | 3.20             |
| Jag1             | 3.19             |
| Tatdn1           | 3.19             |
| 4933421E11Rik    | 3.18             |
| Vwa5a            | 3.18             |
| Msh2             | 3.17             |
| Gapvd1           | 3.14             |
| Srrm1            | 3.13             |
| Adsl             | 3.10             |
| Dnajc2           | 3.06             |
| Kpna4            | 3.06             |
| Tnpo1            | 3.04             |
| LOCC100044979    | 3.04             |
| Cyld             | 3.01             |
| N/A              | 3.00             |
| Crls1            | 2.97             |
| E330009J07Rik    | 2.97             |
| Gapvd1           | 2.94             |
| Atp13α3          | 2.93             |
| Nipa2            | 2.90             |
| Brca2            | 2.90             |
| N/A              | 2.90             |
| Rcor1            | 2.90             |
| Gm608            | 2.89             |
| Hnrdpl           | 2.88             |
| Hnrrnpa0         | 2.87             |
| Pten             | 2.83             |
| Slc35a2          | 2.82             |
| Taf1d            | 2.82             |
| Ddit4l           | 2.80             |
| Hnrdpl           | 2.88             |
| Hnrrnpa0         | 2.87             |
| Slc35a2          | 2.77             |
| Tlk1             | 2.77             |
| LOC100044979     | 2.77             |
| Eid1             | 2.77             |
| Slc35a2          | 2.77             |
| Gene ID   | Log2 Fold Change | Gene Symbol | Description                                      |
|----------|-----------------|-------------|--------------------------------------------------|
| 1419495_at | 2.76            | Imp2l       | IMP2 inner mitochondrial membrane peptidase-like |
| 1454846_at | 2.76            | Utp15       | UTP15, U3 small nucleolar ribonucleoprotein, homolog |
| 1455483_at | 2.75            | Zfp148      | zinc finger protein 148                          |
| 1454831_at | 2.75            | Foxn2       | forkhead box N2                                  |
| 1418988_at | 2.75            | Pex7        | peroxisomal biogenesis factor 7                  |
| 1451649_a_at | 2.74        | Wdr75       | WD repeat domain 75                              |
| 1455513_at | 2.74            | Taf1        | TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor |
| 1424156_at | 2.73            | Rbl1        | retinoblastoma-like 1 (p107)                     |
| 1415863_at | 2.73            | Eif4g2      | eukaryotic translation initiation factor 4, gamma 2 |
| 1429438_at | 2.72            | Bcor        | BCL6 interacting corepressor                     |
| 1440187_at | 2.71            | Taf3        | TAF3 RNA polymerase II, TATA box binding protein (TBP)-associated factor |
| 1424495_a_at | 2.70      | Cklf        | chemokine-like factor                            |
| 1429043_at | 2.70            | Smndc1      | survival motor neuron domain containing 1       |
| 1429796_at | 2.70            | Kalrn       | kalirin, RhoGEF kinase                           |
| 1442757_at | 2.70            | Lrch1       | leucine-rich repeats and calponin homology (CH) domain containing 1 |
| 1416860_s_at | 2.70       | Ing1        | inhibitor of growth family, member 1             |
| 1415890_at | 2.69            | Papss1      | 3'-phosphadenosine 5'-phosphosulfate synthase 1  |
| 1451641_at | 2.68            | Dbr1        | debranching enzyme homolog 1                     |
| 1415791_at | 2.67            | Rnf34       | ring finger protein 34                           |
| 1449175_at | 2.66            | Gpr65       | G-protein coupled receptor 65                    |
| 1428087_at | 2.64            | Dnm1l       | dynamin 1-like                                   |
| 1435821_s_at | 2.63        | Ppp1r8      | protein phosphatase 1, regulatory (inhibitor) subunit 8 |
| 1417770_s_at | 2.63        | Psmc6       | proteasome (prosome, macropain) 265 subunit, ATPase, 6 |
| 1422857_at | 2.63            | Trip4       | thyroid hormone receptor interactor 4            |
| 1439994_at | 2.63            | 1810013D10Rik | RIKEN cDNA 1810013D10 gene                         |
| 1454998_at | 2.63            | 1200011J18Rik |                                               |
| 1451374_x_at | 2.63        | Cklf        | chemokine-like factor                            |
| 1415737_at | 2.62            | Rfk         | riboflavin kinase                                |
| 1451745_a_at | 2.62        | Znhit1      | zinc finger, HIT domain containing 1             |
| 1434418_at | 2.62            | Ces6        | ceramide synthase 5                              |
| 1426977_at | 2.60            | Usp47       | ubiquitin specific peptidase 47                  |
| 1438055_at | 2.60            | Rarres1     | retinoic acid receptor responder (tazarotene induced) 1 |
| 1454938_at | 2.59            | Snx13       | sorting nexin 13                                 |
| 1456886_at | 2.59            | N/A         | N/A                                              |
| 1451000_at | 2.59            | Tmem126a    | transmembrane protein 126A                       |
| 1416653_at | 2.58            | Stxbp3a     | syntaxin binding protein 3A                      |
| 1448234_at | 2.55            | Dnajb6      | Dnaj (Hsp40) homolog, subfamily B, member 6      |
| 1435901_at | 2.55            | Usp40       | ubiquitin specific peptidase 40                  |
| 1424782_at | 2.55            | Tmem77      | transmembrane protein 77                         |
| 1428552_at | 2.54            | 2610001J05Rik | RIKEN cDNA 2610001J05 gene                             |
| 1450012_x_at | 2.53        | Ywhag       | tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide |
| 1418180_at | 2.53            | Sp1         | trans-acting transcription factor 1              |
| Probe ID      | Log2 Fold Change | Gene ID/Name                                                                                                                                 |
|--------------|-----------------|---------------------------------------------------------------------------------------------------------------------------------------------|
| 1416952_at   | 2.53            | Atp6v1d, ATPase, H+ transporting, lysosomal V1 subunit D                                                                                  |
| 1448589_at   | 2.53            | Ndufb5, NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5                                                                             |
| 1459657_s_at | 2.53            | Polr1d, predicted gene, OTTMUSG00000008305                                                                                                 |
| 1417564_at   | 2.53            | Med7, mediator complex subunit 7                                                                                                           |
| 1444328_at   | 2.52            | Clta, clathrin light chain 3                                                                                                               |
| 1429559_at   | 2.52            | Gnaq, guanine nucleotide binding protein, alpha q polypeptide                                                                              |
| 1423707_at   | 2.52            | Tmem50b, transmembrane protein 50B                                                                                                         |
| 1417507_at   | 2.51            | Cyb561, cytochrome b-561                                                                                                                  |
| 1455915_at   | 2.51            | Galnt4, UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 4                                              |
| 1452130_at   | 2.50            | Txndc14, thiorexin domain containing 14                                                                                                    |
| 1425074_at   | 2.49            | Wrn, Werner syndrome homolog                                                                                                               |
| 1452152_at   | 2.48            | Clint1, clathrin interactor 1                                                                                                              |
| 1435093_at   | 2.48            | Zfyve20, zinc finger, FYVE domain containing 20                                                                                             |
| 1440310_at   | 2.48            | Runx1t1, runt-related transcription factor 1; translocated to, 1 (cyclin D-related)                                                         |
| 1441315_s_at | 2.47            | Slc19a2, solute carrier family 19 (thiamine transporter), member 2                                                                         |
| 1436796_at   | 2.47            | Matr3, matrin 3                                                                                                                              |
| 1451195_a_at | 2.46            | Tmx1, thioredoxin-related transmembrane protein 1                                                                                           |
| 1436227_at   | 2.45            | Rab3gap2, RAB3 GTPase activating protein subunit 2                                                                                           |
| 1436945_x_at | 2.45            | Stim1, stromal interaction molecule 1                                                                                                       |
| 1435754_at   | 2.45            | Zyg11b, zyg-ll homolog B                                                                                                                   |
| 1419208_at   | 2.45            | Map3k8, mitogen-activated protein kinase kinase kinase 8                                                                                     |
| 1426842_at   | 2.45            | Ythdf3, YTH domain family 3                                                                                                                 |
| 1417365_a_at | 2.43            | Calm1, calmodulin 1                                                                                                                       |
| 1435284_at   | 2.43            | Rtn4, reticulon-4                                                                                                                          |
| 1447776_x_at | 2.43            | Rab6, RAB6, member RAS oncogene family                                                                                                     |
| 1434389_at   | 2.43            | Sos1, son of sevenless homolog 1                                                                                                           |
| 1415963_at   | 2.42            | Hnrrph2, heterogeneous nuclear ribonucleoprotein H2                                                                                          |
| 1438306_at   | 2.42            | Rnf180, ring finger protein 180                                                                                                             |
| 1419453_at   | 2.41            | Uchl5, ubiquitin carboxyl-terminal esterase L5                                                                                               |
| 1422748_at   | 2.41            | Zeb2, zinc finger E-box binding homeobox 2                                                                                                  |
| 1429436_at   | 2.40            | Prpf40a, pre-mRNA processing factor 40 homolog A                                                                                             |
| 1455252_at   | 2.39            | Tsc1, tuberous sclerosis 1                                                                                                                  |
| 1423994_at   | 2.38            | Kif1b, kinesin family member 1B                                                                                                            |
| 1455505_at   | 2.37            | Gatad2a, GATA zinc finger domain containing 2A                                                                                                |
| 1416009_at   | 2.35            | Tspan3, tetraspanin 3                                                                                                                      |
| 1454602_s_at | 2.34            | Cnot2, CCR4-NOT transcription complex, subunit 2                                                                                                |
| 1455658_at   | 2.33            | Cggbp1, CGG triplet repeat binding protein 1                                                                                               |
| 1448733_at   | 2.32            | Bmi1, Bmi1 polycomb ring finger oncogene                                                                                                   |
| 1424147_at   | 2.32            | Ahsa1, AHA1, activator of heat shock protein ATPase homolog 1                                                                             |
| 1433898_at   | 2.32            | N/A, N/A                                                                                                                                   |
| 1433668_at   | 2.32            | Pnrc1, proline-rich nuclear receptor coactivator 1                                                                                           |
| 1424111_at   | 2.32            | Igf2r, insulin-like growth factor 2 receptor                                                                                               |
| 1434437_x_at | 2.32            | Rrm2, ribonucleotide reductase M2                                                                                                          |
| ProbeID   | log2FC | Description                                                  |
|-----------|--------|--------------------------------------------------------------|
| 1424043_at| 2.31   | Ppil4 peptidylprolyl isomerase (cyclophilin)-like 4          |
| 1439345_at| 2.31   | Gpnmb glycoprotein (transmembrane) nmb                      |
| 1439463_x_at| 2.31 | LOC637733 predicted gene, EG665056                         |
| 1428252_at| 2.31   | Chmp2b chromatin modifying protein 2B                        |
| 1451971_at| 2.30   | Cul4a cullin 4A                                             |
| 1422449_s_at| 2.30 | Rcn2 reticulocalbin 2                                       |
| 1417478_a_at| 2.30 | Ppp2r3c protein phosphatase 2, regulatory subunit B'', gamma |
| 1451356_at| 2.26   | Anp32e acidic (leucine-rich) nuclear phosphoprotein 32 family, member E |
| 1437386_at| 2.26   | Lingo1 leucine rich repeat and Ig domain containing 1       |
| 1434352_at| 2.25   | B630005N14Rik RIKEN cDNA B630005N14 gene                    |
| 1416267_at| 2.24   | Scoc short coiled-coil protein                              |
| 1423069_at| 2.24   | Adnp activity-dependent neuroprotective protein             |
| 1437892_at| 2.17   | Zkscan3 zinc finger with KRAB and SCAN domains 3            |
| 1434928_at| 2.17   | Gas2I1 growth arrest-specific 2 like 1                      |
| 1416637_at| 2.16   | Slc4a2 solute carrier family 4 (anion exchanger), member 2 |
| 1436732_s_at| 2.16 | Fbxw8 F-box and WD-40 domain protein 8                      |
| 1458662_at| 2.15   | Daam1 RIKEN cDNA 1700008003 gene                            |
| 1453109_at| 2.15   | Arsk arylsulfatase K                                        |
| 1421910_at| 2.15   | Tcf20 transcription factor 20                               |
| 1416247_at| 2.14   | Dctn3 dynactin 3                                            |
| 1454863_at| 2.12   | Ankrd11 ankyrin repeat domain 11                            |
| 1417995_at| 2.12   | Ptnp22 protein tyrosine phosphatase, non-receptor type 22   |
| 1418736_at| 2.11   | B3galnt1 UDP-GalNAc:betaGlcNAc beta 1,3-galactosaminyltransferase, polypeptide 1 |
| 1433740_at| 2.10   | Tmem87b transmembrane protein 87B                           |
| 1455312_at| 2.09   | Phc3 polyhomeotic-like 3                                    |
| 1416477_at| 2.08   | Ube2d2 ubiquitin-conjugating enzyme E2D 2                   |
| 1427134_at| 2.08   | Sfrs12 splicing factor, arginine/serine-rich 12              |
| 1438769_a_at| 2.07 | Thyn1 thymocyte nuclear protein 1                            |
| 1425018_at| 2.07   | Mcts1 malignant T cell amplified sequence 1                  |
| 1457069_at| 2.06   | Ascc3 activating signal cointegrator 1 complex subunit 3    |
| 1428949_at| 2.05   | Xpot exportin, tRNA (nuclear export receptor for tRNAs)
| EntrezGene ID | log2FoldChange | Description |
|---------------|----------------|-------------|
| 1429588_at    | 2.04           | RIKEN cDNA 2810474O19 gene |
| 1448509_at    | 2.04           | Fam107b family with sequence similarity 107, member B |
| 1422675_at    | 2.03           | Smarce1 SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1 |
| 1448398_s_at  | 2.03           | Rpl22 ribosomal protein L22 |
| 1435988_x_at  | 2.02           | Ik cytokine |
| 1417390_at    | 2.02           | Gpn1 GPN-loop GTPase 1 |
| 1448215_a_at  | 2.01           | Dpp3 dipeptidylpeptidase 3 |
| 1421965_s_at  | 2.01           | Notch3 Notch gene homolog 3 |
| 1424842_a_at  | 2.01           | Arhgap24 Rho GTPase activating protein 24 |
| 1434014_at    | 2.01           | Atg4c autophagy-related 4C |
| 1435347_at    | 1.99           | Stau1 staufen (RNA binding protein) homolog 1 |
| 1421077_at    | 1.99           | Sertad3 SERTA domain containing 3 |
| 1428461_at    | 1.99           | Ppp2r5e protein phosphatase 2, regulatory subunit B (B56), epsilon isoform |
| 1436165_at    | 1.98           | Luc7l2 LUC7-like 2 |
| 1452895_at    | 1.98           | Fbxo45 F-box protein 45 |
| 1455728_at    | 1.98           | Pten phosphatase and tensin homolog |
| 1456617_a_at  | 1.97           | Elf2s2 eukaryotic translation initiation factor 2, subunit 2 |
| 1436977_at    | 1.97           | N/A |
| 1448527_at    | 1.97           | Pdcd10 programmed cell death 10 |
| 1458414_at    | 1.96           | D2Ertd93e DNA segment, Chr 2, ERATO Doi 93, expressed |
| 1420528_at    | 1.95           | Gpatch2 G patch domain containing 2 |
| 1460615_at    | 1.95           | Nt5dc1 S'-nucleotidase domain containing 1 |
| 1449085_at    | 1.95           | Phf10 PHD finger protein 10 |
| 1431694_a_at  | 1.95           | Ctnnbip1 catenin beta interacting protein 1 |
| 1416840_at    | 1.93           | Mid1ip1 Mid1 interacting protein 1 |
| 1449694_s_at  | 1.93           | Commd5 COMM domain containing 5 |
| 1455432_at    | 1.92           | Taok1 TAO kinase 1 |
| 1434561_at    | 1.92           | Asxl1 additional sex combs like 1 |
| 1426844_a_at  | 1.92           | Pdcd2l programmed cell death 2-like |
| 1416998_at    | 1.91           | Rrs1 RRS1 ribosome biogenesis regulator homolog |
| 1427156_s_at  | 1.88           | Ascc2 activating signal cointegrator 1 complex subunit 2 |
| 1424389_at    | 1.86           | Nupl1 nucleoporin like 1 |
| 1435389_at    | 1.86           | Reps2 RALBP1 associated Eps domain containing protein 2 |
| 1423167_at    | 1.86           | Mobki3 MOB1, Mps One Binder kinase activator-like 3 |
| 1453104_at    | 1.86           | Mapk1 mitogen-activated protein kinase 1 |
| 1455403_at    | 1.84           | Manea mannosidase, endo-alpha |
| 1436346_at    | 1.82           | Cd109 CD109 antigen |
| 1438771_at    | 1.81           | LOC100045983 bromodomain containing 1 |
| 1455644_at    | 1.80           | Vps53 vacuolar protein sorting 53 |
| 1424991_s_at  | 1.80           | Tyms-ps thymidylate synthase |
| 1441942_x_at  | 1.80           | Snupn snurportin 1 |
| 1427317_at    | 1.79           | Kin antigenic determinant of rec-A protein |
| 1428254_at    | 1.79           | Purb purine rich element binding protein B |
| 1426840_at    | 1.78           | Ythdf3 YTH domain family 3 |
| Gene Symbol | Ratio |
|-------------|-------|
| Msl2        | 1.77  |
| Ppp2r5c     | 1.77  |
| Ttc35       | 1.77  |
| Actr8       | 1.75  |
| Lrwd1       | 1.74  |
| Rab9        | 1.73  |
| Uba2        | 1.70  |
| Ggta1       | 1.66  |
| Rab9        | 1.64  |
| N/A         | 1.63  |
| Mtrr        | 1.62  |
| Hnrnpul2    | 1.61  |
| Grpel2      | 1.60  |
| Speg        | 1.59  |
| Gna13       | 1.56  |
| A930001N09Rik | 1.55 |
| Cpsf3l      | 1.55  |
| Ctsl        | 1.54  |
| Lig1        | 1.53  |
| AI314976    | 1.52  |
| Trim65      | 1.51  |
| Ddx6        | 1.51  |
| LOC100046401| 1.50  |
| 1110003E01Rik | 1.45 |
| 4933433G19Rik | 1.38 |
| Sec141l     | 1.36  |
| Cln6        | 1.36  |
| Ctsl        | 1.12  |
| Npc2        | 1.05  |
| Adcy3       | -1.32 |
| Tmem20      | -1.37 |
| Mup5        | -1.37 |
| N/A         | -1.38 |
| Sic35a5     | -1.38 |
| Kank4       | -1.39 |
| 2900083I11Rik | -1.40 |
| Pou2f2      | -1.40 |
| Zfp446      | -1.45 |
| Agb13       | -1.45 |
| N/A         | -1.47 |
| N/A         | -1.51 |
| Ern1        | -1.51 |

**Gene Descriptions:**
- **Msl2**: male-specific lethal 2 homolog
- **Ppp2r5c**: protein phosphatase 2, regulatory subunit B (B56), gamma isoform
- **Ttc35**: tetratricopeptide repeat domain 35
- **Actr8**: ARP8 actin-related protein 8 homolog
- **Lrwd1**: leucine-rich repeats and WD repeat domain containing 1
- **Rab9**: RAB9, member RAS oncogene family
- **Uba2**: ubiquitin-like modifier activating enzyme 2
- **Ggta1**: glycoprotein galactosyltransferase alpha 1, 3
- **Hnrnpul2**: heterogeneous nuclear ribonucleoprotein U-like 2
- **Mtrr**: 5-methyltetrahydrofolate-homocysteine methyltransferase reductase
- **Hnrnpul2**: heterogeneous nuclear ribonucleoprotein U-like 2
- **Grpel2**: GrpE-like 2, mitochondrial
- **Speg**: SPEG complex locus
- **Gna13**: guanine nucleotide binding protein, alpha 13
- **Cpsf3l**: cleavage and polyadenylation specific factor 3-like
- **Ctsl**: cathepsin L
- **Lig1**: ligase I, DNA, ATP-dependent
- **Al314976**: expressed sequence AI314976
- **Trim65**: tripartite motif-containing 65
- **Ddx6**: DEAD (Asp-Glu-Ala-Asp) box polypeptide 6
- **LOC100046401**: ferric-chelate reductase 1
- **1110003E01Rik**: RIKEN cDNA 1110003E01 gene
- **4933433G19Rik**: RIKEN cDNA 4933433G19Rik
- **Sec141l**: SEC14-like 1 (S. cerevisiae)
- **Cln6**: ceroid-lipofuscinosis, neuronal 6
- **Ctsl**: cathepsin L
- **Npc2**: Niemann Pick type C2
- **Adcy3**: adenylate cyclase 3
- **Tmem20**: transmembrane protein 20
- **Mup5**: major urinary protein 5
- **N/A**: Not available
- **Sic35a5**: solute carrier family 35, member A5
- **Kank4**: KN motif and ankyrin repeat domains 4
- **2900083I11Rik**: RIKEN cDNA 2900083I11 gene
- **Pou2f2**: POU domain, class 2, transcription factor 2
- **Zfp446**: zinc finger protein 446
- **Agb13**: ATP/GTP binding protein-like 3
- **N/A**: Not available
- **Ern1**: endoplasmic reticulum (ER) to nucleus signalling 1
| Probe ID       | Ratio | Description                                      |
|---------------|-------|--------------------------------------------------|
| 1446354_at    | -1.53 | C130098B18Rik RIKEN cDNA C130098B18 gene         |
| 1429560_at    | -1.53 | 100043468 predicted gene, 100043468             |
| 1440722_at    | -1.55 | D19Erd386e                                      |
| 1459324_at    | -1.58 | Fermt2 fermitin family homolog 2                |
| 1419214_at    | -1.59 | Tnrsf11a tumor necrosis factor receptor superfamily, member 11a |
| 1438529_at    | -1.60 | Tmem201 transmembrane protein 201               |
| 1445175_at    | -1.61 | 100042940 predicted gene, 100042940             |
| 1443785_x_at  | -1.62 | Pdlim7 PDZ and LIM domain 7                     |
| 1438529_at    | -1.63 | RIKEN cDNA 4933428L12 gene                      |
| 1452764_at    | -1.65 | Socs6 suppressor of cytokine signaling 6         |
| 14458000_at   | -1.66 | 2310061A09Rik RIKEN cDNA 2310061A09 gene        |
| 1443785_x_at  | -1.66 | Rnf32 ring finger protein 32                     |
| 1458747_at    | -1.67 | 4933425M03Rik RIKEN cDNA 4933425M03 gene        |
| 1432260_at    | -1.67 | Gpr39 G protein-coupled receptor 39              |
| 1432875_at    | -1.68 | Tctn2 tectonic family member 2                  |
| 1454294_at    | -1.68 | Frap1 FK506 binding protein 12-rapamycin associated protein 1 |
| 1447789_x_at  | -1.69 | Ddx6 DEAD (Asp-Glu-Ala-Asp) box polypeptide 6    |
| 1430373_at    | -1.70 | Ctda3 cell division cycle associated 3           |
| 1459307_at    | -1.71 | N/A N/A                                          |
| 1432260_at    | -1.72 | 4632409D06Rik RIKEN cDNA 4632409D06 gene        |
| 1455961_at    | -1.72 | Mme membrane metallo endopeptidase              |
| 1447884_x_at  | -1.73 | Fam134c family with sequence similarity 134, member C |
| 1458661_at    | -1.73 | N/A N/A                                          |
| 1430137_at    | -1.74 | 1300002E11Rik RIKEN cDNA 1300002E11 gene         |
| 1439515_at    | -1.75 | Setd5 SET domain containing 5                   |
| 1456045_at    | -1.76 | 1700106N22Rik RIKEN cDNA 1700106N22 gene         |
| 1439339_at    | -1.76 | N/A N/A                                          |
| 1432875_at    | -1.77 | Ddx42 DEAD (Asp-Glu-Ala-Asp) box polypeptide 42  |
| 1432719_x_at  | -1.78 | Tctn2 tectonic family member 2                  |
| 1437594_x_at  | -1.78 | Tctn2 tectonic family member 2                  |
| 14458000_at   | -1.78 | Tctn2 tectonic family member 2                  |
| 1447789_x_at  | -1.79 | N/A N/A                                          |
| 1430373_at    | -1.79 | N/A N/A                                          |
| 1423224_at    | -1.82 | Tctn2 tectonic family member 2                  |
| 1437594_x_at  | -1.82 | Ddx42 DEAD (Asp-Glu-Ala-Asp) box polypeptide 42  |
| 1457639_at    | -1.83 | Gm428                                            |
| 1430996_at    | -1.85 | Etnk1 ethanolamin kinase 1                      |
| 1437594_x_at  | -1.86 | Dhx58 DEXH (Asp-Glu-X-His) box polypeptide 58    |
| 1429560_at    | -1.87 | Nfatc2 nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 |
| 1453090_x_at  | -1.88 | N/A N/A                                          |
| 1421990_at    | -1.90 | Syt1 synaptotagmin 1                            |
| 1452920_a_at  | -1.96 | Ppi2 peptidylprolyl isomerase (cyclophilin)-like 2 |
| 1423953_at    | -1.96 | Cdkal1 CDK5 regulatory subunit associated protein 1-like 1 |
| 1432711_at    | -1.96 | 4933425M03Rik RIKEN cDNA 4933425M03 gene        |
| 1457629_at    | -1.97 | Atmin ATM interactor                             |
| 1415748_a_at  | -1.97 | Dctn5 polo-like kinase 1                        |
| 1430358_at    | -1.99 | Becn1 beclin 1                                  |
| 1430373_at    | -1.99 | 5430427O19Rik RIKEN cDNA 5430427O19 gene         |
| Gene ID   | Ratio | Description                                                                 |
|----------|-------|-----------------------------------------------------------------------------|
| 1447820_x_at | -2.00 | Cpt2, carnitine palmitoyltransferase 2                                     |
| 1437762_at  | -2.04 | Rab39, RAB39, member RAS oncogene family                                    |
| 1416787_at  | -2.07 | Acvr1, activin A receptor, type 1                                            |
| 1446422_at  | -2.12 | A530081L18Rik, membrane-associated ring finger (C3HC4) 3                   |
| 1447267_at  | -2.14 | C8S319, expressed sequence C8S319                                           |
| 1454219_at  | -2.15 | Dnajc2, DnaJ (Hsp40) homolog, subfamily C, member 2                         |
| 1430772_at  | -2.22 | 4932438H23Rik, RIKEN cDNA 4932438H23 gene                                   |
| 1447768_at  | -2.24 | Got2, glutamate oxaloacetate transaminase 2, mitochondrial                 |
| 1446001_at  | -2.25 | N/A                                                                         |
| 1442954_at  | -2.33 | N/A                                                                         |
| 1444873_at  | -2.35 | Hip1, huntingtin interacting protein 1                                       |
| 1424595_at  | -2.37 | F11r, F11 receptor                                                          |
| 1455284_x_at| -2.41 | Pigx, phosphatidylinositol glycan anchor biosynthesis, class X              |
| 1420948_s_at| -2.42 | Atrx, alpha thalassemia/mental retardation syndrome X-linked homolog       |
| 1441784_at  | -2.46 | N/A                                                                         |
| 1446211_at  | -2.55 | Erbb4, v-erb-a erythroblastic leukemia viral oncogene homolog 4             |
| 1433020_at  | -2.65 | 5830413G11Rik, RIKEN cDNA 5830413G11 gene                                   |
| 1456808_at  | -2.68 | 4933426M11Rik, RIKEN cDNA 4933426M11 gene                                   |
| 1453534_at  | -2.71 | 2810004i08Rik                                                              |
| 1446004_at  | -2.95 | N/A                                                                         |
| 1452240_at  | -3.25 | Brunol4, bruno-like 4, RNA binding protein                                   |