Conditional Knockout of Macrophage PPARγ Increases Atherosclerosis in C57BL/6 and Low-Density Lipoprotein Receptor–Deficient Mice

Vladimir R. Babaev, Patricia G. Yancey, Sergey V. Ryzhov, Valentina Kon, Matthew D. Breyer, Mark A. Magnuson, Sergio Fazio, MacRae F. Linton

Objective—Peroxisome proliferator-activated receptor gamma (PPARγ) is highly expressed in macrophage-derived foam cells of atherosclerotic lesions, and its expression may have a dramatic impact on atherosclerosis.

Methods and Results—To investigate the contribution of macrophage PPARγ expression on atherogenesis in vivo, we generated macrophage-specific PPARγ knockout (MacPPARγKO) mice. C57BL/6 and low-density lipoprotein (LDL) receptor–deficient (LDLR−/−) mice were reconstituted with MacPPARγKO or wild-type marrow and challenged with an atherogenic diet. No differences were found in serum lipids between recipients reconstituted with MacPPARγKO and wild-type marrow. In contrast, both C57BL/6 and LDLR−/− mice transplanted with MacPPARγKO marrow had significantly larger atherosclerotic lesions than control recipients. In addition, MacPPARγKO→LDLR−/− mice had higher numbers of macrophages in atherosclerotic lesions compared with controls. Peritoneal macrophages isolated from the MacPPARγKO mice had decreased uptake of oxidized but not acetylated LDL and showed no changes in either cholesterol efflux or inflammatory cytokine expression. Macrophages from MacPPARγKO mice had increased levels of migration and CC chemokine receptor 2 (CCR2) expression compared with wild-type macrophages.

Conclusion—Thus, macrophage PPARγ deficiency increases atherosclerosis under conditions of mild and severe hypercholesterolemia, indicating an antiatherogenic role for PPARγ, which may be caused, at least in part, by modulation of CCR2 expression and monocyte recruitment. (Arterioscler Thromb Vasc Biol. 2005;25:1647-1653.)

Key Words: ABCA1 ■ atherosclerosis ■ CCR2 expression ■ cholesterol efflux ■ macrophages ■ scavenger receptor CD36

Peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear transcription factor that regulates a large number of genes important in lipid metabolism and inflammation.1 The receptor is highly expressed in macrophages and macrophage-derived foam cells of atherosclerotic lesions,2–4 and its expression may critically affect macrophage functions that impact atherosclerosis, including activation, cytokine production, recruitment, and transformation into foam cells.

Several studies have shown that the administration of PPARγ agonists inhibits the development of atherosclerosis in low-density lipoprotein (LDL) receptor–deficient (LDLR−/−)5,6 and apolipoprotein E-deficient (apoE−/−) mice.7 Consistent with this, mice transplanted with bone marrow from a PPARγ−/− chimera mouse exhibit a significant increase in atherosclerosis.8 These data all support an antiatherogenic role for macrophage PPARγ in atherosclerotic lesion development.

It has been assumed that the antiatherogenic effects of macrophage PPARγ expression may derive from activation of genes responsible for cholesterol efflux, thus shifting the balance from lipid loading to lipid efflux.8 Recent studies, however, have not confirmed the role of PPARγ ligands in cholesterol efflux by macrophages.9,10 PPARγ may also exert antiinflammatory effects in macrophages directly,11 or through LXRα12 by negatively interfering with the AP-1, NFκB, and STAT signaling pathways,13 or by reducing tumor necrosis factor-α, IL-1, and IL-6 secretion.14 However, the loss of PPARγ expression in macrophages derived from embryonic stem cells does not appear to alter basal or stimulated levels of cytokine secretion.15,16

In vitro studies have demonstrated that PPARγ expression by human and murine monocytes directly inhibits CC chemokine receptor 2 (CCR2) expression and suppresses MCP-1–mediated chemotaxis.17 In addition, pretreatment of monocytes with PPARγ agonists reduced their adhesion to vascular endothelium18 and their transendothelial migration.8 Based on these data, it appears that at least some of the antiatherogenic effects of macrophage PPARγ may be attributed to inhibition of 1647

Original received January 5, 2005; final version accepted May 27, 2005.
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Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org
DOI: 10.1161/01.ATV.0000173413.31789.1a
of macrophage recruitment and migration; however, the physiological relevance of the inhibitory processes in vivo remains unclear.

Targeted disruption of the PPARγ gene in mice causes early embryonic lethality19–21 and thus presents an obstacle to a systematic study of the gene’s role in atherogenesis. Therefore, we generated mice with a macrophage-specific PPARγ knockout (MacPPARγKO) using the Cre-loxP recombination system approach under the control of the murine M lysozyme promoter. C57BL6 and LDLR knockout (MacPPARγKO) mice were lethally irradiated, reconstituted with marrow from MacPPARγKO or wild-type mice, and challenged with atherogenic diets. Mice reconstituted with MacPPARγKO marrow exhibited significantly larger atherosclerotic lesions with increased numbers of macrophages. Macrophages from these mice also expressed higher levels of CCR2, suggesting that an increase in monocyte recruitment may be responsible for the accelerated atherosclerosis seen in these mice.

Methods

Animal Procedures

Mice with the “floxed” PPARγ (PPARγfl) gene,22 a mouse Cre line under the control of the murine M lysozyme promoter,23 and transgenic ROSA26R24 were at the sixth or more backcross into C57BL/6 background. ROSA26 mice,25 recipient C57BL/6, and LDLR−/− mice on C57BL/6 background were purchased from Jackson Laboratories Inc (Bar Harbor, Me). All mice were main- tained in micro-isolator cages on a rodent chow diet containing 4.5% fat (PMI number 5010) and autoclaved acidified (pH 2.8) water. A Western diet consisted of 21% fat (Intracel Corp, Rockville, Md) at 37°C for 4 hours and analyzed under a fluorescent microscope or by fluorescence-activated cell sorter (FACS) flow cytometry.

Cholesterol Loading and Efflux

Macrophages were cultured in DMEM containing 1% fetal bovine serum, 3 μCi/mL of 1H-cholesterol, and 70 μg/mL of human AcLDL for 48 hours. Cholesterol pools were equilibrated overnight in 0.1% bovine serum albumin–DMEM. Then cells were incubated with human apolipoprotein AI (20 μg/mL) or high-density lipoprotein (50 μg/mL) for up to 7 hours. For each time point, 1H-cholesterol was measured in aliquots of media. The cell lipids were extracted and used for measurement of 1H cholesterol.

Ligand Treatment and Real-Time Polymerase Chain Reaction

Macrophages were cultured in DMEM media supplemented with 5% lipoprotein-deficient fetal bovine serum (Intracel) with or without 10 μmol/L per mL rosiglitazone for 24 hours. Total RNA was isolated from peritoneal macrophages using the Trizol reagent (Invitrogen, Carlsbad, Calif) purified by RNA Easy kit (Qiagen). Relative quantitation of the target mRNA were performed on the ABI Prism 7000 Sequence Detection System (Applied Biosystems) and normalized to β-actin or 18S ribosomal RNA. Probes for PPARγ, CD36, apoE, ABCA1, MCP-1, Gro1, and CCR2 were provided by Applied Biosystems.

Migration Assay

In vitro assays were performed in a 96-well modified Boyden chamber with a 3-μm filter pore size (Millipore). Cell solution (100 μL) was added to each well in the top filter plate portion of the assembly, and MCP-1 (0.1 μg/mL) or media was added to the bottom feeder wells. A 1 hour, the upper portion was removed and cell numbers were counted.

Statistical Analysis

The statistical differences in mean serum lipids and aortic lesion areas between the groups were determined using the SigmaStat V.2 software (SPSS Inc, Chicago, Ill) by Student t test and the Mann–Whitney rank sum test, respectively.

Results

Macrophage-Specific PPARγ Knockout

Using the Cre-loxP recombination system, we generated MacPPARγKO mice on the C57BL/6 background. These mice were viable and fertile with no notable differences in body weight or plasma lipid levels when compared with PPARγfl littermates. The level of PPARγ RNA was dramatically decreased in peritoneal macrophages from MacPPARγKO mice compared with that of wild-type macrophages as analyzed by RT-PCR (Figure 1A) or quantitative real-time PCR normalized to 18S ribosomal RNA (Figure 1B). Deficiency in PPARγ RNA was specific for macrophages and not observed in kidney, adipose, or liver tissue (data not shown).

To monitor the effectiveness of Cre recombinase, we crossed MacPPARγKO mice with transgenic ROSA26R mice. This strain has a “floxed” STOP codon at the 5’ end of the lacZ gene driven by the β-actin promoter.24 In MacPPARγKO/ROSA26R mice, Cre recombinase excised the STOP codon, releasing lacZ expression in the majority (93% to 96%) of peritoneal macrophages. The intensity of lacZ expression in these cells, as measured by a FACS assay, was significantly higher when compared with macrophages
Role of Macrophage PPARγ in Atherosclerosis

To examine the impact of MacPPARγKO on atherosclerosis, 7-week-old female C57BL/6 and 8-week-old female LDLR−/− mice were lethally irradiated and transplanted with marrow from female MacPPARγKO (n=16 for each experimental group) or PPARγfl/fl mice (n=15 for each control group). Six or 4 weeks after transplantation, recipient mice were challenged with the butterfat or the Western diets for 16 or 8 weeks, respectively.

Serum lipid levels did not differ significantly between the control and experimental groups of mice on either the chow or the atherogenic diets with exception that the triglycerides were higher in MacPPARγKO→C57BL/6 mice after 12 weeks of the butterfat diet (Tables I and II, available online at http://atvb.ahajournals.org). Similarly, serum lipoprotein profiles did not differ significantly between experimental and control groups of the recipient mice (Figure Ia and Ib, available online at http://atvb.ahajournals.org). In contrast, the extent of atherosclerotic lesions in the proximal aortas of C57BL/6 and LDLR−/− recipients reconstituted with MacPPARγKO macrophages was significantly greater (48% and 84%) compared with PPARγfl/fl →C57BL/6 (37 715±3010 versus 25 512±2660 μm²; P=0.005) and PPARγfl/fl→LDLR−/− mice (125 120±9540 versus 67 498±6205 μm²; P<0.001), respectively (Figure 2A and 2B). MacPPARγKO→LDLR−/− recipients had larger (46%) lesion area in the distal aortas analyzed en face compared with PPARγfl/fl→LDLR−/− mice (0.19±0.01 versus 0.13±0.02%; P=0.031; Figure 2C). Thus, macrophage PPARγ expression plays a protective role in atherosclerotic lesion formation.

Modified LDL Uptake and Cholesterol Efflux by Macrophages From MacPPARγKO Mice

In an effort to better understand the molecular basis of these effects, we first analyzed the impact of the macrophage PPARγ gene deletion on uptake of modified LDL. Peritoneal macrophages were isolated and incubated with DiI-labeled oxidized and acetylated LDL. Microscopic analysis showed that PPARγ−/− macrophages accumulated significantly less oxidized LDL than wild-type macrophages (Figure 3A). FACS analysis demonstrated that PPARγ−/− macrophages had a reduced (43% to 57%) levels of oxidized LDL uptake but not AcLDL uptake, compared with wild-type macrophages (Figure 3B and 3C). To test cholesterol efflux, macrophages were loaded with human AcLDL cholesterol
and incubated with high-density lipoprotein or apolipoprotein AI. No differences were noted in either high-density lipoprotein- or apolipoprotein AI-mediated cholesterol efflux from PPARγ/H9253/H11002/H11002/ and wild-type macrophages (Figure 4A and 4B). However, real-time PCR analysis revealed that wild-type macrophages treated with a PPARγ ligand, rosiglitazone, expressed significantly higher levels of CD36 (269%) and ABCA1 (125%) but not apoE RNA compared with control nontreated cells (Figure 4C to 4F). In PPARγ/H9253/H11002/H11002/macrophages, these stimulation effects for the CD36 and ABCA1 genes were lost.

Inflammatory Cytokine Gene Profiles and CCR2 Expression by Macrophages From MacPPARγKO Mice

To examine the antiinflammatory effects of macrophage PPARγ, mRNA was isolated from lipopolysaccharide-activated macrophages and analyzed using an inflammatory response cytokines gene array kit. For both type of macrophages, expression levels for the majority of cytokines (IL1a, IL1b, IL6, IL12a, IL18, transforming growth factor-β, and tumor necrosis factor-α) were not significantly different, with the exception of the Gro1 oncogene (Table III, available online at http://atvb.ahajournals.org). As confirmed by real-time PCR, PPARγ+− macrophages stimulated by lipopolysaccharide had increased levels of the Gro1 (1.7 fold) but not MCP-1 expression compared with wild-type macrophages (Figure 4A and 4B, available online at http://atvb.ahajournals.org).

Finally, the impact of PPARγ expression by macrophages on the CCR2/MCP-1 pathway was analyzed by real-time PCR. The level of mRNA CCR2 expression was significantly increased (1.7-fold) in PPARγ+− macrophages compared with wild-type macrophages (Figure 5A). In addition, PPARγ+− macrophages had increased levels CCR2 protein expression (107±12 versus 75±5; P<0.05) as detected by FACS (Figure 5B).

Given the pivotal role of the CCR2 pathway in monocyte recruitment, we performed a series of in vitro experiments to determine the ability of peritoneal macrophages to migrate. Macrophages from MacPPARγKO mice migrated significantly faster in both nonstimulated and MCP-1–directed tests compared with macrophages from PPARγfl/fl mice (Figure 5C). In addition, we stained sections from the proximal aorta of LDLR−− recipients using a macrophage-specific antibody and DAPI (Figure 6). MacPPARγKO→LDLR−− mice had a significantly increased (36%) number of macrophages per a
In these studies, we have used a novel macrophage-specific knockdown approach to examine the role of macrophage PPARγ in atherogenesis. To our knowledge, this is the first report of the Lys-M-Cre approach to examine the effects of macrophage-specific gene expression in atherosclerosis in vivo. Although Akiyama et al. previously developed a macrophage-specific knockout of PPARγ driven by the MX1 promoter, they did not report the impact of PPARγ deficiency on atherosclerosis. Furthermore, our approach has an advantage over the MX1 promoter approach in that the LysM-Cre mice do not need induction to develop PPARγ deficiency. Chawla et al. reported similar antiatherogenic effects of macrophage PPARγ expression in LDLR−/− mice reconstituted with PPARγ−/− marrow from a chimeric mouse. Consistent with the results of genetic deletion of macrophage PPARγ, administration of PPARγ agonists to LDLR−/− mice and apoE−/− mice, and in balloon injury experiments, have also demonstrated an antiatherogenic role for PPARγ.

To investigate possible mechanisms by which macrophage PPARγ delivers its antiatherogenic effects, we first focused on the ability of macrophages to take-up modified lipoproteins. We found that MacPPARγKO macrophages have decreased uptake of oxidized but not acetylated LDL and, unlike wild-type macrophages, did not show an increase in CD36 expression in response treatment with PPARγ agonists. These findings are consistent with previous ex vivo studies demonstrating that PPARγ has a critical role in the basal regulation of the CD36 gene in macrophages. Interestingly, Liang et al. recently reported that, in the setting of extreme insulin resistance caused by leptin deficiency found in ob/ob and ob/ob LDLR−/− mice, treatment with a thiazolidinedione results in reduced systemic insulin resistance leading to reduced macrophage CD36 protein, despite an increase in macrophage CD36 gene expression, caused by correction of defective insulin signaling in the macrophage. In contrast, in vivo thiazolidinedione treatment of LDLR−/− mice on a Western diet, a model associated with mild insulin resistance, resulted in a 3-fold increase in macrophage CD36 expression. Thus, the impact of PPARγ agonists on macrophage CD36 protein may vary with the degree of insulin resistance. Given that PPARγ agonists have been reported to reduce atherosclerosis in LDLR-deficient mice, the potentially proatherogenic effects of CD36 upregulation on foam cell formation are apparently outweighed by other antiatherogenic effects of PPARγ agonists in this model.
Next, we found that PPARγ−/− and wild-type macrophages have similar basal levels of cholesterol efflux. However, the agonist treatment increased ABCA1 gene expression levels in wild-type but not in PPARγ−/− macrophages, consistent with previous studies suggesting that the PPARγ-LXRα-ABCA1 pathway may be important in modulating the development of atherosclerosis.5,12

Macrophage PPARγ mediates the activation of a large number of genes that are important in inflammation.13 To test how PPARγ deficiency affects the macrophage’s ability to produce cytokines in response to lipopolysaccharide, inflammatory cytokine gene expression profiles were compared in macrophages from MacPPARγKO and wild-type mice. The majority of cytokines had similar levels of expression for both groups of macrophages, indicating that at least some of the previously described effects of PPARγ agonists on cytokine gene expression are independent of PPARγ gene expression.15,16 At the same time, MacPPARγKO→LDLR−/− mice had increased macrophage numbers in atherosclerotic lesions, augmented macrophage CCR2 expression, and migration. These data suggest that PPARγ modulates CCR2 expression and may affect monocyte recruitment.

Monocyte CCR2 expression is increased in hypercholesterolemic patients.34 Native LDL and oxidative stress increase CCR2 gene expression, whereas antioxidants rapidly inhibit it.35 Treatment with oxidized LDL activates PPARγ expression and PPARγ agonists markedly attenuate CCR2 expression in circulating monocytes.17 Recent in vivo studies have shown that PPARγ agonists suppress the recruitment of inflammatory cells via a PPARγ-dependent mechanism in cases of experimental glomerulonephritis36 and myocardial infarction in rats.37 All these data support the concept that PPARγ-modulated CCR2 expression may impact the development of atherosclerosis through an effect on monocyte recruitment.

Mounting evidence suggests that the MCP-1/CCR2 pathway is important in atherogenesis. Targeted deletion of CCR2 or its ligand MCP-1 significantly decreased macrophage recruitment and atherosclerotic lesion size in apoE−/− mice.38–40 Clinical studies also demonstrated that coronary atherosclerosis is decreased in patients with a polymorphism of the CCR2 gene that reduces its function.41 In addition, PPARγ agonists inhibit CCR2 expression in monocytes and atherosclerosis development in rats.42,43 Thus, PPARγ-mediated CCR2 expression by macrophages may be an important pathway in atherogenesis and provides a novel therapeutic target for prevention or treatment of atherosclerosis.

The role of macrophage PPARγ in atherosclerosis is clearly complex and likely includes important effects on cholesterol homeostasis and inflammatory pathways, which may vary with lesion stage and metabolic factors such as insulin resistance.30 Our conditional macrophage-specific knockout of PPARγ presents a new opportunity to study the role of macrophage PPARγ gene expression in atherogenesis in vivo. C57BL6 and LDLR−/− mice reconstituted with PPARγ−/− macrophages developed significantly larger atherosclerotic lesions compared with control mice in response to atherogenic diets. In the absence of any notable changes in serum lipids between control and experimental mice, the increase in atherosclerosis suggests that macrophage PPARγ is crucial for these antiatherogenic effects. The increase in CCR2 expression by macrophages from MacPPARγKO mice suggests a novel role for PPARγ in monocyte recruitment and the development of atherosclerosis.

Acknowledgments

This work was supported by National Institutes of Health grants HL65405, HL53989, HL 57986, DK59637 (Lipid, Lipoprotein, and Atherosclerosis Core of the Vanderbilt Mouse Metabolic Phenotyping Centers), V.R.B. is supported by an American Heart Association grant (0160160B). The authors thank Lei Ding and Youmin Zhang for excellent technical expertise.

References

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Arterioscler Thromb Vasc Biol. 2005;25:1647-1653; originally published online June 9, 2005; doi: 10.1161/01.ATV.0000173413.31789.1a

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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### Table I and II. Total Serum Cholesterol and Triglyceride Levels in Female C57BL6 (a) and Male LDLR−/− (b) Mice Transplanted with PPARγfl/fl and MacPPARγKO Marrow

<table>
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<tr>
<th>Group of Animals</th>
<th>Serum Lipid</th>
<th>6 weeks</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>12 weeks</th>
<th>16 weeks</th>
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<td></td>
<td></td>
<td>Chow diet</td>
<td>Butterfat diet</td>
<td>Butterfat diet</td>
<td>Butterfat diet</td>
<td>Butterfat diet</td>
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<td><strong>PPARγfl/fl</strong></td>
<td>Cholesterol</td>
<td>95 ± 2</td>
<td>118 ± 5</td>
<td>126 ± 4</td>
<td>134 ± 4</td>
<td>130 ± 4</td>
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<td>n = 16</td>
<td>Triglycerides</td>
<td>62 ± 5</td>
<td>59 ± 3</td>
<td>64 ± 4</td>
<td>81 ± 4</td>
<td>84 ± 4</td>
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<td><strong>MacPPARγ KO</strong></td>
<td>Cholesterol</td>
<td>98 ± 4</td>
<td>124 ± 3</td>
<td>139 ± 6</td>
<td>138 ± 3</td>
<td>138 ± 11</td>
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<td>n = 17</td>
<td>Triglycerides</td>
<td>71 ± 4</td>
<td>64 ± 2</td>
<td>76 ± 9</td>
<td>99 ± 4*</td>
<td>89 ± 5</td>
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</tbody>
</table>

Values are in mg/dl (mean ± SEM). The number of animals in each group is indicated by n. *The differences are statistically significant (p<0.05) compare to the control group at the same time point.

**Fig I.** Lipoprotein distribution in C57BL/6 (A) and LDLR−/− (B) mice transplanted with marrow from PPARγfl/fl (●) and MacPPARγKO mice (○) after 16 or 8 weeks of the diets. The lipoprotein distribution was determined by FPLC followed by cholesterol analysis of each fraction. Data are represented as an average (n=3) of the percent of total cholesterol per fraction. Fractions 14-17 contain VLDL; fractions 18-24 are IDL/LDL; and fractions 25-30 contain HDL. Fractions 31-40 include non-lipoprotein associated proteins.
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Supplemental Data

Figure III. **Representative cytokine gene array hybridization experiment.** Peritoneal macrophages isolated from MacPPARγKO and PPARγ<sup>fl/fl</sup> mice were treated with LPS (50ng/ml) for 5 hours. Total RNA was extracted, labeled with <sup>32</sup>P and hybridized with the Mouse inflammatory response cytokines gene array (SuperArray Bioscience Corp., Frederick, MD).

Figure IV. **Gro1 and MCP-1 expression in peritoneal macrophages.** Macrophages from MacPPARγKO (□) and PPARγ<sup>fl/fl</sup> (■) mice were treated with LPS for 5 hours. Total RNA was extracted and analyzed by quantitative real-time PCR. Note macrophages from MacPPARγKO mice expressed higher levels of *Gro1* (*p*<0.03) but not MCP-1 compared to wild type macrophages.
Table III. Gene expression profile in LPS-induced macrophages from MacPPARgKO and PPARγfl/fl mice, as determined by duplicate measurements using the Mouse Inflammatory Response Cytokines Gene Array (SuperArray kit, Cat No mGEA1013030(Rad))

<table>
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<tr>
<th>Unigene</th>
<th>Genebank</th>
<th>Gene Name</th>
<th>Description</th>
<th>GEA Location</th>
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<tr>
<td>Mm.529</td>
<td>K00083</td>
<td>FNγ</td>
<td>Mouse immune interferon (ifn-gamma) mRNA</td>
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<td>Mm.21013</td>
<td>J04956</td>
<td>Gro1</td>
<td>GRO1 oncogene</td>
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<td>Mm.15534</td>
<td>X01450</td>
<td>IL-1α</td>
<td>Mouse interleukin 1, alpha</td>
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<td>Mm.22150</td>
<td>M15131</td>
<td>IL-1P</td>
<td>Mouse interleukin 1-beta (IL-1-beta) mRNA</td>
<td>(2,A)(2,B)</td>
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<td>Mm.14190</td>
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<td>Mouse interleukin-2</td>
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<td>Mus musculus migration inhibitory factor (10K protein)</td>
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<td>Mm.89123</td>
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<td>MCP-1</td>
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<td>Mm.1293</td>
<td>NM 013693</td>
<td>TNF-a</td>
<td>Mus musculus tumor necrosis factor (Tnf)</td>
<td>(8,A)(8,B)</td>
<td>1.0</td>
</tr>
<tr>
<td>Mm.87778</td>
<td>M16819</td>
<td>TNF-P</td>
<td>Mouse tumor necrosis factor-beta mRNA</td>
<td>(8,0)(8,D)</td>
<td>1.1</td>
</tr>
<tr>
<td>Mm.103618</td>
<td>M12481</td>
<td>Actin</td>
<td>Cytoplasmic beta-actin</td>
<td>(3,G)(4,G)</td>
<td>1.0</td>
</tr>
<tr>
<td>Mm.5289</td>
<td>M32599</td>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>(5,G)(6,G)</td>
<td>(7,G)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pUC18</td>
<td>Bacterial plasmid.</td>
<td>(1,G)(2,G)</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Abbreviation: n.d., not detected