Reversal of Hyperlipidemia With a Genetic Switch Favorably Affects the Content and Inflammatory State of Macrophages in Atherosclerotic Plaques

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Background—We previously showed that the progression of atherosclerosis in the Reversa mouse (Ldlr<sup>−/−</sup>/Apob<sup>100/100</sup>Mttp<sup>fl/fl</sup>Mx1<sup>Cre</sup><sup>+/−</sup>) was arrested when the hyperlipidemia was normalized by inactivating the gene for microsomal triglyceride transfer protein. Here, we tested whether atherosclerosis would regress if the lipid levels were reduced.

Methods and Results—Reversa mice were fed an atherogenic diet for 16 weeks. Plasma lipid levels were then reduced. Within 2 weeks, this reduction led to decreased monocyte-derived (CD68<sup>+</sup>) cells in atherosclerotic plaques and was associated with emigration of these cells out of plaques. In addition, the fall in lipid levels was accompanied by lower plaque lipid content and by reduced expression in plaque CD68<sup>+</sup> cells of inflammatory genes and higher expression of genes for markers of antiinflammatory M2 macrophages. Plaque composition was affected more than plaque size, with the decreased content of lipid and CD68<sup>+</sup> cells balanced by a higher content of collagen. When the reduced lipid level was combined with the administration of pioglitazone to simulate the clinical aggressive lipid management and proliferator-activated receptor-γ agonist treatment, the rate of depletion of plaque CD68<sup>+</sup> cells was unaffected, but there was a further increase in their expression of antiinflammatory macrophage markers.

Conclusion—The Reversa mouse is a new model of atherosclerosis regression. After lipid lowering, favorable changes in plaque composition were independent of changes in size. In addition, plaque CD68<sup>+</sup> cells became less inflammatory, an effect enhanced by treatment with pioglitazone. (Circulation. 2011;123:989-998.)

Key Words: atherosclerosis □ lipoproteins □ macrophage □ murine model

Blocking the progression of atherosclerotic lesions and inducing their regression are highly desirable goals. Although mouse models have provided valuable information about atherosclerosis progression, comparatively little is known from them about the molecular mechanisms underlying regression. We previously allowed plaques to develop in ApoE<sup>−/−</sup> mice and then transplanted segments of the diseased aorta into wild-type mice. Plaque regression (defined as a reduction in monocyte-derived cell content) rapidly ensued. Indeed, there was a remarkable 50% loss from the plaques of the monocyte-derived (CD68<sup>+</sup>) cells within 3 days. This loss involved the migration of these cells to regional and systemic lymph nodes. We hypothesized that because dendritic cells are migratory though a pathway dependent on CCR7<sup>+</sup> the migratory CD68<sup>+</sup> cells during regression acquired some features of dendritic cells. Indeed, we found that during regression the expression of CCR7 was induced in plaque CD68<sup>+</sup> cells and that their migration was dependent on the CCR7-mediated pathway.

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Recently, another model, the Reversa mouse, has given us the opportunity to extend our studies to determine whether the findings in the transplant model were generally applicable to the regression process or were model specific. Reversa mice (Ldlr<sup>−/−</sup>/Apob<sup>100/100</sup>Mttp<sup>fl/fl</sup>Mx1<sup>Cre</sup><sup>+/−</sup>) have severe hypercholesterolemia as a result of homozygosity for the low-density lipoprotein (LDL) receptor knockout allele and an “apolipoprotein (apo) B100−only” allele, but the hypercholesterolemia can be conditionally eliminated by inducing the expression of the Mx1-Cre transgene, which inactivates Mttp (the gene for microsomal triglyceride transfer protein). In mammalian liver, microsomal triglyceride transfer protein is required for the
secretion of atherogenic apoB-containing lipoproteins, so after inactivation of its gene, plasma levels of very low-density lipoprotein and LDL fall. We previously showed that when the Mttp gene was activated early in life, atherosclerosis progression was retarded. We reasoned that the Reversa mouse could be used to study regression if the hypercholesterolemia were reduced after plaques developed. In the present study, we establish the Reversa mouse as a model of atherosclerosis regression and then tested whether the reduction of hypercholesterolemia led not only to plaque depletion of CD68+ cells, as in the transplant model, but also to an alteration of their phenotype to “alternatively activated” M2 macrophages, which are considered antiinflammatory (see, the work by Gordon). We also investigated whether the changes in atherosclerotic plaques occurring after the reduction in plasma cholesterol would be augmented by treatment of mice with the proliferator-activated receptor-γ (PPARγ) agonist pioglitazone, given its ability to reduce cardiovascular risk and its widespread clinical use in combination with lipid-lowering agents.

Methods

Genetically Modified Mice and Experimental Design

All procedures were approved by the Institutional Animal Care and Use Committee. Four- to 5-week-old Reversa mice (n = 86) were weaned onto a Western diet containing 0.15% cholesterol and 21% fat (Research Diets, New Brunswick, NJ) for 16 weeks. Mice were divided into treated and control groups. The treated group was switched to a chow diet, and the expression of the Mxl-Cre transgene was induced with injections of polyinosinic-polycytidylic acid (pIpC) (Sigma, St Louis, MO; 500 μg every other day for a total of 4 injections). The plpC injections led to inactivation of Mttp in the liver, resulting in the elimination of apoB100-containing lipoproteins from the plasma and normalization of plasma cholesterol levels 9 days after the first injection. That time point is considered the baseline or day 0 time point for all experiments. Separate groups of mice were euthanized either at the baseline time point or 3, 6, 14, 28, or 56 days after the baseline time point. The control group mice, which were given injections of normal saline and continued on the Western diet, were euthanized at the same time points. In some experiments, mice treated with plpC were maintained on a chow diet or a chow diet containing pioglitazone (200 ppm), a dose that induces PPARγ target genes in CD68+ cells in plaques.

Aortic transplant experiments were done as previously described, with the only differences being that the donor mice were hyperlipidemic Reversa mice fed the Western diet for 16 weeks and the recipients were normolipidemic Reversa mice. The transplantations were performed 2 weeks after the last plpC injections in the recipients. Five days after the atherosclerotic aortic segment was transplanted, it was harvested from the recipient mouse, frozen in optimum cutting temperature compound, and sectioned, and immunohistochemical and morphometric analyses were conducted as described below.

Lipid and Lipoprotein Analyses

Blood samples were obtained from the retro-orbital plexus. Plasma cholesterol levels were determined by colorimetric enzymatic assays that were adapted to 96-well formats (Infinity Total Cholesterol Reagent or Infinity Triglyceride Reagent, Sigma).

Labeling of Blood Monocytes

Ly6C+ (CCR2 high-expressing) and Ly6C0 (CCR2 low-expressing, CXCR1 high-expressing) monocytes in blood were labeled with fluorescent 0.5-μm microspheres (beads) as previously described. For selective labeling of the Ly6C0 monocytes, 250 μL of liposomes containing clodronate was intravenously injected into mice to transiently deplete monocytes, followed by an injection of 250 μL of fluorescent beads 24 hours later. To selectively label Ly6C+ monocytes, 250 μL of the fluorescent beads was injected intravenously without the clodronate pretreatment. Briefly, circulating monocytes will take up the beads and then enter tissues, including atherosclerotic plaques. Fluorescent beads in the plaque are visualized by fluorescence microscopy and counted in a blinded fashion. Over time, the bead content of the plaque will decrease if the beads left in the same cells that brought them in or if they were transferred to another monocyte-derived cell that then left. In other words, a decrease in bead number indicates that there was emigration of monocyte-derived cells from the plaque.

Tissue Processing, Histology, Immunohistochemistry, and Morphometry

Mice were euthanized, and the hearts with attached aortic roots were collected and frozen in optimum cutting temperature compound as described.4,12 Serial frozen aortic sections (6 μm thick) were prepared and mounted on positively charged slides (Color Frost Plus, Fisher Scientific, Pittsburgh, PA). Every fifth slide was stained with a CD68-specific antibody and used for morphometric analysis and as a guide slide for laser-capture microdissection. Additional slides were stained with Oil Red O, MOMA-2, CCR7, monocyte chemoattractant protein (MCP-1), or arginase I. For some studies, immunofluorescence microscopy was used. Collagen content of lesions was assessed with Sirius Red–stained slides under polarizing light. Intimal lesions and CD68-immunostained areas were quantified by computer-aided morphometric analysis of digitized images (ImagePro Plus 3.0 software, Media Cybernetics, Silver Spring, MD).

Laser Capture Microdissection and RNA Extraction

For laser-capture microdissection, all procedures were performed under RNase-free conditions. Sections were fixed in 70% ethanol for 1 minute, washed in H2O, stained with Mayer hematoxylin (WVR Scientific, Radnor, PA) for 1 minute, washed in H2O, incubated in PBS for 15 seconds (to develop the blue color), washed in H2O, partially dehydrated in 70% ethanol followed by 95% ethanol, stained in eosin Y (WVR Scientific) for 5 seconds, washed in 95% ethanol, and completely dehydrated in 100% ethanol (30 seconds) and xylene (7 minutes). After air drying for 10 minutes, foam cells could be identified by light microscopy and were verified by CD68 staining of guiding slides. Samples from 3 mice from the same treatment group and time point were pooled, and RNA was extracted with the QIAGEN microRNA kit with on-column DNase I treatment. The concentration of RNA was determined by the Ribogreen RNA Quantitation kit (Molecular Probes, Carlsbad, CA), and RNA quality was verified with the Agilent 2100 Bioanalyzer.

Quantitative Real-Time Polymerase Chain Reaction

RNA abundance was determined by quantitative real-time polymerase chain reaction (qRT-PCR) with 100 pg of total RNA. The primer and probe sequences have been described previously.15–17 All data were normalized to cyclophilin A and expressed as fold change compared with controls. For tissue samples generated by laser-capture microdissection, results were obtained from 2 to 3 independent samples, each pooled from plaques of 3 mice.

Statistics

All data are expressed as average ± SEM. For studies involving atherosclerotic lesions, the number of mice is indicated in the figure legends. Unless otherwise indicated, for RNA analyses, 2 pooled samples were used for each experiment. PRISM software (GraphPad,
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CD68

plaque CD68 points in the control group. Within each treatment group, 11 mg/dL). In addition, using laser-captured

/H11006 averaged 80

pIpC group was negligible by day 56 (total cholesterol then

Content of CD68 Reversal of the Hyperlipidemia Decreases the

Statistical analysis within each treatment group was by ANOVA followed by the Dunnett test to compare results at each time point to those on day 0. \( +P<0.03 \) or \( P<0.0001 \) vs day 0. B, Immunohistochemical staining of CD68. By 56 days, CD68+ cells (red) in plpC-treated mice were virtually undetectable.

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Figure 1. Correcting hyperlipidemia in Reversa mice reduces the content of CD68+ cells in plaques. A, CD68+ cell content in plaques of Reversa mice after plasma lipid normalization. Reversa mice (n=13 per group) were fed a Western diet for 16 weeks, at which point their high plasma lipid levels were either maintained (saline group) or normalized (pIpC group), as described in Methods. CD68+ cell content, quantified by morphometric techniques, was significantly lower 14 days after plasma lipids stabilized at the reduced levels. Statistical analysis within each treatment group was by ANOVA followed by the Dunnett test to compare results at each time point to those on day 0. \( +P<0.03 \) or \( P<0.0001 \) vs day 0. B, Immunohistochemical staining of CD68. By 56 days, CD68+ cells (red) in plpC-treated mice were virtually undetectable.

San Diego, CA) was used to analyze statistical differences typically by 1-way ANOVA with the Bonferroni posttest for differences between selected pairs of means. In some cases, as noted in Results or the figure legends, an unpaired \( t \) test or ANOVA with the Dunnett posttest was used to evaluate statistical differences. Values of \( P<0.05 \) were considered significant.

Results

Reversal of the Hyperlipidemia Decreases the Content of CD68+ Cells and Lipids in Plaques

Reversa mice (Ldlr−/−Apob100/100Mttp100/100Mx1Cre+/+) were placed on Western diet for 16 weeks, allowing them to develop severe hypercholesterolemia (1115±215 mg/dL) and advanced atherosclerotic plaques. Nine days after the administration of pIpC, plasma cholesterol levels fell to 86±5 mg/dL. That time point was designated day 0 or the baseline time point, and mice were euthanized 3, 6, 14, 28, or 56 days later. Plasma cholesterol levels at these time points were measured, and the reduction in hyperlipidemia was sustained for the duration of the experiment (Table I in the online-only Data Supplement).

The plaque area of CD68+ cells (predominately macrophages and macrophage-f organizing cells) at each time point is graphically summarized in Figure 1A. Based on ANOVA followed by the Bonferroni test, this area in pIpC-treated mice was reduced significantly at days 6, 14, and 28 (\( P<0.0001 \)) compared with mice at the corresponding time points in the control group. Within each treatment group, plaque CD68+ content significantly decreased by day 3 after hyperlipidemia was reduced, whereas it significantly increased by day 6 in the control group (Figure 1A), as determined by Dunnett testing to compare results at day 0 with those at the other time points.

As shown in Figure 1B, plaque CD68+ cell content in the pIpC group was negligible by day 56 (total cholesterol then averaged 80±11 mg/dL). In addition, using laser-captured CD68+ cells from baseline and regressing plaques, we found that there was no significant difference in CD68 mRNA levels (Figure IA in the online-only Data Supplement). In bone marrow–derived M1 and M2 macrophages, there was also no significant difference in CD68 mRNA abundance (data not shown). These results suggested that the decrease in CD68+ content in the pIpC-treated group was due to reduced cell number and not reduced cell expression. This would also be consistent with the immunostaining of plaque sections of another known macrophage marker, MOMA-2.

Sections from only a subset of the mice were available for this analysis, but as shown in Figure 1B in the online-only Data Supplement, we found a high degree of correlation (\( r=0.96 \)) between the areas stained by CD68 and MOMA-2 in these samples.

To exclude as possible explanations for the results in Figure 1 either nonspecific effects of pIpC on the immune system or the loss of MTP activity in cells of the arterial wall, we performed transplantation studies using hyperlipidemic Reversa mice as donors and pIpC-treated (normolipidemic) Reversa mice as recipients. The aortic grafts were harvested 5 days after transplantation. Note that the donors were not treated with pIpC, so it could not exert any effects on the immune system or cause the inactivation of the MTP gene in any tissue. In addition, the recipients were used 2 weeks after the last injection of pIpC, well past the 48-hour duration of its action in vivo.\(^{18}\)

The results are summarized in Figure 2. First, they show that as in apoe−/− mice,\(^3,4\) transfer of atherosclerotic aortas from another hyperlipidemic model to normolipidemic recipients causes decreased CD68+ cell content of plaques. Second, the percent reduction (\( \approx 50\% \)) found 5 days after exposure to the normolipidemic environment is quite comparable to that shown in Figure 1A after approximately the same duration of normolipidemia. Thus, the results demonstrate that the reduction in plaque CD68+ cells after hyperlipidemia is reduced is independent of the atherosclerotic mouse model, of MTP expression in the arterial wall, and of the effects of plpC on the immune system. Although transplantation of an aortic arch to the abdominal aorta exposes the plaques to hemodynamic changes and to potential traumatic damage and...
inflammatory stimulation, given the similar results for the decreased content of CD68\(^{+}\) cells in the transplantation (Figure 2A) and the nonsurgical experiments (Figure 1A), it is unlikely that these factors were significant confounders in the present study.

As shown in Figure 3A, the plaque content of neutral lipids in both baseline and plpC-treated Reversa mice was mostly found in CD68\(^{+}\) areas. The lipid content of plaques in the plpC-treated mice was significantly reduced by \(\geq 50\%\) at day 14 after baseline (Figure 3B and 3C), similar to the reduction in CD68\(^{+}\) content at that time point (Figure 1A). In contrast, in the saline-treated control group, at the same time point, there was an increase (\(\approx 3\) times compared with baseline) in the lipid content of the plaque as assessed by Oil Red O staining (data not shown). There was a concomitant increase in CD68\(^{+}\) area. Taken together, the changes in neutral lipid content (presumably cholesteryl ester) were directly related to the plaque CD68\(^{+}\) cell content, reflecting that this population includes the cholesterol-laden macrophage-foam cells.

Expression of the Chemokine Receptor CCR7 Is Upregulated in CD68\(^{+}\) Cells in Plaques After Reversing Hyperlipidemia

We previously showed the depletion of CD68\(^{+}\) cells from apoe\(^{-/-}\) plaques 3 days after transplanting aortas into

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**Figure 2.** Transplantation of aortic segments from hyperlipidemic Reversa mice into normolipidemic Reversa mice leads to CD68\(^{+}\) cell depletion. A, Male hyperlipidemic Reversa mice were fed a Western diet for 16 weeks, and aortic segments transplanted into normolipidemic Reversa (ie, treated with plpC and used 2 weeks thereafter). The transplanted aortic segments were then harvested 5 days later, and morphometric analysis of plaque CD68 content was performed. \(P=0.0045\) vs baseline by unpaired 2-tailed \(t\) test; \(n=6\) per group. B, Representative sections demonstrating a decrease in CD68\(^{+}\) cells as assessed by immunohistochemistry.

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**Figure 3.** Correcting hyperlipidemia in Reversa mice lowers neutral lipid content in aortic atherosclerotic plaques. A, Immunohistochemistry and colocalization studies were performed in baseline mice and plpC-treated Reversa mice after 16 weeks of Western diet feeding and 14 days after reduction of plasma lipids, respectively. The pictures are representative images showing Oil Red O staining (neutral lipid, presumably cholesteryl ester). Note that although the staining is less for lipid and CD68\(^{+}\) cells in the plpC-treated mouse, in both cases, there is colocalization of the 2 plaque components as assessed by confocal immunofluorescence microscopy. B, Oil Red O staining of the aortas of Reversa mice at baseline and 14 days after reduction of the plasma lipids (by plpC injection). Reversa mice treated with plpC had visibly reduced amounts of Oil Red O staining. C, Morphometric analysis of Oil Red O staining. L indicates the lumen of the artery. \(P<0.05\) vs baseline. \(n=8\) per group.
wild-type mice and that this reduction was associated with the CCR7-dependent migration of monocyte-derived cells from the aorta to either regional lymph nodes or the systemic circulation. To evaluate the importance of CCR7 in the regression of atherosclerosis in Reversa mice, we measured its expression in CD68 cells laser captured from plaques 14 days after normalization of the plasma lipid levels. As shown in Figure 4A, CCR7 expression was increased significantly in plaque cells captured from pIpC-treated mice (relative to those from saline-injected control mice). These findings were confirmed by immunohistochemistry (Figure 4B).

To investigate the possibility that pIpC, independently of reducing plasma lipid levels, may be inducing CCR7, we performed additional control experiments. Ldlr mice maintained on a Western diet for 16 weeks were injected with pIpC, and the aortas were examined by immunohistochemistry. We did not observe significant CCR7 staining of plaques in those mice, suggesting that the changes that we observed in the Reversa mice were regression related and not an artifact of pIpC treatment (data not shown).

Both Ly6Chi- and Ly6Clo-Derived Macrophages Emigrate From Atherosclerotic Plaques

The changes in CCR7 expression suggested that plaque CD68+ cell depletion could be mediated by cell emigration. There are 2 major circulating mouse monocyte subpopulations, CCR2+ CX3CR1+ Ly6Chi and CCR2+ CX3CR1+ Ly6Clo; both are normally recruited to atherosclerotic plaques. To determine whether the plaque macrophages derived from either subset emigrated during regression, we took advantage of the ability to differentially label the subsets of circulating monocytes and then follow the plaque content of labeled cells (which are CD68+; Figure II in the online-only Data Supplement) after injections of pIpC or normal saline. The labeling of the Ly6Chi population requires the use of clodronate to first deplete the circulating monocytes. To address whether clodronate treatment itself may affect monocyte/macrophage trafficking, we performed morphometric analysis on histological sections from mice treated with pIpC and clodronate and compared the CD68 areas with the corresponding values obtained when only pIpC was used. As shown in Figure III in the online-only Data Supplement, it is clear that clodronate had no effect on plaque CD68+ cell content independently of the reduction of plasma lipids.

As shown in Figure 4C and 4D, macrophages derived from both the Ly6Chi (CCR2+) and Ly6Clo (CX3CR1+) subsets emigrated from plaques, as assessed by the numbers of beads remaining in the plaque 14 days after reversal of hyperlipidemia.

Collagen Content Is Increased in Atherosclerotic Plaques After Reversal of Hyperlipidemia

As shown in Figure 5A, after the reduction in hyperlipidemia, the change in total plaque area did not follow the monotonic decline in CD68+ content (Figure 1A). We therefore predicted that other plaque components, particularly collagen, increased over time, given that human atherosclerosis plaque stabilization is pictured to involve both macrophage depletion and increased fibrosity (eg, see elsewhere for a recent review). Indeed, we found a significant increase in collagen content in plaques after reversal of hyperlipidemia (Figure 5B). As shown in Figure 5C, there is mainly a diffuse mesh...
of collagen with a fibrous cap observed under the regression conditions.

**Lipid Lowering Promoted Decreased Inflammation in Plaque CD68 \(^+\) Cells, an Effect Enhanced by PPAR\(\gamma\) Activation**

Increased expression of inflammatory genes in macrophages has been shown to be associated with plaque progression (reviewed by Williams et al\(^2\)). We have previously determined that the expression of 2 inflammation-related genes, vascular cell adhesion molecule-1 and MCP-1, are decreased in plaque CD68 \(^+\) cells in the transplant model of regression.\(^4\) We found consistent findings in the Reversa mouse model. As shown in Figure 6, transcripts for vascular cell adhesion molecule-1 and MCP-1, as well as intercellular adhesion molecule-1 and tumor necrosis factor-\(\alpha\), were reduced in plpC-treated Reversa mice at day 14.

We also examined gene expression markers for M2 macrophages. Whereas M1 macrophages are activated cells that produce mainly proinflammatory cytokines such as tumor necrosis factor-\(\alpha\), interleukin-6, and interleukin-12,\(^8,25\) M2 macrophages (“alternatively activated”) dampen inflammatory responses by producing antiinflammatory factors such as interleukin-10, transforming growth factor-\(\beta\), and interleukin-1 receptor antagonist.\(^8\) Interestingly, markers of M2 macrophages (arginase I, mannose receptor, CD163, C-lectin, Fizz-1) in CD68 \(^+\) cells within plaques were significantly increased in Reversa mice after lipid lowering (Figure 7). These markers were further increased in mice treated during the regression phase with pioglitazone (Figure 7). Although the effects of pioglitazone on gene-expression were unequivocal, we were unable to detect any additional effect of the drug on the content of CD68 \(^+\) cells within plaques (Figure 8A).

**The Expression of Plasminogen Activator Inhibitor-1 Is Reduced in Regressing Plaques and Is Enhanced by PPAR\(\gamma\) Activation**

One marker of the atherothrombotic state is an increased plasma level of plasminogen activator inhibitor-1 (PAI-1).\(^26\)

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**Figure 5.** Correction of the hyperlipidemia in Reversa mice has little effect on the size of plaques but increases their collagen content. A, Plaque areas at baseline (day 0) and at various time points after the mice were treated with plpC or normal saline as measured by morphometric analysis of hematoxylin and eosin-stained sections. Statistical analysis of the means in the plpC group was based on \(n=13\) for each time point; \(^*P<0.01\) for the comparison between day 0 and any other time point; NS indicates no statistical difference among the other time points. B, Quantification of collagen content in plaques at baseline (day 0) or 14 days after saline or plpC treatment as judged by morphometric analysis of Sirius Red-stained sections examined under polarizing light. Statistical analysis of the means was based on \(n=11\) for each group; \(^*P<0.0001\) for the comparison of the plpC group to either the baseline (day 0) or the saline treatment group. C, Representative section showing a diffuse intimal mesh and a subendothelial collection of collagen under regression conditions from a plpC-treated mouse used for the analysis shown in B. L and M indicate the lumen and medial layer of the artery, respectively.

**Figure 6.** Correction of the hyperlipidemia in Reversa mice is associated with reduced expression of inflammatory markers in plaque CD68 \(^+\) cells. CD68 \(^+\) cells in plaques were obtained by laser-capture microdissection 14 days after baseline. Levels of gene expression were compared in CD68 \(^+\) cells from plpC- and saline-treated Reversa mice. Total RNA was isolated, and transcript levels of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), MCP-1, and tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)) were measured by qRT-PCR. Data were from 2 pools of RNA, each from 3 mice, and are expressed as fold change compared with samples obtained from mice at baseline. \(^*P<0.05\).
which is expressed by macrophages and other cell types. During regression, PAI-1 gene expression in plaque CD68⁺ cells decreased, with a further reduction observed in the pioglitazone group (Figure 8B). The latter result is consistent with a report that PPARγ agonists reduce PAI-1 gene expression.27

**Discussion**

The underlying cause of hyperlipidemia in the Reversa mouse is LDL receptor deficiency, resulting in elevated plasma LDL levels, the statistically strongest risk factor for atherosclerotic disease in people. It is also this lipoprotein fraction that is most affected by the conditional ablation of the Mttp gene. That the Reversa mouse is a clinically relevant model is suggested, then, by its characteristics mimicking both the common finding of elevated plasma LDL in hyperlipidemic patients and the treatment strategy to lower these levels to reduce the risk of atherosclerotic disease. We previously showed that correction of hyperlipidemia in Reversa mice early in life retarded the development of atherosclerosis.6 In the present study, we show that reversal of hyperlipidemia after atherosclerosis develops to an advanced stage leads to lower levels of CD68⁺ cells (which are mainly monocyte-derived macrophages and foam cells) in plaques. There were also phenotypic changes in plaque CD68⁺ cells, including evidence for migratory behavior in vivo, decreased expression of genes encoding inflammatory and prothrombotic factors, and enrichment in markers of the M2 macrophage state. The activation of PPARγ by pioglitazone treatment did not further decrease the plaque CD68⁺ cell content but led to other presumably beneficial effects such as an enhancement of the M2 state and a further reduction in PAI-1 expression levels. Furthermore, the regression process was characterized by reduced plaque lipid and increased content of collagen.

In earlier work in which aortas from apoe⁻/⁻ mice were transplanted into wild-type mice, the reversal of dyslipidemia also reduced plaque CD68⁺ cell content.1,3,4,28 As in the present study, this reduction was accompanied by increased CCR7 mRNA in lesional CD68⁺ cells and by evidence of their migration out of plaques. We have now extended these results to show that this emigration was independent of the regression model and the subset of circulating monocytes from which the plaque CD68⁺ cells were derived. There also may have been decreased ongoing recruitment of monocytes into plaques after lipid reduction, but this was not examined in the present study. The slower kinetics in the depletion of the CD68⁺ cells in the Reversa mice may relate to the more gradual course of lipid reduction or the reduced high-density lipoprotein levels after inactivating Mttp in the liver.6

A probable link between lipid lowering and CD68⁺ cell emigration is suggested by the structure of the mouse and human CCR7 promoters. Both contain potential sterol response elements (SREs), which are functional in vitro and promote chemotaxis of macrophages in response to the CCR7 ligands CCL19 or 21 (J.E. Feig, L. Shang, M.J. Garabedian, and E.A. Fisher, submitted manuscript). The finding of reduced Oil Red O staining raises the possibility

Figure 8. PPARγ activation does not increase the rate of CD68⁺ cell depletion but further reduces PAI-1 expression in Reversa mouse plaques after hyperlipidemia was corrected. A, Reversa mice were treated with piPC to reverse the hyperlipidemia as in Figure 1; one half of the mice received pioglitazone in their diet. Plaque CD68⁺ cell content was measured by morphometric analysis of sections stained by immunohistochemistry. n=8 per group. B, Reversa mice were treated as in Figure 1, and CD68⁺ cells were obtained by laser-capture microdissection. The level of PAI-1 transcripts was measured by qRT-PCR and normalized to cyclophilin A. Data are from 3 pools of RNA, each from 3 mice. **P<0.05 vs piPC; ***P<0.05 vs baseline mice; **P<0.05 vs mice treated with piPC alone.
that the “regression environment” within the arterial wall might be associated with CCR7 gene expression through its SRE. This would be consistent with our finding of increased nuclear localization of SREBP2 in regressing plaques (J.E. Feig, L. Shang, M.J. Garabedian, and E.A. Fisher, submitted manuscript). This regulation is also consistent with a recent report that loading of THP-1 human monocytes with oxidized LDL suppresses CCR7 expression.29

The finding that correcting hyperlipidemia also led to an enrichment of markers of M2 macrophages in plaque CD68+ cells was striking. Macrophage heterogeneity in human and mouse atherosclerotic plaques has been documented,17 but to the best of our knowledge, this is the first report of dynamic changes during regression. Interestingly, as shown in Figure IV in the online-only Data Supplement, we found that there is some colocalization to the same plaque area of markers of M1 and M2 macrophages but also spatial distinction between the markers. In a recent study30 examining macrophage plasticity in progressing atherosclerotic lesions, the authors concluded that M2 cells can convert to the M1 state. The present results suggest that in addition to a possible conversion of the M1 to M2 state, another source of M2 cells in regressing plaques may be the continued recruitment of circulating monocytes3 that become M2 cells, a possibility we are currently testing.

Classically activated M1 macrophages have long been recognized to be inflammatory.31–33 In contrast, the M2 state is thought to represent an antiinflammatory state8 associated with tissue remodeling and repair, which certainly occur during plaque regression. Although the mechanisms for reduced inflammatory markers and increased M2 markers remain to be explored, these changes may be related to recent findings by others.34,35 They found that increased macrophage sterol content led to heightened macrophage activation in vitro and in vivo. Moreover, this effect was reversed by efflux of sterols from cells. The reduced Oil Red O staining of plaques in our studies may indicate that there was a sufficient loss of lipid from the plaques in the regression environment not only to activate CCR7 through its SRE but also to have an antiinflammatory effect on CD68+ cells.

That pioglitazone treatment increased the expression of the M2 markers is consistent with recent studies showing that PPARγ activation promotes the polarization of macrophages to an antiinflammatory state.17,37 Although this would generally be considered to be beneficial, Thorp and colleagues37 have shown that PPARγ activation in Ldlr−/− mice with advanced atherosclerosis had a negative impact on plaque macrophages by increasing cell death. If those effects had occurred in the present study, we would have expected an increased rate of CD68+ cell loss from plaques and histological evidence of necrosis, but neither was observed. The 2 studies can be reconciled by recognizing the effects of the lipid burden of the cells because once they become foam cells, PPARγ activation can no longer skew them toward the M2 phenotype.17 Thus, in the present study, it is likely that the reversal of hyperlipidemia and the consequent decrease in plaque lipid content allowed the M2-polarizing activity of PPARγ. Another potentially protective benefit of PPARγ activation was the reduction of PAI-1 expression in CD68+ cells, consistent with the ability of PPARγ to repress PAI-1 gene transcription.27

After correction of the hyperlipidemia, the plaques of Reversa mice had an increased content of collagen. This finding is consistent with observations from the transplantation model, in which regression was accompanied by increased connective tissue in plaques.2 We suspect that these observations are likely due to decreased matrix degradation because we have observed reduced matrix metalloproteinase-2 and -9 mRNA levels in CD68+ cells after reversal of hyperlipidemia (J.E. Feig, E.A. Fisher, unpublished data). Additionally, there may have been some stimulation of collagen synthesis related to the increase in arginase I expression.38

We should note that the reduction in the plasma level of LDL particles is clinically achieved usually by increasing their clearance with statins. New strategies based on decreasing hepatic apoB lipoprotein production akin to the strategy taken in the Reversa mouse, however, have been shown to be effective hypolipidemic therapies in clinical trials (eg, see the work by Kastelein et al39 and Samaha et al40). Although end-point studies are not available, based on the present results and the analyses of clinical trial data showing event reduction that was independent of the means of LDL lowering (eg, the study by LaRosa et al41), it is reasonable to expect benefits on plaques in patients treated with the novel agents.

Conclusions

We have introduced a new model of atherosclerosis regression in which we identified significant changes in the composition of plaques after correcting hyperlipidemia, including reduced numbers of CD68+ cells and increased collagen content. Besides quantitative changes in the numbers of CD68+ cells, there were also effects on their gene expression, with evidence for the induction of migratory machinery, enrichment in the M2 state, and reduced expression of inflammatory and atherothrombotic genes. Pioglitazone treatment enhanced these changes, implying additive clinical benefits of aggressive lipid management and PPARγ stimulation. Continued study of this model should lead to an improved understanding of plaque regression at the molecular level.

Acknowledgments

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

The ultimate cure for atherosclerosis would be the regression of arterial plaques. Discovery research toward this goal has been hampered by limited and sometimes cumbersome animal models. The Reversa mouse combines a standard model of human atherosclerosis, the hyperlipidemic low-density lipoprotein receptor-deficient mouse, with a genetic switch that electively shuts off low-density lipoprotein production. In the present study, arterial plaques were allowed to develop in Reversa mice to a stage mimicking advanced human coronary artery disease, and then the elevated low-density lipoprotein level was severely reduced, thereby simulating aggressive lipid management. The major findings after such lipid reduction were decreases in the content and inflammatory state of the central cell of plaques, macrophages, with the change in total plaque size more modest because of compensatory increases in collagen content. The improvement in macrophage inflammatory status was augmented by treatment with pioglitazone, consistent with the effects of peroxisome proliferator-activated receptor-γ agonists on macrophages in vitro. The results may explain why plaque volume decreases have been modest in recent statin trials despite significant reduction in events and may provide one basis for the cardioprotective effects of pioglitazone in clinical studies. Continued study of this convenient model should lead to an improved understanding of plaque regression at the molecular level.
Reversal of Hyperlipidemia With a Genetic Switch Favorably Affects the Content and Inflammatory State of Macrophages in Atherosclerotic Plaques

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Supplemental Table 1. Total plasma cholesterol levels at 3, 6, 14, 28, and 56 days after the “baseline” time point (nine days after the first pIpC injection).

<table>
<thead>
<tr>
<th>Time</th>
<th>Saline Treated Group (mg/dL)</th>
<th>pIpC Treated Group (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 days</td>
<td>1111±188</td>
<td>88±9</td>
</tr>
<tr>
<td>6 days</td>
<td>1002±191</td>
<td>81±10</td>
</tr>
<tr>
<td>14 days</td>
<td>1125±104</td>
<td>77±9</td>
</tr>
<tr>
<td>28 days</td>
<td>1029±166</td>
<td>79±7</td>
</tr>
<tr>
<td>56 days</td>
<td>1114±191</td>
<td>80±11</td>
</tr>
</tbody>
</table>
Supplemental Figure 1. Expression of CD68 is not significantly different under baseline and regression conditions and is highly correlated with that of macrophage marker MOMA-2. Mice were treated as in Figure 1. (A) CD68+ cells were laser captured from plaques from 6 mice/group, RNA isolated, and qRT-PCR performed to measure relative levels of CD68 mRNA. An unpaired two-tailed t-test showed no statistically significant significance (p=0.688); (B) Consecutive sections of regressing plaques from pIpC injected Reversa mice were stained with CD68 and MOMA-2 antibodies. Morphometric analysis of the lesions demonstrated excellent correlation between CD68+ and MOMA-2+ areas (correlation coefficient = 0.96) and a Bland-Altman test showed no evidence of any bias in measurements between the immunopositive area as measured by MOMA and as measured by CD68.

Supplemental Figure 2. Fluorescent beads are located in CD68+ rich areas. Using immunofluorescent microscopy, sections were stained for CD68 (red) and cell nuclei (blue). Arrows indicate the fluorescent beads that entered within the circulating monocytes that were recruited to the plaque. Note, as expected, that the beads co-localized with CD68+ cells. “L” indicates the arterial lumen.

Supplemental Figure 3. Clodronate does not affect CD68 content in regressing plaques. One week before pIpC injections, hyperlipidemic Reversa mice were treated with clodronate to deplete the circulating monocytes in order to label the LY6hi (CCR2 positive) population. Fourteen days later, the aortic roots were sectioned, and the CD68+
area quantified. The results were compared to mice that were only treated with pIpC. *n = 8/group*

**Supplemental Figure 4. Spatial relationship between M1 and M2 macrophages.**

Frozen sections of atherosclerotic plaques were incubated with a mix of primary antibodies, biotin anti-mouse MCP-I (Biolegend) and rabbit polyclonal Arg I (Santa Cruz) followed by alkaline phosphatase and FITC-conjugated anti-rabbit secondary antibody, respectively. MCP-1(red) and Arg I (arginase I; green) are highly expressed by M1 and M2 macrophages, respectively. Though there are areas in which both markers are present, progressing lesions from saline injected Reversa mice tended to show significant amounts of MCP-1 positive cells near the lumen and some Arg I+ cells deeper in the intima. In contrast, the regressing plaque (from a pIpC-injected Reversa mouse) is characterized by fewer MCP-1+ cells and a larger amount of cells expressing Arg I that were diffusely distributed. “L” indicates the arterial lumen.

**REFERENCE**

Supplemental Figure 1

A) Relative mRNA/28s

B) MOMA Positive Area (μm²) vs. CD68 Positive Area (μm²)
Supplemental Figure 2
Supplemental Figure 3

P=NS

CD68 Area (mm²)

plpC (2 weeks)

plpC+clodronate (2 weeks)
Supplemental Figure 4

Arg I  MCP-1  Overlay

saline

Arg I  MCP-1  Overlay

plpC