Macrophage Mal1 Deficiency Suppresses Atherosclerosis in Low-Density Lipoprotein Receptor–Null Mice by Activating Peroxisome Proliferator-Activated Receptor-γ–Regulated Genes

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Accessibility
Macrophage Mal1 deficiency suppresses atherosclerosis in LDL-receptor null mice by activating PPARγ-regulated genes

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Abstract

Objective—The adipocyte/macrophage fatty acid-binding proteins, aP2 (FABP4) and Mal1 (FABP5), are intracellular lipid chaperones that modulate systemic glucose metabolism, insulin sensitivity and atherosclerosis. Combined deficiency of aP2 and Mal1 has been shown to reduce the development of atherosclerosis, but the independent role of macrophage Mal1 expression in atherogenesis remains unclear.

Methods and Results—Here we transplanted wild type (WT), Mal1⁻/⁻ or aP2⁻/⁻ bone marrow into LDLR⁻/⁻ mice and fed them a Western diet for 8 weeks. Mal1⁻/⁻→LDLR⁻/⁻ mice had significantly reduced (36%) atherosclerosis in the proximal aorta compared to control WT→LDLR⁻/⁻ mice. Interestingly, peritoneal macrophages isolated from Mal1-deficient mice displayed increased PPARγ activity and up-regulation of a PPARγ-related cholesterol trafficking gene CD36. Mal1⁻/⁻ macrophages showed suppression of inflammatory genes such as COX-2 and IL6. Mal1⁻/⁻→LDLR⁻/⁻ mice had significantly decreased macrophage numbers in the aortic atherosclerotic lesions compared to WT→LDLR⁻/⁻ mice, suggesting that monocyte recruitment may be impaired. Indeed, blood monocytes isolated from Mal1⁻/⁻→LDLR⁻/⁻ mice on a high-fat diet had decreased CC chemokine receptor 2 (CCR2) gene and protein expression levels compared to WT monocytes.

Conclusion—Taken together our results demonstrate that Mal1 plays a pro-atherogenic role by suppressing PPARγ activity, which increases expression of CCR2 by monocytes promoting their recruitment to atherosclerotic lesions.

Keywords

Atherosclerosis; Macrophages; PPARγ; CD36; CCR2 receptor

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Disclosures None.
Introduction

Fatty acid-binding proteins (FABPs) play important roles in FA transport, cellular signaling, gene transcription and cytoprotection\(^1\). FABPs belong to a family of 14- to 15-kDa proteins that bind with high affinity to hydrophobic ligands such as saturated and unsaturated long-chain fatty acids, eicosanoids and other lipids\(^2\). The adipocyte/macrophage FABPs, aP2 (FABP4) and Mal1 (FABP5), are intracellular lipid chaperones that modulate systemic metabolism of glucose and lipids, insulin sensitivity and atherosclerosis\(^2\). We have previously demonstrated that either deficiency of aP2 or combined deficiency of aP2 and Mal1 genes significantly attenuates atherosclerosis in apoE\(^{-/-}\) mice both on normal chow or high-fat diets\(^3-5\). Bone marrow transplantation studies demonstrated that the anti-atherogenic effect of aP2-deficiency is predominantly, if not entirely, related to its actions in the macrophage and is independent of the impact of aP2 on insulin sensitivity\(^2,5\). However, the independent role of macrophage Mal1 expression in atherogenesis has not been studied yet.

Previous reports have shown that macrophage aP2-deficiency significantly enhances the nuclear hormone peroxisome proliferator-activated receptor gamma (PPAR\(\gamma\)) activity in macrophages increasing both CD36-mediated uptake of oxidized low-density lipoprotein, and ABCA1-mediated cholesterol efflux in the cells\(^6\). In addition, aP2\(^{-/-}\) macrophages have reduced I\(\kappa\)B kinase activity and NF-xB-related inflammatory gene expression\(^6\). These aP2-related changes in macrophage cholesterol trafficking and inflammation have a dramatic impact on the development of atherosclerosis. Macrophages express the aP2 and Mal1 FABP isoforms at a ratio of approximately 1:1\(^5\). These two proteins have 52% amino-acid similarity and bind various fatty acids and synthetic compounds with similar selectivity and affinity\(^7\). The high degree of homology in structure and ligand affinity between aP2 and Mal1 suggests that Mal1 may have similar, and possibly redundant, roles to aP2 in macrophage biology and atherogenesis.

Recent studies demonstrated that FABPs acts as chaperons facilitating transport of fatty acids from the plasma membrane to different intracellular compartments\(^2\). Mal1 expression modulates systemic insulin sensitivity in two models of obesity and insulin resistance\(^2,8\). This may induce basal and insulin-stimulated phosphorylation of Akt in adipose and muscle tissues specific for aP2\(^{-/-}\)/Mal1\(^{-/-}\) mice\(^9\). Akt is a key regulator of macrophage survival and inflammatory responses, and several studies have indicated an important role for macrophage Akt signaling in atherosclerosis\(^10,11\). However, the impact of Mal1 expression on macrophage Akt expression and the development of atherosclerosis have not been previously examined.

To study the role of macrophage Mal1 in early atherosclerosis, we generated chimeric LDLR\(^{-/-}\) mice with Mal1-deficient hematopoietic cells and challenged them with a Western diet for 8 weeks. Recipient mice reconstituted with Mal1\(^{-/-}\) marrow had significantly smaller (36%) atherosclerotic lesions compared to control mice transplanted with WT marrow. In addition, Mal1\(^{-/-}\) macrophages displayed a significant increase in PPAR\(\gamma\) activity and affected expression of the PPAR\(\gamma\)–regulated gene, CD36, and genes involved in inflammation, including suppression of CCR2 levels in monocytes, which likely reduces their recruitment to atherosclerotic lesions.

Methods

Animal Procedures

The Mal1-deficient mice were developed using homologous recombination in embryonic stem cells, as described\(^12\), and backcrossed 10 or more generations onto C57BL/6 background\(^13\). All recipient LDLR\(^{-/-}\) mice and corresponding wild type controls were
purchased from the Jackson Laboratories (Bar Harbor, ME). Mice were maintained in microisolator cages on a rodent chow diet containing 4.5% fat (PMI 5010, St. Louis, MO) or on a Western type diet containing 21% milk fat and 0.15% cholesterol Teklad, Madison, WI). Animal care and experimental procedures were performed according to the regulations of Vanderbilt University’s Animal Care Committee.

**Genotyping and bone marrow transplantation**

To identify the Mal1 genotype, we generated a set of primers (GAC GAT ATA AGC GCA GAT GG, AAC TGA GGG GCT GTT TGT AG and TCG CCT TCT ATC GCC TTC TTG AC) producing a 610-bp band specific for the Mal1 targeted allele and a 435-bp band specific for the wild type allele by PCR analysis. Recipient 8-week-old female LDLR−/− mice were lethally irradiated (9Gy) from a cesium gamma source and transplanted with 5×10^6 bone marrow cells from female Mal1−/−, aP2−/− or WT donor mice as described.

**Serum Lipids and Lipoprotein Distribution Analyses**

Mice were fasted for 4 hours, and then serum total cholesterol and triglycerides were measured by enzymatic methods using the reagents from Raichem (San-Diego, CA) and the SoftMax Pro5 software (Molecular Devices). Fast performance liquid chromatography (FPLC) was performed on an HPLC system model 600 (Waters, Milford, MA) using a Superose 6 column (Pharmacia, Piscataway, NJ).

**Analysis of Aortic Lesions**

Aortas were flushed through the left ventricle and cryosections of the proximal aorta were analyzed using the Imaging System KS 300 (Kontron Electronik GmbH) as described.

**Peritoneal macrophages: isolation and treatment**

Thioglycollate-elicited peritoneal macrophages were isolated from WT and Mal1−/− mice. Macrophages were treated with 0.5 mM palmitic acid complexed to BSA as described, with human oxidized LDL (OxLDL; 100μg/ml) or acetylated LDL (AcLDL; 100μg/ml; Intracel Corp, Rockville, MD) plus an ACAT inhibitor, Sandoz 58035 (10μg/ml; Sigma) as described, or with PPARγ agonist, ciglitazone (Cayman Chemicals) or PPARγ antagonist, GW9662 (Sigma).

**Modified LDL Uptake**

Macrophages were incubated with DiI-labeled human AcLDL or OxLDL (Intracel) at 37°C for 2 hrs and analyzed under a fluorescent microscope or by fluorescence-activated cell sorter (FACS) flow cytometry as described.

**RNA Isolation and real-time PCR**

Total RNA was isolated from macrophages using a Trizol reagent (Life Technologies, Inc.) and purified by the RNA Easy kit (Qiagen, Valencia, CA). Relative quantitation of the target mRNA was performed using primers, probes and the Sequence Detection System (Applied Biosystems, ABI) and normalized with 18S ribosomal RNA as described.

**Blood monocyte analyses**

Blood was collected from mice in the presence of 5U heparin and the opaque layer of mononuclear cells was isolated by Histopaque-1077 (Sigma) gradient. Then cells were kept in 6-well plate at 37°C for 30 min and washed with PBS. CCR2 protein expression was detected by a rabbit monoclonal antibody to CCR2 (Epitomics, Burlingame, CA) and analyzed by FACS (Becton Dickinson) as described.
**Western blotting**

Cells were treated with a cell lysis buffer (Cell Signaling Technology, Danvers, MA) with a protease (Sigma) and a phosphatase inhibitor (Pierce) cocktail. Proteins (20–100μg/lane) were resolved by NuPAGE Bis-Tris electrophoresis (Invitrogen) and transferred onto polyvinylidene difluoride nitrocellulose membranes (Amersham Bioscience). Blots were probed with rabbit antibodies to PPARγ (cat# ab27649; Abcam, Inc. Cambridge, MA), Akt and p-Akt (from Cell Signaling Technology), c-Rel, NF-κB p65 and IκBα (Santa Cruz Biotechnology), β-actin (Abcam) and goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Upstate Cell Signaling, Lake Placid, NY). Proteins were visualized with ECL western blotting detection reagents (GE healthcare) on X-ray films. To quantify the bands obtained via Western blot analysis, we applied ImageJ software (http://rsb.info.nih.gov/ij/).

**Statistical Analysis**

The statistical differences in mean serum lipids and aortic lesion areas between the groups were determined by One Way ANOVA test and t-test.

**Results**

**Mal1-deficiency in hematopoietic cells does not affect serum lipid levels but suppresses early atherosclerosis**

To study the impact of macrophage Mal1 expression on early atherosclerosis, we used bone marrow transplantation to generate LDLR<sup>−/−</sup> mice with hematopoietic cells from Mal1<sup>−/−</sup> mice (n=15), aP2<sup>−/−</sup> mice (n=15) or control wild type mice (WT, n=13). Four weeks post-transplantation, recipient mice were challenged with a Western diet for 8 weeks. There was a steady increase in body weight with no differences between the groups (Figure 1A). No significant differences were found in serum total cholesterol and triglyceride levels between the groups either on chow or Western diet for 4 and 8 weeks (Table 1). Similarly, size exclusion chromatography analyses of plasma lipoproteins revealed no differences between mice reconstituted with WT, Mal1<sup>−/−</sup> or aP2<sup>−/−</sup> marrow (Figure 1B). However, recipient mice receiving Mal1<sup>−/−</sup> or aP2<sup>−/−</sup> bone marrow cells had significantly reduced size (36% and 21%) of atherosclerotic lesions in the proximal aorta compared to mice transplanted with WT cells (Figure 1C).

**Mal1-deficiency increases PPARγ activity in macrophages**

Recent studies have implicated enhanced PPARγ activity in aP2<sup>−/−</sup> macrophages as the mechanism responsible for altering expression of genes that regulate cholesterol homeostasis and inflammation resulting in inhibition of atherosclerosis<sup>6</sup>. Since Mal1 has many similarities to aP2 in both structure and function, we hypothesized that the anti-atherogenic effects of Mal1-deficiency may result from a similar mechanism. To test this hypothesis, we isolated peritoneal macrophages from WT and Mal1<sup>−/−</sup> mice, and incubated them with DMEM media containing 10% lipoprotein-deficient serum overnight. Then cells were treated with fresh media alone (control) or with a potent PPARγ agonist, ciglitazone, with or without the selective PPARγ antagonist, GW9662. Real-time PCR analysis indicated that PPARγ activation increased expression of the PPARγ gene significantly higher in Mal1<sup>−/−</sup> macrophages than in WT cells and these effects were completely reversed by addition of the PPARγ antagonist (Figure 2A). Similarly, the ligand treatment significantly (1.5-fold) increased expression of the CD36 mRNA in both WT and Mal1<sup>−/−</sup> macrophages (Figure 2B). There was a similar trend that was not statistically significant for an increase in expression of the ABCA1 and ABCG1 genes (data not shown), which are regulated indirectly by PPARγ through LXRα. Interestingly, the expression of the CD36, ABCA1, and

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ABCG1 genes were significantly increased in Mal1−/− macrophages compared to WT cells (Supplement Material, Figure I), when they were loaded with OxLDL or free cholesterol by incubating them with AcLDL together with the ACAT inhibitor, Sandoz 58035, as described17. These data strongly suggest that the PPARγ pathway is up-regulated in Mal1 null macrophages.

Next, WT and Mal1−/− macrophages were incubated with increasing doses of ciglitazone. Then proteins were extracted from the cells and analyzed by Western blot. The ligand treatment significantly increased PPARγ protein expression in both types of cells but was always higher in Mal1−/− macrophages than WT cells (Figure 2C, D). Similarly, the ciglitazone treatment significantly (1.5-fold) increased CD36 protein expression levels in both WT and Mal1−/− macrophages (Figure 2E, F). Then, to verify the role of PPARγ in mediating the increase in CD36, we made use of the PPARγ antagonist GW9662, which covalently modifies a cysteine residue of PPARγ resulting in complete loss of ligand binding ability of PPARγ19. Interestingly, treatment cells with the PPARγ antagonist GW9662 in conjunction with ciglitazone abolished the increase of CD36 expression in both types of macrophages (Figure 2E,F).

Finally, we examined whether activation of scavenger receptor CD36, which is directly regulated by PPARγ has an effect on uptake of modified LDL. WT and Mal1−/− macrophages were incubated with Dil-labeled AcLDL and Dil-labeled OxLDL for 2 hours and then analyzed visually and by flow cytometry. Compared to WT cells, Mal1−/− macrophages displayed increased uptake of OxLDL (Figure 3A). FACS analysis demonstrated that Mal1−/− macrophages had increased OxLDL uptake (69-125%) but only slightly increased AcLDL (13-26%) uptake (Figure 3C). Taken together these data indicate that Mal1-deficiency activates the PPARγ pathway in macrophages enhancing expression of the PPARγ-regulated gene, CD36, and results in up-regulation of CD36-mediated uptake of oxLDL.

**Mal1-deficiency increases Akt phosphorylation, and suppresses COX2 and IL6 gene expression in macrophages**

Since FABP expression may change Akt activity in different types of cells9, we compared Mal1−/− and WT peritoneal macrophages in their response to a lipotoxic factor, palmitic acid (0.5mM) complexed with BSA (PA-BSA), used to induce endoplasmic reticulum (ER) stress-related signaling16. Mal1−/− cells had significantly increased levels of Akt activation (Figure 4A; p-Akt/β-actin ratios were 2.2, 1.6 and 1.7 vs 1.0, 1.4 and 1.2, respectively in WT cells), a survival factor that is capable of modulating inflammatory pathways including NF-κB. In contrast, the expression levels of Akt and β-actin were not significantly different in these two types of cells (Figure 4A; Akt/β-actin ratios were 1.0-1.2 and 1.0-1.1, respectively). Similarly, treatment with PA-BSA resulted in significantly less IκBα protein in Mal1−/− macrophages compared to WT cells (Fig 4B, 0.73, 0.81 and 0.53 vs. 1.0, 1.35 and 1.7, respectively). The levels of other NF-κB-related proteins such as p-65 and c-Rel were not significantly different in these cells (Fig 4B). Interestingly, Mal1−/− macrophages had significantly higher levels of basal and LPS-mediated (50ng/ml) Akt-1 and c-Jun gene expression than WT cells (Figure 4C,D). In contrast, LPS induced less COX-2 and IL6 gene expression in Mal1−/− macrophages compared to WT cells (Figure 4E, F). These data indicate that Mal1-deficiency increases Akt activity and suppresses IκBα protein, COX2 and IL6 gene expression levels in macrophages.
Mal1-deficiency decreases macrophage cell numbers in atherosclerotic lesions and suppresses CCR2 expression by blood monocytes

To test whether macrophage Mal1-deficiency affects cell density in atherosclerotic lesions, we analyzed the number of nuclei (stained by DAPI) in the macrophage (MOMA-2 positive) area of atherosclerotic lesions in the proximal aorta. Compared to control LDLR−/− mice transplanted with WT marrow (Fig 5A–C), LDLR−/− mice reconstituted with Mal1−/− bone marrow (Figure 5D–F) had significantly (36%) lower numbers of macrophages in the lesion area (Figure 5G). These data suggest that Mal1−/− macrophages undergo decreased recruitment to atherosclerotic lesions.

Next, we lethally irradiated and transplanted male 10-week-old LDLR−/− mice with Mal1−/− (n=5) or WT (n=5) marrow. Eight weeks later, recipient mice were fed with the Western diet for 12 weeks. Blood monocytes were isolated from the recipient mice and CC chemokine receptor 2 (CCR2) gene and protein levels were analyzed by real-time PCR and FACS. Real-time PCR demonstrated that Mal1−/− monocytes expressed significantly lower (64%) levels of levels of CCR2 mRNA compared to WT cells (Figure 5H). Compared to control WT control monocytes, the Mal1−/− monocytes also had suppressed (28%) levels of CCR2 protein expression as detected by FACS (Figure 5I). Similar suppression of CCR2 gene and protein expression levels were noted in Mal1−/−/apoE−/− monocytes compared to apoE−/− cells (Supplement Material; Figure II). Taken together, the results indicate that up-regulation of the PPARγ pathway in Mal1−/− mice suppresses CCR2 expression in blood monocytes and this likely affects their recruitment to atherosclerotic lesions.

Discussion

The adipocyte/macrophage FABPs aP2 and Mal1 link features of the metabolic syndrome including insulin resistance and atherosclerosis. Studies with aP2−/− or aP2−/−/Mal1−/− mice have shown that the elimination of these proteins in total body or exclusively in hematopoietic cells significantly suppresses atherosclerotic lesion formation in apoE−/− mice. Remarkably, aP2 and Mal1 have additive effects with regard to insulin sensitivity, as aP2−/− mice show improved insulin sensitivity only in the setting of dietary or genetic obesity, whereas aP2−/−/Mal1−/− 2KO mice show improved insulin sensitivity on the apoE-deficient background even when lean and on a normal chow diet. Expression of aP2 and Mal1 by both adipocytes and macrophages contributes to insulin resistance. However, macrophage aP2 expression promotes atherosclerosis independent of its impact on insulin sensitivity. Since both proteins, aP2 and Mal1, share a high degree of homology and are expressed by macrophages in similar proportions, we examined the hypothesis that macrophage Mal1 expression influences the development of atherosclerosis. Here we demonstrate that Mal1-deficiency in hematopoietic cells significantly inhibits (36%) early atherosclerotic lesion formation in LDLR−/− mice when compared to control mice reconstituted with WT bone marrow. This effect is not mediated by differences in serum lipids levels or lipoprotein distributions.

Previous studies have shown that macrophage aP2-deficiency significantly enhances PPARγ activity and suppresses atherosclerosis formation. Therefore, to examine mechanisms underlying the impact of macrophage Mal1 expression on atherogenesis, we analyzed PPARγ gene and protein expression levels in peritoneal macrophages isolated from Mal1−/− and WT mice. We demonstrate that treatment with the PPARγ agonist ciglitazone increases PPARγ gene and protein expression more in Mal1−/− macrophages than in WT cells, and the effect on PPARγ gene expression is reversed by addition of a PPARγ antagonist (Figure 2A). In addition, we show in a dose response study with ciglitazone that PPARγ protein expression increases to a greater extent in Mal1−/− macrophages than in WT cells (Figure 2C,D). Our results suggest an interesting possibility that the promoter of the PPARγ gene
may contain functional PPRE sites. Interestingly, a gene database search of the mouse and human PPARγ gene promoters revealed three potential PPRE sites. The site with the highest score (AGGGCAAAGGCCT) is 100% conserved between human and mouse, has 10/13 (76.9%) of identity with the consensus PPRE sequence, and reveals high similarity with known functional PPREs in PPAR-gamma target genes. (Supplement Material; Figure III). We also demonstrate that expression of CD36, which is directly regulated by PPARγ, is increased to a greater extent in Mal1−/− macrophages than in WT cells (Figure 3B) and the ciglitazone-related increase in CD36 protein expression was abolished by the PPARγ antagonist, GW9662 (Figure 2E,F). There was also a trend for an increase in expression of ABCA1 and ABCG1 gene expression in ciglitazone treated Mal1−/− macrophages but it was not statistically significant (data not shown). It is important to note, that PPARγ regulates ABCA1 through LXRα, and our failure to see a significant increase in ABCA1 may be due to the conditions of the experiment in that the cells were not loaded with cholesterol. Indeed, Mal1−/− macrophages showed increased expression of CD36, ABCA1 and ABCG1 in response to OxLDL and free cholesterol loading (Supplemental Figure I). These findings are consistent with earlier studies reporting that FABPs bind PPAR ligands and that FABP over-expression inhibits lipid-mediated signaling to the PPARs21. A similar PPARγ-active phenotype has been described in aP2−/− macrophages and it was associated with an anti-inflammatory status6. Previous studies have demonstrated that activation of the PPARγ-LXRα pathway reciprocally regulates inflammation and lipid metabolism22, stimulating genes involved in cholesterol homeostasis23 and antagonizing genes encoding inflammatory proteins8. Recent studies have suggested that free cholesterol accumulation may induce a pro-inflammatory phenotype in macrophages24. Indeed, the increased cellular free cholesterol and lipid raft contents in ABCA1−/− macrophages enhanced expression of pro-inflammatory cytokines and activation of the NF-κB pathway25. Taken together, these data indicate that Mal1-deficiency activates the PPARγ pathway in macrophages protecting them against pro-inflammatory and pro-atherogenic changes.

We also noted that Mal1-deficiency significantly increases basal and stimulated Akt activity in macrophages. Interestingly, a similar increase of basal and insulin-stimulated phosphorylation of Akt was noted in adipose and muscle tissues of aP2−/−/Mal1−/− mice9. The Akt activation was higher in the presence of shorter chain (12:0 and 14:0) fatty acids and strongly inhibited in the presence of longer chain (16:0 and 18:0) fatty acids9. Akt signaling promotes cell survival but also modulates inflammatory responses and stimulates transport and metabolism of glucose and amino acids26. It is important to note that macrophages constitutively express p-Akt and inhibition of the pathway significantly accelerates their apoptosis11. Consistent with these data, Akt null macrophages are more susceptible to apoptotic stimuli27.

Macrophage aP2-deficiency has been shown to reduce the activity of IκB, which may, at least in part, underlie the alterations in cytokine expression6. Similarly, we found that Mal1-deficiency suppresses COX-2, IL6 mRNA and IκB protein expression in macrophages although to a lesser degree than is seen in aP2−/− macrophages6. Next, we demonstrated that LDLR−/− mice reconstituted with Mal1−/− bone marrow had decreased numbers of macrophages in their atherosclerotic lesions compared to control mice transplanted with WT bone marrow (Figure 5, A-G). This strongly supports the hypothesis that the recruitment of Mal1−/− monocytes to atherosclerotic lesions may be impaired, leading to diminished cell numbers in the atherosclerotic lesions. CCR2 is known as a receptor for monocyte chemoattractant protein 1, which plays pivotal roles in immune responses and atherosclerosis28. CCR2 is necessary for efficient monocyte recruitment from the blood to inflamed tissue and to atherosclerotic lesions28. A recent study has identified PPARγ as a critical signaling molecule in determining macrophage phenotype in vitro, and treatment with a PPARγ agonist enhances the anti-inflammatory properties of macrophages29. In this
regard, PPARγ activation by oxidized LDL inhibits CCR2 expression in human and mouse monocytes\(^3\). In contrast, macrophage-specific deficiency in PPARγ significantly accelerates CCR2 expression and atherosclerosis in LDLR\(^{-/-}\) mice\(^1\). These findings suggest the hypothesis that reduced expression of CCR2 by Mal1\(^{-/-}\) monocytes may contribute to the reduction in atherosclerosis in the Mal1\(^{-/-}\)→LDLR\(^{-/-}\) mice. We did not see an impact of MCP1 on macrophage migration in an in vitro transwell assay comparing unstimulated Mal1\(^{-/-}\)and WT peritoneal macrophages (data not shown). However, these results do not rule out an important role for reduced CCR2 expression by Mal1\(^{-/-}\) monocytes in reducing recruitment to atherosclerotic lesions in vivo. We provide evidence from Mal1\(^{-/-}\)→LDLR\(^{-/-}\) mice fed a high fat diet that Mal1\(^{-/-}\) monocyte CCR2 gene and protein expression levels are reduced in vivo compared to WT cells from control Mal1\(^{+/+}\)→LDLR\(^{-/-}\) mice. The relevance of this finding is supported by our data showing that monocyte deficiency of Mal1\(^{-/-}\) reduces CCR2 expression in vivo in a second genetic model of atherosclerosis, apoE\(^{-/-}\) mice (Supplemental Figure 2). Although the impact of CCR2 expression on atherogenesis is not limited to its role in recruitment\(^3\) we believe that our findings of reduced expression of CCR2 in Mal1 deficient monocytes coupled with the in vivo evidence that the atherosclerotic lesions of Mal1\(^{-/-}\)→LDLR\(^{-/-}\) mice have reduced numbers of macrophages, support the hypothesis that reduced CCR2 expression by Mal1 monocytes contributes to the reduced atherosclerosis in the Mal1\(^{-/-}\)→LDLR\(^{-/-}\) mice, likely due to an impact on recruitment. Together these data show that Mal1 expression regulates inflammatory activity in macrophages and likely affects monocyte recruitment to atherosclerotic lesions.

In conclusion, our results demonstrate that macrophage Mal1 plays an important role in early atherosclerosis. As a key regulator of PPARγ activity and inflammatory responses in macrophages, Mal1 modulates monocyte recruitment and the development of atherosclerotic lesions. These findings support macrophage Mal1 as a potential therapeutic target for the prevention of atherosclerosis. The potential relevance of these findings is supported by the previous studies demonstrating that a small molecule inhibitor of aP2 is able to retard the development of diabetes and atherosclerosis in murine models\(^3\).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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activated receptors and localize to the nucleus as well as the cytoplasm. J Lipid Res. 2000; 41:1740–1751. [PubMed: 11060343]


Figure 1.
Changes in body weight (A), serum lipoprotein profiles (B) and atherosclerotic lesion area in the proximal aorta (C) of LDLR−/− mice reconstituted with wild type (●), Mal1−/− (○), or aP2−/− (●) bone marrow cells. Data from FPLC analysis (B) are represented as the average (n=3 per group) of the percent of total cholesterol for each fraction. Fractions 14-17 contain VLDL; fractions 18-24 contain IDL/LDL; and fractions 25-30 contain HDL. Note the differences (* p<0.05) between mice reconstituted with WT marrow vs. mice transplanted with Mal1−/− or aP2−/− marrow determined by One Way ANOVA.
Figure 2.
Expression of PPARγ (A) and CD36 (B) genes. PPARγ (C,D) and CD36 (E,F) protein expression levels in WT and Mal1⁻/⁻ macrophages treated with the PPARγ agonist, ciglitazone (A-E) alone or together with the specific PPARγ antagonist, GW9662 (A). A, B. Thioglycollate-elicited WT (■) and Mal1⁻/⁻ (□) macrophages were treated with lipid-free DMEM media containing the PPARγ agonist, ciglitazone (Cigl., 15 μM), alone or together with the specific PPARγ antagonist, GW9662 (GW, 10 μM) at 37°C for 24 hours. Then RNA was extracted from the macrophages and analyzed by real-time PCR. Graphs represent data (Mean ± SEM) of analysis the same number (n=3) of mice per group (*p<0.05 between untreated and treated with the ciglitazone macrophages of the same group)
C-F. WT and Mal1⁻/⁻ macrophages were treated with ciglitazone (15μM), alone or together with GW9662 (30μM) for 24 hours. Extracted proteins (50μg/well) were resolved and analyzed by Western Blot. The data of PPARγ/β-actin and CD36/β-actin ratios are presented as average (Mean±SEM) assay of three separate experiments. *Differences between the groups with the same dose (D; p<0.05) or between control cells and treated with ciglitazone macrophages (F; p<0.001).
Figure 3.
Visualization (A) and quantified uptake of human DiI-OxLDL (B) or DiI-AcLDL (C) by macrophages from WT (■) and Mal1−/− (□) mice.
A. Peritoneal macrophages were incubated with human DiI-oxLDL (10μg/mL) or DiI-acLDL (10μg/mL) for 2 hours and examined under the microscope (Olympus BX-40) (Magnification x20; insert x60).
B, C. Peritoneal macrophages were incubated with indicated doses of human DiI-OxLDL or DiI-AcLDL for 1 hour and analyzed by FACS.
Figure 4.
Treatment with PA-BSA (A,B) or LPS (C-F) significantly increases activity of Akt and slightly suppresses NF-κB-related protein and genes in Mal1 deficient macrophages. A, B. Macrophages were incubated with media alone or with palmitic acid complexed with PBS (PA-BSA, 0.5mM) for 3 and 6 hours. Extracted proteins were resolved (100 μg/well) and analyzed by Western Blot using antibodies to Akt, p-Akt or β-actin (A); or extracted proteins (20 μg/well) were analyzed by Western Blot using antibodies to p-65, c-Rel, IκBα or β-actin (B).
C-F. Peritoneal macrophages from WT (●) and Mal1−/− (○) mice were treated with LPS (50ng/ml) for the indicated time periods and the expression of Akt-1 (C), c-Jun(D), COX-2 (E) or IL6 (F) genes were analyzed by real-time PCR.
Figure 5.
Mal 1 deficiency decreases the number of nuclei in MOMA-2+ area of atherosclerotic lesions (A-G), and suppresses CCR2 gene (H) and protein (I,K) expression levels in blood monocytes.

A-F. Serial sections from the proximal aorta of LDLR-/- mice reconstituted with WT (A-C; ■) and Mal1-/- (D-F; □) marrow and fed the Western diet for 12 weeks. Sections were stained with antibodies to mouse macrophage, MOMA-2 (A,D) and nuclear stain, DAPI (B,E). After merging of the images, the number of nuclei was analyzed in MOMA-2-positive area. Note, number of nuclei per standard lesion area (G; Mean ± SEM; *p<0.05 between mice with WT and Mal1-/- marrow).

A-C. Blood monocytes were isolated from WT→LDLR-/-(■) and Mal1-/-→LDLR-/-(□) mice on the Western diet. CCR2 gene (H) and protein (I) expression levels were analyzed by real-time PCR and FACS. Graphs represent data (Mean ± SEM) analysis of the same number (n=3) mice per group (*p<0.05 between WT and Mal1-/- cells).
### Table 1

Total serum cholesterol and triglyceride levels in LDLR\(^{-/-}\) mice reconstituted with wild type, Mal1\(^{-/-}\) or aP2\(^{-/-}\) bone marrow.

<table>
<thead>
<tr>
<th>Type of marrow reconstituted</th>
<th>Serum Lipid</th>
<th>Baseline</th>
<th>4 weeks Western diet</th>
<th>8 weeks Western diet</th>
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<tr>
<td>WT cells n = 13</td>
<td>Cholesterol</td>
<td>201 ± 9</td>
<td>476 ± 26</td>
<td>597 ± 26</td>
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<tr>
<td></td>
<td>Triglycerides</td>
<td>95 ± 8</td>
<td>198 ± 11</td>
<td>207 ± 21</td>
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<tr>
<td>Mal1(^{-/-}) cells n = 14</td>
<td>Cholesterol</td>
<td>208 ± 3</td>
<td>433 ± 16</td>
<td>584 ± 18</td>
</tr>
<tr>
<td></td>
<td>Triglycerides</td>
<td>91 ± 5</td>
<td>194 ± 16</td>
<td>228 ± 14</td>
</tr>
<tr>
<td>aP2(^{-/-}) cells n = 15</td>
<td>Cholesterol</td>
<td>202 ± 5</td>
<td>472 ± 15</td>
<td>601 ± 26</td>
</tr>
<tr>
<td></td>
<td>Triglycerides</td>
<td>97 ± 5</td>
<td>177 ± 12</td>
<td>209 ± 12</td>
</tr>
</tbody>
</table>

Values are in mg/dl (Mean ± SEM). The number of recipient mice in each group is indicated by \(n\). The differences are not statistically significant between the groups at either time point.