

Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2011 June ; 31(6): 1283–1290. doi:10.1161/ATVBAHA.111.225839.

Macrophage Mal1 deficiency suppresses atherosclerosis in LDL-receptor null mice by activating PPAR γ -regulated genes

Vladimir R. Babaev¹, Robert P. Runner¹, Daping Fan¹, Lei Ding¹, Youmin Zhang¹, Huan Tao¹, Ebru Erbay², Cem Z. Görgün², Sergio Fazio^{1,3}, Gökhan S. Hotamisligil², and MacRae F. Linton^{1,4}

¹Department of Medicine, Vanderbilt University Medical Center, Nashville, TN 37232

²Department of Genetics & Complex Diseases, Harvard School of Public Health, Boston, MA, 02115 U.S.A

³Department of Pathology, Vanderbilt University Medical Center, Nashville, TN 37232

⁴Department of Pharmacology, Vanderbilt University Medical Center, Nashville, TN 37232

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 PPAR γ activity and up-regulation of a PPAR γ -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 PPAR γ activity, which increases expression of CCR2 by monocytes promoting their recruitment to atherosclerotic lesions.

Keywords

Atherosclerosis; Macrophages; PPAR γ ; CD36; CCR2 receptor

Address correspondence to Vladimir Babaev (Sergio Fazio, or MacRae F. Linton), Department of Cardiovascular Medicine, Vanderbilt University School of Medicine, 312 PRB, 2220 Pierce Avenue, Nashville, Tennessee 37232-6300, Phone: (615) 936-1450, Fax: (615) 936-3486, vladimir.babaev (sergio.fazio or macrae.linton)@vanderbilt.edu.

Disclosures None.

Introduction

Fatty acid-binding proteins (FABPs) play important roles in FA transport, cellular signaling, gene transcription and cytoprotection¹. 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 lipids². The adipocyte/macrophage FABPs, aP2 (FABP4) and Mal1 (FABP5), are intracellular lipid chaperones that modulate systemic metabolism of glucose and lipids, insulin sensitivity and atherosclerosis². 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 diets³⁻⁵. 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 γ) activity in macrophages increasing both CD36-mediated uptake of oxidized low-density lipoprotein, and ABCA1-mediated cholesterol efflux in the cells⁶. In addition, aP2^{-/-} macrophages have reduced I κ B kinase activity and NF- κ B-related inflammatory gene expression⁶. 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 1:1⁵. These two proteins have 52% amino-acid similarity and bind various fatty acids and synthetic compounds with similar selectivity and affinity⁷. 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 compartments². 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^{-/-} mice⁹. 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 γ activity and affected expression of the PPAR γ -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 described¹², and backcrossed 10 or more generations onto C57BL/6 background¹³. 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 described¹⁴.

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 Suprose 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 Elektronik GmbH.) as described¹⁵

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 described¹⁶, 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 described¹⁷, or with PPAR γ agonist, ciglitazone (Cayman Chemicals) or PPAR γ 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 described¹⁵.

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 described¹⁸.

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 described¹⁵.

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 PPAR γ (cat# ab27649; Abcam, Inc. Cambridge, MA), Akt and p-Akt (from Cell Signaling Technology), c-Rel, NF- κ B p65 and I κ B α (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 $^{-/-}$ mice with hematopoietic cells from Mal1 $^{-/-}$ mice (n=15), aP2 $^{-/-}$ 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 $^{-/-}$ or aP2 $^{-/-}$ marrow (Figure 1B). However, recipient mice receiving Mal1 $^{-/-}$ or aP2 $^{-/-}$ 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 PPAR γ activity in macrophages

Recent studies have implicated enhanced PPAR γ activity in aP2 $^{-/-}$ macrophages as the mechanism responsible for altering expression of genes that regulate cholesterol homeostasis and inflammation resulting in inhibition of atherosclerosis⁶. 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 $^{-/-}$ 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 PPAR γ agonist, ciglitazone, with or without the selective PPAR γ antagonist, GW9662. Real-time PCR analysis indicated that PPAR γ activation increased expression of the PPAR γ gene significantly higher in Mal1 $^{-/-}$ macrophages than in WT cells and these effects were completely reversed by addition of the PPAR γ antagonist (Figure 2A). Similarly, the ligand treatment significantly (1.5-fold) increased expression of the CD36 mRNA in both WT and Mal1 $^{-/-}$ 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 PPAR γ through LXRA. Interestingly, the expression of the CD36, ABCA1, and

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 PPAR γ 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 PPAR γ 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 PPAR γ in mediating the increase in CD36, we made use of the PPAR γ antagonist GW9662, which covalently modifies a cysteine residue of PPAR γ resulting in complete loss of ligand binding ability of PPAR γ ¹⁹. Interestingly, treatment cells with the PPAR γ 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 PPAR γ has an effect on uptake of modified LDL. WT and Mal1^{-/-} macrophages were incubated with DiI-labeled AcLDL and DiI-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 PPAR γ pathway in macrophages enhancing expression of the PPAR γ -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- κ B. 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 I κ B α 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- κ B-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 I κ B α 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 PPAR γ 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 atherosclerosis². 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^{-/-} mice³⁻⁵. 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^{4, 5, 13}. Expression of aP2 and Mal1 by both adipocytes and macrophages contributes to insulin resistance²⁰. However, macrophage aP2 expression promotes atherosclerosis independent of its impact on insulin sensitivity³. Since both proteins, aP2 and Mal1, share a high degree of homology and are expressed by macrophages in similar proportions⁵, 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 PPAR γ activity and suppresses atherosclerosis formation⁶. Therefore, to examine mechanisms underlying the impact of macrophage Mal1 expression on atherogenesis, we analyzed PPAR γ gene and protein expression levels in peritoneal macrophages isolated from Mal1^{-/-} and WT mice. We demonstrate that treatment with the PPAR γ agonist ciglitazone increases PPAR γ gene and protein expression more in Mal1^{-/-} macrophages than in WT cells, and the effect on PPAR γ gene expression is reversed by addition of a PPAR γ antagonist (Figure 2A). In addition, we show in a dose response study with ciglitazone that PPAR γ protein expression increases to a greater extent in Mal1^{-/-} macrophages than in WT cells (Figure 2 C,D). Our results suggest an interesting possibility that the promoter of the PPAR γ gene

may contain functional PPRE sites. Interestingly, a gene database search of the mouse and human PPAR γ 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 PPAR γ , 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 PPAR γ 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 PPAR γ regulates ABCA1 through LXR α , 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 PPAR γ -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 PPAR γ -LXR α 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- κ B pathway²⁵. Taken together, these data indicate that Mal1-deficiency activates the PPAR γ 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 I κ B, which may, at least in part, underlie the alterations in cytokine expression⁶. Similarly, we found that Mal1-deficiency suppresses COX-2, IL6 mRNA and I κ B 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 PPAR γ as a critical signaling molecule in determining macrophage phenotype in vitro, and treatment with a PPAR γ agonist enhances the anti-inflammatory properties of macrophages²⁹. In this

regard, PPAR γ activation by oxidized LDL inhibits CCR2 expression in human and mouse monocytes³⁰. In contrast, macrophage-specific deficiency in PPAR γ significantly accelerates CCR2 expression and atherosclerosis in LDLR^{-/-} mice¹⁵. 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 recruitment³¹ 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 PPAR γ 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 models³².

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Sources of Funding This work was supported by National Institutes of Health grants HL65405, HL105375, HL53989, DK064360, ADA(7-02-RA-38), 0555323B, HL57986, DK59637 (Lipid, Lipoprotein and Atherosclerosis Core of the Vanderbilt Mouse Metabolic Phenotype Centers).

References

1. Zimmerman AW, Veerkamp JH. New insights into the structure and function of fatty acid-binding proteins. *Cell Mol Life Sci.* 2002; 59:1096–1116. [PubMed: 12222958]
2. Furuhashi M, Hotamisligil G. Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets. *Nature Reviews: Drug Discovery.* 2008; 7:489–503.
3. Boord JB, Maeda K, Makowski L, Babaev VR, Fazio S, Linton MF, Hotamisligil G. Adipocyte fatty acid-binding protein, aP2, alters late atherosclerotic lesion formation in severe hypercholesterolemia. *Arterioscler Thromb Vasc Biol.* 2002; 22:1686–1691. [PubMed: 12377750]
4. Boord JB, Maeda K, Makowski L, Babaev VR, Fazio S, Linton MF, Hotamisligil G. Combined adipocyte-macrophage fatty acid-binding protein deficiency improves metabolism, atherosclerosis, and survival in apolipoprotein E-deficient mice. *Circulation.* 2004; 110:1492–1498. [PubMed: 15353487]

5. Makowski L, Boord JB, Maeda K, Babaev VR, Uysal KT, Morgan MA, Parker RA, Suttles J, Fazio S, Hotamisligil GS, Linton MF. Lack of macrophage fatty-acid-binding protein aP2 protects mice deficient in apolipoprotein E against atherosclerosis. *Nat Med*. 2001; 7:699–705. [PubMed: 11385507]
6. Makowski L, Brittingham KC, Reynolds JM, Suttles J, Hotamisligil G. The fatty acid-binding protein, aP2, coordinates macrophage cholesterol trafficking and inflammatory activity. Macrophage expression of aP2 impacts peroxisome proliferator-activated receptor gamma and IkappaB kinase activities. *J Biol Chem*. 2005; 280:12888–12895. [PubMed: 15684432]
7. Haunerland NH, Spener F. Fatty acid-binding proteins--insights from genetic manipulations. *Progr Lipid Res*. 2004; 43:328–349.
8. Welch JS, Ricote M, Akiyama TE, Gonzalez FJ, Glass CK. PPARgamma and PPARdelta negatively regulate specific subsets of lipopolysaccharide and IFN-gamma target genes in macrophages. *Proc Natl Acad Sci USA*. 2003; 100:6712–6717. [PubMed: 12740443]
9. Maeda K, Cao H, Kono K, Gorgun CZ, Furuhashi M, Uysal KT, Cao Q, Atsumi G, Malone H, Krishnan B, Minokoshi Y, Kahn BB, Parker RA, Hotamisligil GS. Adipocyte/macrophage fatty acid binding proteins control integrated metabolic responses in obesity and diabetes. *Cell Metabolism*. 2005; 1:107–119. [PubMed: 16054052]
10. Babaev VR, Chew JD, Ding L, Davis S, Breyer MD, Breyer RM, Oates JA, Fazio S, Linton MF. Macrophage EP4 Deficiency Increases Apoptosis and Suppresses Early Atherosclerosis. *Cell Metabolism*. 2008; 8:492–501. [PubMed: 19041765]
11. Liu H, Perlman H, Pagliari LJ, Pope RM. Constitutively activated Akt-1 is vital for the survival of human monocyte-differentiated macrophages. Role of Mcl-1, independent of nuclear factor (NF)-kappaB, Bad, or caspase activation. *J Exp Med*. 2001; 194:113–126. [PubMed: 11457886]
12. Bronson SK, Smithies O. Altering mice by homologous recombination using embryonic stem cells. *J Biol Chem*. 1994; 269:27155–27158. [PubMed: 7961620]
13. Maeda K, Uysal KT, Makowski L, Gorgun CZ, Atsumi G, Parker RA, Bruning J, Hertz AV, Bernlohr DA, Hotamisligil GS. Role of the Fatty Acid Binding Protein mal1 in Obesity and Insulin Resistance. *Diabetes*. 2003; 52:300–307. [PubMed: 12540600]
14. Linton MF, Atkinson JB, Fazio S. Prevention of atherosclerosis in apolipoprotein E-deficient mice by bone marrow transplantation. *Science*. 1995; 267:1034–1037. [PubMed: 7863332]
15. Babaev VR, Yancey PG, Ryzhov SV, Kon V, Breyer MD, Magnuson MA, Fazio S, Linton MF. Conditional knockout of macrophage PPAR-gamma increases atherosclerosis in C57BL/6 and low-density lipoprotein receptor-deficient mice. *Arterioscler Thromb Vasc Biol*. 2005; 25:1647–1653. [PubMed: 15947238]
16. Borradaile NM, Han X, Harp JD, Gale SE, Ory DS, Schaffer JE. Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death. *J Lipid Res*. 2006; 47:2726–2737. [PubMed: 16960261]
17. Yao PM, Tabas I. Free cholesterol loading of macrophages induces apoptosis involving the fas pathway. *J Biol Chem*. 2000; 275:23807–23813. [PubMed: 10791964]
18. Babaev VR, Ishiguro H, Ding L, Yancey PG, Dove DE, Kovacs WJ, Semenkovich CF, Fazio S, Linton MF. Macrophage expression of peroxisome proliferator-activated receptor-alpha reduces atherosclerosis in low-density lipoprotein receptor-deficient mice. *Circulation*. 2007; 116:1404–1412. [PubMed: 17724261]
19. Leesnitzer LM, Parks DJ, Bledsoe RK, Cobb JE, Collins JL, Consler TG, Davis RG, Hull-Ryde EA, Lenhard JM, Patel L, Plunket KD, Shenk JL, Stimmel JB, Therapontos C, Willson TM, Blanchard SG. Functional Consequences of Cysteine Modification in the Ligand Binding Sites of Peroxisome Proliferator Activated Receptors by GW9662. *Biochemistry*. 2002; 41:6640–6650. [PubMed: 12022867]
20. Furuhashi M, Fucho R, Gorgun CZ, Tuncman G, Cao H, Hotamisligil GS. Adipocyte/macrophage fatty acid-binding proteins contribute to metabolic deterioration through actions in both macrophages and adipocytes in mice. *J Clin Invest*. 2008; 118:2640–2650. [PubMed: 18551191]
21. Helledie T, Antonius M, Sorensen RV, Hertz AV, Bernlohr DA, Ivraa S, Kristiansen K, Mandrup S. Lipid-binding proteins modulate ligand-dependent trans-activation by peroxisome proliferator-

- activated receptors and localize to the nucleus as well as the cytoplasm. *J Lipid Res.* 2000; 41:1740–1751. [PubMed: 11060343]
22. Zelcer N, Tontonoz P. Liver X receptors as integrators of metabolic and inflammatory signaling. *J Clin Invest.* 2006; 116:607–614. [PubMed: 16511593]
 23. Chawla A, Boisvert WA, Lee CH, Laffitte BA, Barak Y, Joseph SB, Liao D, Nagy L, Edwards PA, Curtiss LK, Evans RM, Tontonoz P. A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. *Molecular Cell.* 2001; 7:161–171. [PubMed: 11172721]
 24. Li Y, Gerbod-Giannone M-C, Seitz H, Cui D, Thorp E, Tall AR, Matsushima GK, Tabas I. Cholesterol-induced apoptotic macrophages elicit an inflammatory response in phagocytes, which is partially attenuated by the Mer receptor. *J Biol Chem.* 2006; 281:6707–6717. [PubMed: 16380374]
 25. Zhu X, Lee J-Y, Timmins JM, Brown JM, Boudyguina E, Mulya A, Gebre AK, Willingham MC, Hiltbold EM, Mishra N, Maeda N, Parks JS. Increased cellular free cholesterol in macrophage-specific Abca1 knock-out mice enhances pro-inflammatory response of macrophages. *J Biol Chem.* 2008; 283:22930–22941. [PubMed: 18552351]
 26. Plas DR, Thompson CB. Akt-dependent transformation: there is more to growth than just surviving. *Oncogene.* 2005; 24:7435–7442. [PubMed: 16288290]
 27. Fernandez-Hernando C, Ackah E, Yu J, Suarez Y, Murata T, Iwakiri Y, Prendergast J, Miao RQ, Birnbaum MJ, Sessa WC. Loss of Akt1 leads to severe atherosclerosis and occlusive coronary artery disease. *Cell Metabolism.* 2007; 6:446–457. [PubMed: 18054314]
 28. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med.* 2006; 354:610–621. [PubMed: 16467548]
 29. Bouhrel MA, Derudas B, Rigamonti E, Dievart R, Brozek J, Haulon S, Zawadzki C, Jude B, Torpier G, Marx N, Staels B, Chinetti-Gbaguidi G. PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell Metabolism.* 2007; 6:137–143. [PubMed: 17681149]
 30. Han KH, Chang MK, Boullier A, Green SR, Li A, Glass CK, Quehenberger O. Oxidized LDL reduces monocyte CCR2 expression through pathways involving peroxisome proliferator-activated receptor gamma. *J Clin Invest.* 2000; 106:793–802. [PubMed: 10995790]
 31. Gautier EL, Jakubzick C, Randolph GJ. Regulation of the Migration and Survival of Monocyte Subsets by Chemokine Receptors and Its Relevance to Atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2009; 29:1412–1418. [PubMed: 19759373]
 32. Furuhashi M, Tuncman G, Gorgun CZ, Makowski L, Atsumi G, Vaillancourt E, Kono K, Babaev VR, Fazio S, Linton MF, Sulsky R, Robl JA, Parker RA, Hotamisligil GS. Treatment of diabetes and atherosclerosis by inhibiting fatty-acid-binding protein aP2. *Nature.* 2007; 447:959–965. [PubMed: 17554340]

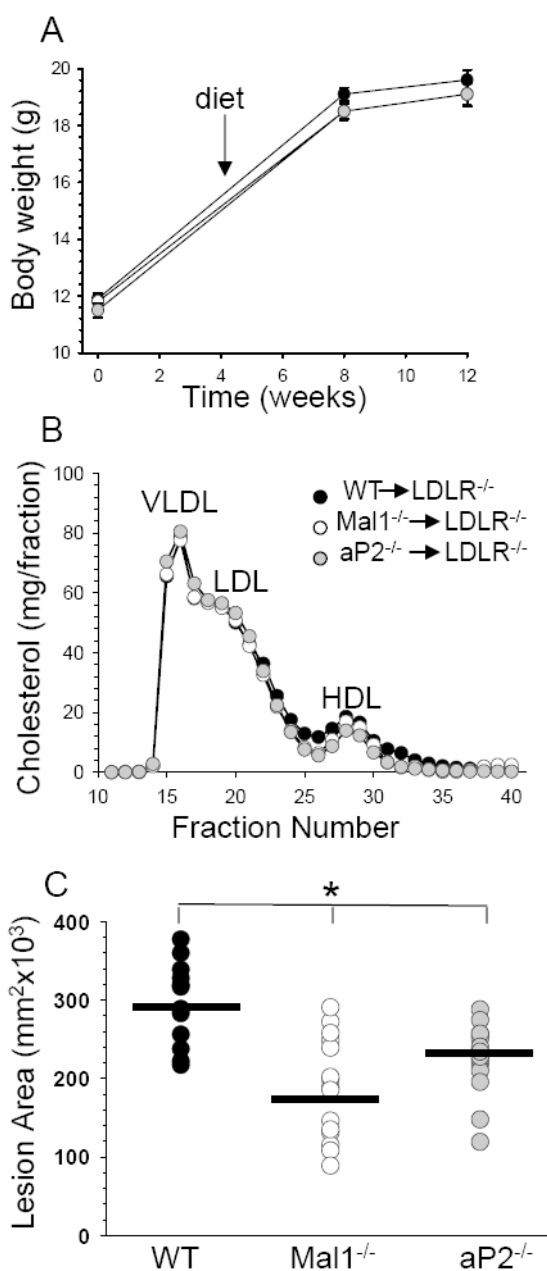
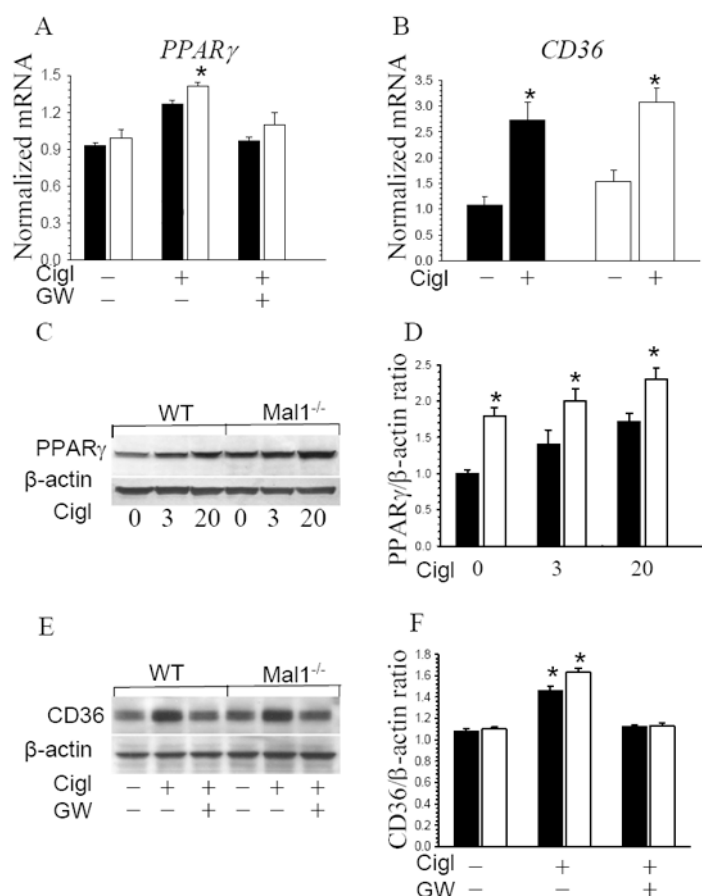


Figure 1.

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 PPAR γ (A) and CD36 (B) genes, PPAR γ (C,D) and CD36 (E,F) protein expression levels in WT and Mal1^{-/-} macrophages treated with the PPAR γ agonist, ciglitazone (A-E) alone or together with the specific PPAR γ antagonist, GW9662 (A).

A, B. Thioglycollate-elicited WT (■) and Mal1^{-/-} (□) macrophages were treated with lipid-free DMEM media containing the PPAR γ agonist, ciglitazone (Cigl., 15 μ M), alone or together with the specific PPAR γ antagonist, GW9662 (GW, 10 μ M) at 37°C for 24 hours. Then RNA was extracted from the macrophages and analyzed by real-time PCR. Graphs represent data (Mean $\pm$ 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 (15 μ M), alone or together with GW9662 (30 μ M) for 24 hours. Extracted proteins (50 μ g/well) were resolved and analyzed by Western Blot. The data of PPAR γ /β-actin and CD36/β-actin ratios are presented as average (Mean $\pm$ 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).

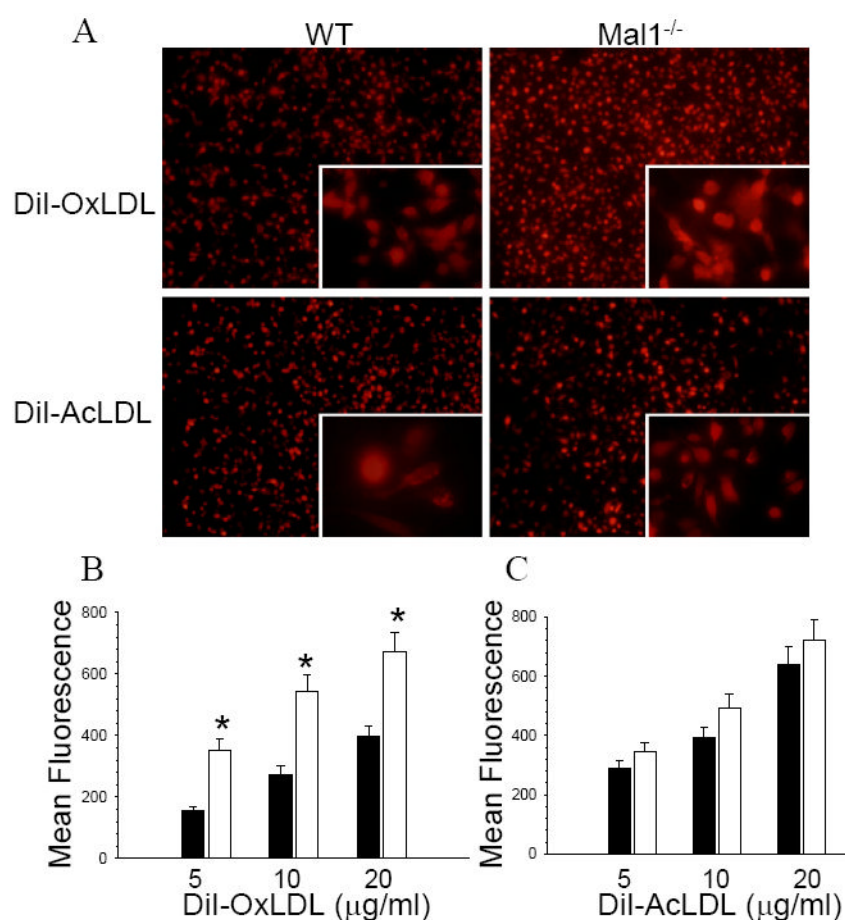


Figure 3.

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.

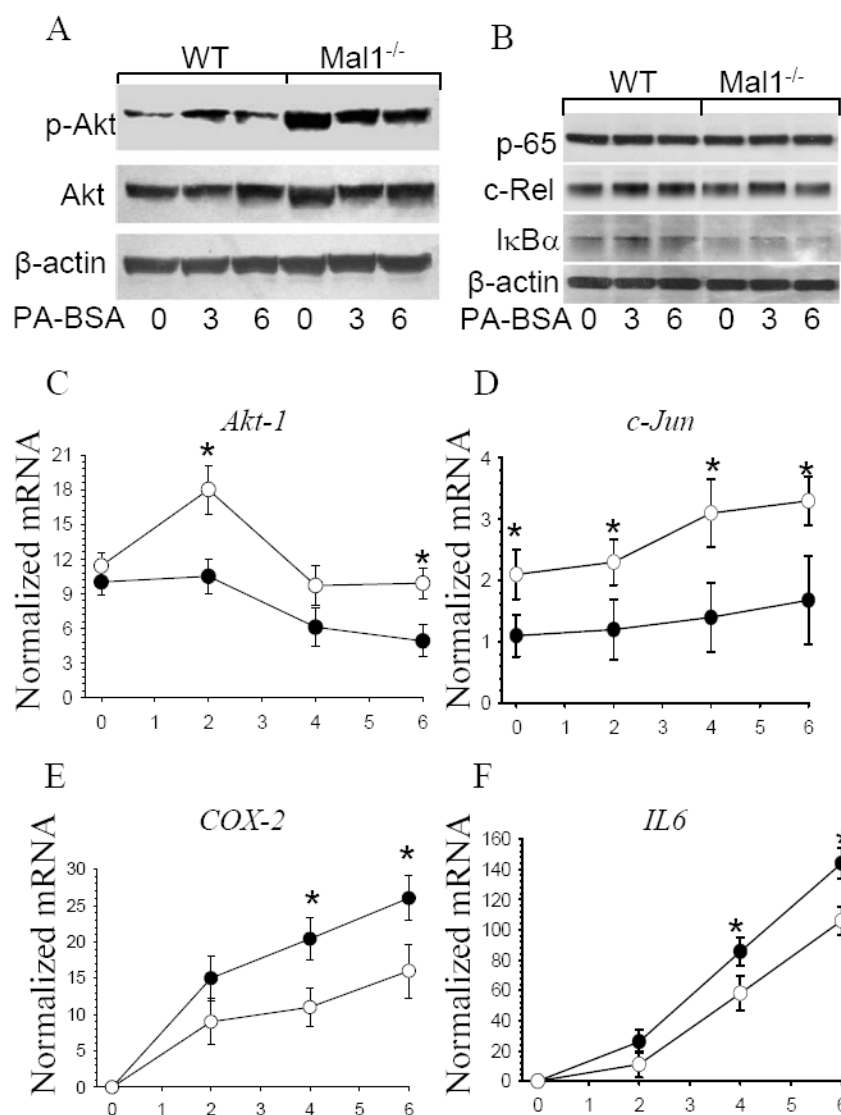


Figure 4.

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 (100μg/well) and analyzed by Western Blot using antibodies to Akt, p-Akt or β-actin (A); or extracted proteins (20μg/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

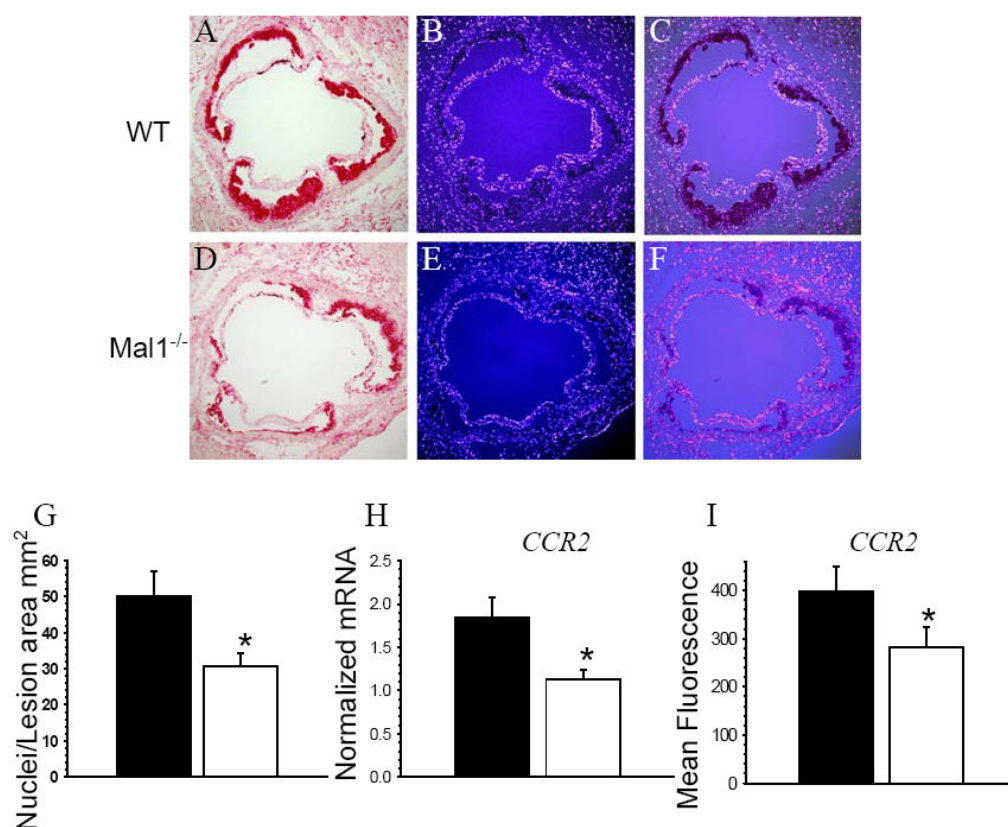


Figure 5.

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,K) 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.

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

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.