Caveolin-1 Plays a Critical Role in the Differentiation of Monocytes into Macrophages

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Objective—Monocyte to macrophage differentiation is an essential step in atherogenesis. The structure protein of caveolae, caveolin-1, is increased in primary monocytes after its adhesion to endothelium. We explore the hypothesis that caveolin-1 plays a role in monocyte differentiation to macrophages.

Methods and Results—Both phorbol myristate acetate–induced THP-1 and colony stimulating factor–induced primary monocyte differentiation was associated with an increase in cellular caveolin-1 expression. Overexpression of caveolin-1 by transfection increased macrophage surface markers and inflammatory genes, whereas caveolin-1 knockdown by small interfering RNA or knockout reduced these. Also, caveolin-1 knockdown inhibited the differentiation—induced nuclear translocation of early growth response factor 1 (EGR-1) through ERK phosphorylation, further decreased the binding of EGR-1 to CD115 promoter, thus decreasing EGR-1 transcriptional activity. In functional assays, caveolin-1 inhibited transmigration but promoted phagocytosis in the monocyte–macrophage lineage. Decreasing caveolin-1 inhibited the uptake of modified low-density lipoprotein and reduced cellular lipid content. Finally, we showed that caveolin-1 knockout mice displayed less monocyte differentiation than wild-type mice and that EGR-1 transcription activity was also decreased in these mice because of the inhibition of ERK phosphorylation.

Conclusion—Caveolin-1 promotes monocyte to macrophage differentiation through the regulation of EGR-1 transcriptional activity, suggesting that phagocytic caveolin-1 may be critical for atherogenesis. (Arterioscler. Thromb. Vasc. Biol. 2012;32:00-00.)

Key Words: caveolin-1 ■ monocyte differentiation ■ macrophages ■ early growth response factor 1

Atherosclerosis, a chronic inflammatory disease, remains one of the leading causes of premature death in the developed world. Circulating monocytes play a key role in the pathogenesis of atherosclerosis, adhering to the endothelium upon vascular insult and transmigrating into the subendothelial space where they differentiate into macrophages, which take up cholesterol, converting into foam cells and forming fatty streaks.1,2

The differentiation of monocytes into macrophages is dependent on macrophage–colony stimulating factor (M-CSF) as evidenced by the finding of reduced circulating monocytes and a subsequent reduction in atherosclerotic lesions in M-CSF and apolipoprotein E (apoE) double knockout (KO) mice compared with apoE KO mice.3 This finding also highlights that monocyte to macrophage differentiation is a pivotal process in atherogenesis and a powerful therapeutic target in the prevention and treatment of atherosclerosis.

Caveolae are 50- to 100-nm flask-shaped invaginations of the plasma membrane.4,5 They have been identified in a wide variety of tissues and cell types and have been implicated in lipid regulation, mechanotransduction, and endocytosis.6 As the location for proteins related to cholesterol metabolism and cell signaling, caveolae have been linked to cholesterol homeostasis and signal transduction.7 Caveolin-1, a major structural protein of caveolae, has been reported to be involved in monocyte/macrophage inflammation,8 adhesion,9 and phagocytosis.10 Interestingly, primary monocytes display an increase in caveolin-1 expression after a 2-hour adhesion to the endothelium.12 Moreover, caveolin-1 is associated with the nuclear translocation of transcription factors13 and integrin signals,14 2 major regulators in monocyte differentiation.15

In the current study, we explore the hypothesis that caveolin-1 influences monocyte to macrophage differentiation. Here, we report that caveolin-1 positively regulates monocyte differentiation to macrophages via the transcriptional activity of the transcription factor, early gene response factor 1 (EGR-1).

Materials and Methods

THP-1 monocyctic cell lines, human primary monocytes, and mouse blood mononuclear cells were studied in the in vitro experiments, whereas wild-type and caveolin-1 KO mice on C57BL/6 background were used in the in vivo studies. Detailed methodology used can be found in the online-only Data Supplement.
Results

Caveolin-1 Expression Increases After Monocyte Differentiation into Macrophage

THP-1 monocytes can be activated and differentiated into macrophages by phorbol myristate acetate (PMA), and it is widely applied as a cell model for studies on monocyte differentiation. In the current study, THP-1 monocytes were treated with PMA in time- and dose-dependent patterns. Caveolin-1 expression was shown to increase by Western blot (Figure 1A and 1B in the online-only Data Supplement) after PMA treatment. To confirm that THP-1 cells were activated and differentiated by PMA, the macrophage marker CD68 was assessed by flow cytometry and confirmed to increase with PMA 100 nmol/L at 72 hours (Figure IC in the online-only Data Supplement).

To validate that the findings from the immortalized THP-1 cells were also true of primary cells, human and mouse primary monocytes were stimulated to differentiate. Caveolin-1 expression was increased by M-CSF treatment (Figure 1A), and CD68 was likewise elevated confirming the differentiation of human primary monocytes to macrophages (Figure ID in the online-only Data Supplement). Likewise, mouse peripheral blood mononuclear cells stimulated by granulocyte M-CSF demonstrated an increase in caveolin-1 and CD68 by real-time polymerase chain reaction (Figure 1B). Although it has been reported that peripheral blood mononuclear cells rarely express caveolin-1 with the exception of those cells in the monocyte/macrophage lineage, the caveolin-1 expression detected in these studies may be construed to be relevant to this lineage. Moreover, flow analysis displayed the decrease of monocyte marker ly6C and increase of macrophage markers, CD115 and F4/80 on CD11b+ peripheral blood mononuclear cells, indicating mouse monocyte differentiation into macrophage (Figure IE in the online-only Data Supplement). These experiments demonstrate that the expression of caveolin-1 was elevated after monocyte to macrophage differentiation in both immortalized and primary cells.

Increasing Caveolin-1 in THP-1 Monocytes Promotes Differentiation into Macrophages

To examine whether caveolin-1 can promote the differentiation of monocytes into macrophages, we increased caveolin-1 expression in THP-1 cells by transfection with pcDNA3-caveolin-1 expression plasmid or pcDNA3 as control using Western blots to confirm transfection efficiency (Figure IIA in the online-only Data Supplement). Monocyte inflammatory and differentiation gene expression levels were then assessed by real-time polymerase chain reaction. Caveolin-1 transfection significantly increased the expression of the inflammatory genes, CD11b and intercellular cell adhesion molecule-1 (Figure IIB in the online-only Data Supplement), as well as those of the macrophage markers CD68 and CD36 (Figure 2A). As expected, application of the positive control PMA induced increased expression of these genes (Figure 2A; and Figure IIB in the online-only Data Supplement). These experiments show that caveolin-1 expression positively differentiates monocytes into macrophages albeit to a lesser extent than observed with PMA.

Knockdown of Caveolin-1 Inhibits Monocyte to Macrophage Differentiation

To further explore the role of caveolin-1 in PMA--induced monocyte differentiation, THP-1 monocytes were transfected with control or caveolin-1 small interfering RNA plasmids and then treated with PMA at 100 nmol/L for 72 hours. The knockdown of caveolin-1 was confirmed by Western blot. Whereas untreated cells did not display a significant knockdown of caveolin-1 because of the low basal level of expression (Figure IIC in the online-only Data Supplement), caveolin-1 knockdown decreased PMA--induced monocyte inflammatory (Figure IID in the online-only Data Supplement), CD11b and intercellular cell adhesion molecule-1 and differentiation (Figure 2B: CD68, CD36, and CD14) gene expression. The finding was validated in mouse primary cells where peripheral blood mononuclear cells from wild-type (WT) and caveolin-1 KO mice were induced to differentiate by granulocyte M-CSF. The morphology of the differentiated cells from 2 different mouse types displayed a significant difference, that is, a more elongated phenotype in the WT group as compared with the rounded appearance of the KO group (Figure 2C). Moreover, the granulocyte M-CSF--induced CD68 expression was inhibited in KO cells compared with WT cells using real-time polymerase chain reaction (Figure 2D). Similarly, flow analysis showed the granulocyte M-CSF--induced macrophage markers CD115 and F4/80 in KO CD11b+...
cells were decreased compared with the WT group (Figure 2E). These results further emphasize the critical role of caveolin-1 in monocyte to macrophage differentiation.

**Nucleus Location of EGR-1 and Its Target Genes Are Regulated by Caveolin-1 Expression During Monocyte Differentiation into Macrophage**

Several transcription factors have been reported to be involved in monocyte differentiation and macrophage maturation. Among these, EGR-1 transcription activity has been demonstrated to be inhibited by caveolin-1 knockdown in fibroblasts. We, therefore, hypothesized that caveolin-1 influenced monocyte differentiation into macrophage through regulation of EGR-1 transcriptional activity.

THP-1 monocytes were transfected with control or caveolin-1 small interfering RNA plasmids, and then treated with PMA (100 nmol/L) for 72 hours. PMA increased the expression of EGR-1, and although caveolin-1 knockdown did not affect the PMA–induced EGR-1 expression in whole cell fraction, it inhibited its nuclear location (Figure 3A). Moreover, decreasing caveolin-1 expression also blocked PMA–induced ERK phosphorylation (Figure 3A), which was related to EGR-1 nuclear translocation. Pretreatment with U0126, the MEK1/2 inhibitor, inhibited the PMA–induced ERK phosphorylation and nuclear translocation of EGR-1 but had no effect on caveolin-1 expression (Figure III in the online-only Data Supplement). These results indicated that caveolin-1 affects monocyte differentiation through ERK phosphorylation.
phosphorylation and the nuclear translocation of EGR-1. Because CD115 and tumor necrosis factor-α
are target genes of EGR-1 in monocyte to macrophage differentiation, we explored their expression levels and showed that both were elevated after PMA–induced monocyte differentiation into macrophage but inhibited by knockdown of caveolin-1 (Figure 3B). To further explore the effect of caveolin-1 on EGR-1 and its target genes during differentiation, EGR-1 DNA-binding ability was assessed using chromatin immunoprecipitation assay. PMA increased the binding of EGR-1 to its target sequence on CD115 promoter but this was inhibited by caveolin-1 knockdown (Figure 3C). These results demonstrate that inhibition of nuclear translocation of EGR-1 by caveolin-1 knockdown further decreases the binding of EGR-1 to its target promoter sequence inhibiting its transcription efficiency.

**Caveolin-1 Regulates the Physiological Functions of Monocyte and Macrophage**

Migration and phagocytosis are 2 major functions of monocyte/macrophage cells. Here, we explore the influence of
caveolin-1 in transmigration (Figure 4A) and phagocytosis (Figure 4B) of monocytes/macrophages. In nontransfected cells, PMA-treated cells (macrophages) displayed lower transmigration and higher phagocytosis abilities than untreated cells (monocytes), consistent with the macrophage phenotypes expected. Caveolin-1 overexpression inhibited transmigration and promoted the phagocytosis in untreated THP-1 cells, whereas knockdown of caveolin-1 inhibited the phagocytosis without effect on transmigration in PMA-induced cells. These results demonstrate that caveolin-1 is positively associated with phagocytosis, a macrophage function, and negatively with transmigration, a monocytic function.

In atherosclerosis, monocyte-derived macrophages form foam cells through the uptake of modified low-density lipoprotein (LDL). Here, we measured the ability of acetylated LDL endocytosis on both THP-1 monocytic cells and mouse macrophages. As shown in Figure 4C (including Figure IVA in the online-only Data Supplement), PMA–treated THP-1 cells (macrophages) display more acetylated LDL uptake than untreated THP-1 monocytes, which was inhibited by caveolin-1 knockdown, consistent with the findings in the phagocytosis assay. WT mouse peritoneal macrophages took up more acetylated LDL than cells from KO mice (Figure 4D; and Figure IVB in the online-only Data Supplement). These experiments indicate that caveolin-1 plays a critical role in the modified LDL endocytosis of macrophages to form foam cells. To further explore the role of caveolin-1 in macrophage–derived foam cell formation, LDL–treated mouse peritoneal macrophages were collected for lipid extraction and determination. As expected, LDL treatment increased cholesteryl ester and triglyceride content (2 of the major lipid species in foam cells) in macrophages. Caveolin-1 KO cells displayed significantly less lipid content in comparison (Figure 4E–4F) implying that decreasing caveolin-1 reduces foam cell formation.

**Monocyte Differentiation to Macrophage Decreases in Caveolin-1 KO Mice**

To confirm the influence of caveolin-1 in monocyte to macrophage differentiation in vivo, caveolin-1 KO mice were used. At baseline, there are significantly less CD115+/CD11b+ macrophages in KO mice compared to WT mice (Figure 4). A and B, THP-1 cells without transfection (NT), transfected with pcDNA3 (ctrl) or pcDNA3-caveolin-1 (CAV), or transfected with pcDNA6.2-GW/EmGFPC-control-miR (ctrl) or pcDNA6.2-GW/EmGFPC-caveolin-1-miR (CAV) were treated with vehicle or phorbol myristate acetate (PMA) (100 nmol/L) for 72 hours. A, THP-1 cells with different treatments were added to confluent EA.Hy926 endothelial cells. After a 6-hour incubation, THP-1 cells in lower chamber containing monocyte chemoattractant protein-1 (100 ng/mL) were collected and lysed for DNA measurement by CyQUANT GR dye. Graph displays the amount of DNA of migrating THP-1 cells in the different groups. Data are from 4 independent experiments. (*P<0.05)

**Figure 4. Caveolin-1 (CAV-1) regulates monocyte and macrophage functions.**

A and B, THP-1 cells without transfection (NT), transfected with pcDNA3 (ctrl) or pcDNA3-caveolin-1 (CAV), or transfected with pcDNA6.2-GW/EmGFPC-control-miR (ctrl) or pcDNA6.2-GW/EmGFPC-caveolin-1-miR (CAV) were treated with vehicle or phorbol myristate acetate (PMA) (100 nmol/L) for 72 hours. A, THP-1 cells with different treatments were added to confluent EA.Hy926 endothelial cells. After a 6-hour incubation, THP-1 cells in lower chamber containing monocyte chemoattractant protein-1 (100 ng/mL) were collected and lysed for DNA measurement by CyQUANT GR dye. Graph displays the amount of DNA of migrating THP-1 cells in the different groups. Data are from 4 independent experiments. (*P<0.05)

B. The fluorescein isothiocyanate (FITC)-anti-rabbit IgG latex beads were added to different treated THP-1 cells. After a 4-hour incubation, the uptake of beads by cells was detected by flow cytometry. Graph shows the group data of FITC geo-means under different conditions. Data are from 4 independent experiments. (*P<0.05)

C. THP-1 cells were transfected with control small interfering RNA (siRNA) or caveolin-1 siRNA, and then treated with vehicle or phorbol myristate acetate (PMA) (100 nmol/L) for 72 hours. C, Peritoneal macrophages were isolated from wild-type (WT) and caveolin-1 KO (KO) mice. C and D, acetylated low-density lipoprotein (10 µg/mL) was added to treated cells for 24 hours. Graphs are group data of DiI fluorescence intensities, and the intensities of PMA–treated THP-1 cells with control siRNA (C) and WT macrophages (D) are set as 100%. (*P<0.05; n=3) E and F, LDL– (2 nmol/L) activated mouse peritoneal macrophages were treated with or without LDL (50 µg/mL) for 24 hours. Lipids content of treated cells were extracted and determined for cholesteryl esters (CEs) (E) and triglycerides (TGs) (F). Graphs show the amount of all CEs and top 10 abundant TGs in treated cells. (*P<0.05; n=3). KO indicates knockout.
cells as a percentage of all peritoneal cells in caveolin-1 KO mice compared with WT (WT 55% versus KO 39%). However, there is no difference in the number of F4/80+/CD11b+ cells between WT and KO mice. When mice were administered 2.9% thioglycollate, the quantity of macrophages, including CD115+/CD11b+ (from 55%–69%) and F4/80+/CD11b+ (from 27%–66%) cells in the peritoneal cavity, was increased in WT mice. However, no significant elevation of these cells was found in KO animals (Figure 5A) demonstrating that reduced monocyte to macrophage differentiation in the peritoneal cavity occurs in vivo in mice lacking caveolin-1 in response to inflammatory stimulation, implying that KO of caveolin-1 delays or inhibits differentiation. As peritoneal macrophages are derived from monocytes in the blood and bone marrow, we measured the levels of CD11b+ and ly6C+ cells (monocytes) from both these sources. Consistent with the low conversion of monocytes to macrophages observed in the peritoneal cavity, we observed higher levels of blood monocytes in KO mice than WT (WT 11% versus KO 19%). In addition, thioglycollate injection, which had no influence on the differentiation of monocytes to macrophages in KO animals, did not influence blood monocyte levels (WT 12% versus KO 19%; Figure VA in the online-only Data Supplement). WT mice were observed to have higher levels of bone marrow–derived monocytes than KO animals (WT 54% versus KO 48%). This may be because of the demand to replenish peripheral monocytes in these animals. After thioglycollate injection, a fall in bone marrow monocytes was observed possibly because of immediate mobilization of these cells into the periphery (WT 48% versus KO 57%; Figure VB in the online-only Data Supplement).

To validate the influence of caveolin-1 on ERK phosphorylation and EGR-1 nuclear translocation in vivo, we showed using Western blot that peritoneal cells from KO mice displayed reduced ERK phosphorylation and nuclear location of EGR-1 compared with WT cells (Figure 5B). Moreover, chromatin immunoprecipitation analysis also showed that caveolin-1 KO decreases the EGR-1 binding to its target site on CD115 promoter in peritoneal cells (Figure 5C). These in vivo experiments are consistent with our in vitro finding that caveolin-1 plays a critical role in monocyte to macrophage differentiation.

Figure 5. Caveolin-1 knockout (KO) mice display lower monocyte to macrophage differentiation. A, Peritoneal cells were isolated from wild-type (WT) and caveolin-1 KO mice with or without thioglycollate injection. PE-CD115 or PE-F4/80 antibodies were applied with APC-CD11b antibodies to detect the isolated cells by flow cytometry. The dots gated by square frames in representative flow analysis are macrophages (CD11b and F4/80). The bar graphs group the percentage of gated cells (*P<0.05; n=8–9). B and C, Peritoneal cells were isolated from injected mice (WT and KO). B, Proteins in nuclear fraction (NP) and whole cell lysate (WCL) were detected by Western blot. Bar graphs display the ratios of EGR-1 in NP to WCL fraction and phosphorylated ERK to total ERK, respectively (*P<0.05; n=6). C, Graph shows the relative quantitative ratio of EGR-1 binding sequence on CD115 promoter in precipitated group to input group, and anti-EGR-1 antibody precipitated group in KO mice is set as 1. Rabbit IgG was applied to exclude the nonspecific antibody binding (*P<0.05; n=6). pERK indicates phosphorylated ERK; t-ERK, total ERK.
Monocyte to macrophage differentiation is a pivotal step in the pathogenesis of atherosclerosis, culminating in the formation of foam cells in atherosclerotic lesions. In this study, we show for the first time that caveolin-1 plays a critical role in the ability of monocytes to differentiate into macrophages through phosphorylation of ERK and EGR-1 nuclear translocation in vitro and in vivo. Consistent with this, we also show that caveolin-1 is critical for normal macrophage function, inhibiting cellular transmigration but promoting phagocytosis and that KO or knockdown of caveolin-1 decreases modified LDL uptake and lipid content, consistent with the inhibition of foam cell formation. These findings suggest that monocytes caveolin-1 may be a novel therapeutic target for atherosclerosis.

THP-1 monocytes are widely used as a cell model to study monocyte differentiation into macrophages. Distinct from other monocytic cell lines, increased caveolin-1 was found in PMA-treated, differentiated THP-1 cells compared with undifferentiated ones, a finding validated in human and mouse primary monocytes in the current study. Because a high correlation (0.78 or 0.84 correlation coefficient) has been reported to exist between THP-1 and primary monocytes, or between PMA-induced THP-1 macrophages and human monocyte-derived macrophages, we have continued to use THP-1 cells as a representative of the primary cells in the majority of our in vitro experiments, validating key findings in primary cells (both human and mouse).

The differentiation of monocytes into macrophages is dependent on a developmentally ordered pattern of gene expression regulated by several transcription factors, such as PU.1, C/EBPα, AML1, EGR-1, MaFB, and IRF-8/ICSBP. Through gain-in-function or knockdown strategies, EGR-1 is activated to increase CD115 expression on monocytes, promoting M-CSF signaling. In order to exert their effect, transcription factors need to translocate into nucleus after synthesis in the endoplasmic reticulum. It is known that EGR-1 nuclear translocation is dependent on ERK activation. In the current study, we show that knockdown or KO of caveolin-1 inhibits ERK phosphorylation, thus, impairing EGR-1 nuclear translocation and reducing its transcriptional activity. Caveolin-1 may also affect integrin signaling and other transcriptional factors, which are also related to monocyte differentiation to macrophage. For example, the β2-integrin family of receptors has been reported to be involved in monocyte to macrophage differentiation. Because caveolin-1 has been demonstrated to regulate β1-integrin signaling, we can speculate that caveolin-1 also modulates β2-integrins to influence differentiation. Also, as the transcription factor IRF-8/ICSBP has been identified as a regulator of macrophage maturation and caveolin-1 has been reported to influence its activation through the Janus kinase/signal transducers and activators of transcription signal pathway, this may be another means by which caveolin-1 influences differentiation.

Monocyte-derived macrophages are a key component of foam cell formation, a major cellular fraction in atherosclerotic lesions where LDL is taken up by macrophages which accumulate intracellular lipids. We report that knockdown or KO of caveolin-1 attenuated phagocytosis and acetylated LDL uptake and that LDL-induced lipid accumulation was limited in caveolin-1 KO cells compared with wild-type macrophages. We also show that phagocytosis by macrophages was reduced by caveolin-1 knockdown. These findings are consistent with the premise that caveolin-1 is involved in monocyte to macrophage differentiation and the functional effects of macrophages. CD36 and scavenger receptor class B type I (SR-BI), the 2 key molecules involved in LDL uptake, are colocalized with caveolin-1 together on the cell surface, and caveolin-1 deficiency can disrupt the plasma membrane location and dysfunction CD36 or SR-BI-mediated lipid endocytosis. As shown in Figure 2A–2B, CD36 was observed to increase after PMA-induced differentiation and attenuated by knockdown of caveolin-1 during differentiation implying that CD36 may be involved in the differentiation–increased modified LDL endocytosis (Figure 4C; and Figure IVA in the online-only Data Supplement). Cholesterol and fatty acid in lipoproteins are taken up by macrophages and then metabolized into cholesteryl esters and triglycerides stored in cells. Once loaded, macrophages turn into foam cells. Thus, intracellular cholesteryl esters and triglycerides can be used as markers of foam cell formation. In the current study, we scanned the global profile of free cholesterol, cholesteryl esters, and triglycerides amounts (Table III in the online-only Data Supplement) in LDL-treated cells and found that KO of caveolin-1 reduces the accumulation of cholesteryl ester and triglycerides in cells. However, there was no significant effect on free cholesterol, a major membrane constituent, after LDL loading in both WT and KO cells (data not shown). These findings suggest that caveolin-1 is associated with foam cell formation and that inhibiting monocyte/macrophage caveolin-1 is likely to decrease foam cell formation, potentially diminishing atherosclerotic lesion formation.

To demonstrate that the in vitro findings are physiologically relevant, we performed a series of experiments using the caveolin-1 KO mouse. Here, we report that monocyte to macrophage differentiation was attenuated in caveolin-1 KO mice. The process of monocyte to macrophage differentiation originates from progenitor monocytes in bone marrow to blood monocytes differentiating into macrophages in peripheral tissues (for example in the peritoneal cavity). Under normal conditions, no significant difference was observed on the quantity of peritoneal mature macrophages between WT and KO mice. CD11b+/F4/80+ cells between WT and KO, CD115, a marker of mononuclear phagocytes system and which is regulated at early stages of differentiation, however, was significantly reduced in KO mice. When thioglycollate, which induces peritonitis, was introduced, peritoneal macrophages were observed to increase, as expected, in WT mice. In contrast, this increase was not observed in KO mice, substantiating our in vitro observations that caveolin-1 is involved in monocyte to macrophage differentiation. Concurrently, we also observed that monocyte levels were higher in peripheral blood from KO mice compared with WT, consistent with the low conversion of monocytes to macrophages in these animals. These levels were not affected by thioglycollate, which as described above had no effect on monocyte to macrophage conversion in these animals.
animals. This supposition is substantiated by the finding that the percentage ratio of peritoneal macrophages to blood monocytes is 2.46 (27%/11%) in WT compared with 1.58 (30%/19%) in KO mice.

Also, WT mice were observed to have higher levels of bone marrow–derived monocytes than KO animals, which may be because of the higher demand to replenish peripheral monocytes in these animals. After thioglycollate injection, a fall in bone marrow monocytes in the WT mice was observed possibly because of immediate mobilization of these cells into the periphery. Moreover, hematopoietic caveolin-1 has been demonstrated to be involved in immune modulation, as KO of caveolin-1 promotes regulatory T lymphocyte generation decreasing atherosclerosis in apoE mice. As human CD4+ CD25+ regulatory T cells can exert suppressive effects on monocytes or macrophages, these T cells may be involved in the effects of caveolin-1 on monocyte differentiation.

The implications of the current findings are pronounced. Caveolin-1 is expressed constitutively in various cells, including endothelial cells, smooth muscle cells, and monocytes/macrophages, all of which are involved in atherogenesis. The roles of caveolin-1 in each of these cell types may be different. Caveolin-1 in the endothelium mediates lipid uptake and inhibits NO phosphorylation, thus, exerting a proatherosclerotic influence. Caveolin-1–deficient aortic smooth muscle cells display an increase in cell proliferation, migration, and endothelin–induced signal transduction implying that in smooth muscle cells caveolin-1 has an antiatherosclerotic function.

The current study demonstrates that caveolin-1 mediates monocyte differentiation into macrophage while also influencing macrophage endocytosis of lipids, both essential steps for the formation of macrophage–derived foam cells. Interestingly, this is reflected in caveolin-1 and apoE double KO mice, which display a proatherogenic lipid protein profile, despite the fact that atherosclerotic lesions are smaller when compared with apoE KO mice. Specific cellular down-regulation of caveolin-1, therefore, appears to be a potential way forward toward future therapeutic benefits.

In conclusion, we report for the first time that caveolin-1 is a critical regulator of monocyte differentiation into macrophage and as such that targeted inhibition of monocyctic caveolin-1 may impair differentiation and hence reduce atherosclerosis.

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Disclosures

None.

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Materials and Methods

Cell Isolation and Culture

THP-1 monocytic cell line was purchased from ATCC (Mannassas, VA). Peripheral blood mononuclear cells (PBMCs) were isolated from human or mouse blood through gradient centrifugation using the Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ). Human primary monocytes were then purified from PBMCs using the MACS human monocyte isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of isolated monocytes was more than 85%, validated through expression of CD14 by flow cytometry. Mouse peritoneal macrophages were isolated from thioglycollate-injected mice. All these cells were cultured in RPMI medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (In Vitro Technologies, Noble Park, VIC, Australia). Human M-CSF (50ng/ml, R&D Systems, Minneapolis, MN) and mouse GM-CSF (50ng/ml, R&D Systems, Minneapolis, MN) were respectively used to induce differentiation of human or mouse monocyte to macrophage. EA hy 926 cell line (ATCC, Mannassas, VA) was cultured in DMEM medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum.

Extraction of protein from whole cell and nuclear fraction

Whole cells were lysed in whole cell lysis buffer consisting of 10mM of Tris (pH=7.4), 0.1M of NaCl, 1mM of EDTA, 1mM of EGTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X100, 10% glycerol and proteinase inhibitors (1 tablet in 10ml, Roche Applied Science, Indianapolis, IN). Following 3-second sonication at 14% intensity and 30-minute incubation on ice, the whole cell lysates were
centrifuged at 12,000g for 20 minutes at 4°C and the supernatant was collected as whole cell protein samples for further analysis. Nucleus fraction extract was performed as described in a previous study. Briefly, cells were collected and lysed in membrane disruption buffer including 0.4% NP-40, 10mM of HEPES (pH=7.9), 10mM of KCl, 0.1mM of EDTA and proteinase inhibitors (1 tablet in 10ml). After centrifugation at 15,000g for 5 min at 4°C, the pellet was kept and dissolved in nuclear lysis buffer containing 20mM of HEPES (pH=7.9), 0.4M of NaCl, 1mM of EDTA, 10% Glycerol and proteinase inhibitors (1 tablet in 10ml). Following 30-minute incubation on ice, the dissolved pellet suspension was centrifuged at 15,000g for 15 min, the supernatant collected and served as nuclear fraction protein for subsequent analysis. The protein concentration was measured by Dc Protein Assay (Bio-Rad Laboratories, Hercules, CA).

**Western Blot**

Protein samples were separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Billerica, MA). The membrane was blocked using 5% fat-free milk and respectively incubated with various primary antibodies (1:1000), according to the experimental purpose, including rabbit anti-caveolin-1, β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), pERK, ERK and EGR-1 (Cell Signaling Technology, Danvers, MA) and mouse anti-GAPDH (ABCAM, Cambridge, UK), followed with horseradish peroxidase-conjugated anti-rabbit or -mouse IgG secondary antibodies (1:4000, Bio-Rad Laboratories, Hercules, CA). The protein bands were visualized by use of the enhanced luminal reagents (PerkinElmer, Waltham, MA), and quantified using NIH FIJI software.
Flow cytometry analysis

The purity of isolated human primary monocytes was detected using APC-conjugated anti-human CD14 antibodies (eBioscience, San Diego, CA). Identification of human macrophages differentiated from monocytes was carried out by using FITC-conjugated anti-human CD68 (AbD Serotec, Oxford, UK). Mouse bone marrow monocyte progenitors or monocytes were identified by using PE-conjugated anti-mouse ly6C (eBioscience, San Diego, CA) and mouse peritoneal macrophages by co-labelling of PE-conjugated anti-mouse CD115 (eBioscience, San Diego, CA) and APC-conjugated anti-mouse CD11b (BD Biosciences, San Diego, CA) or PE-conjugated anti-mouse F4/80 (eBioscience, San Diego, CA) and APC-conjugated anti-mouse CD11b. Collected cells (1×10^5 per tube) were respectively incubated, according to experimental purpose, with aforementioned antibodies or their respective isotype controls for 30 minutes on ice. After washing out of the non-binding antibody, labelled cells were examined by FACS on BD FACS Calibur (BD Biosciences, San Diego, CA). For analysis, 10,000 cell counts per tube were collected and analyzed using Cell Quest (BD Biosciences, San Diego, CA) or FlowJo (Tree Star Inc., Ashland, Or).

Construction of caveolin-1 siRNA plasmids

Caveolin-1 siRNA plasmid, pcDNA6.2-GW/EmGFP-miR, was constructed with the use of the BLOCK-iT Pol II miR RNAi Expression Vector Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Briefly, caveolin-1 siRNA oligos (supplemental table I), designed and synthesized by Invitrogen, were ligated into a
vector provided by the kit through T4 DNA ligase to form pcDNA6.2-GW/EmGFP-miR. A control plasmid containing non-specific siRNA oligo, pcDNA6.2-GW/EmGFP-miR-neg, was provided by the kit and applied as a negative control in transfection study. The effect of transfection was validated by measurement of caveolin-1 expression through Western blot.

**Transfection**

Transfection was performed using the Fugene Transfection reagent (Promega Corporation, Madison, WI). THP-1 cells (10^6 per well) were seeded on a 6-well plate. For each well to be transfected, a transfection mixture was made up by mixing of 1.7µg of plasmid DNA, 155µl of optiMEM (Invitrogen, Carlsbad, CA) and then 10µl of Fugene reagent. Following 30-minute incubation, the mixture was added into a well follow by 4-hr incubation at 37°C in cell culture incubator. Then, the culture medium was refreshed and cells were ready for subsequent experiments.

**Extraction of RNA and Real-time PCR**

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA). After RNase-Free DNase (Promega, madison, WI) treatment, 2 µg of total RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Promega, madison, WI). Real-time PCR was performed on 100 ng cDNA using FastStart Universal SYBR Green Master (Roche Applied Science, Indianapolis, IN) on an ABI Prism 7500 Sequence Detection System to determine mRNA levels of the target genes. Primers were designed using Roche’s ProbeFinder software and ordered from Geneworks (sequences are listed in supplemental table II).
ChIP assay
ChIP assay was performed using the Agarose ChIP Kit (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer’s instructions. Briefly, cells were fixed and crosslinked before collection. Collected cells were lysed and digested into protein-DNA fragment complexes by MNase reaction. Rabbit EGR-1 antibody or Rabbit IgG (Cell Signaling Technology, Danvers, MA) was applied to immunoprecipitate protein-DNA complexes. Finally, DNA fragments were enriched and purified from precipitated protein-DNA complexes. EGR-1 binding area on its target gene promoter was amplified by designed primers (supplemental table II) on the Applied Biosystems 7500 Fast Real-Time PCR System.

Transmigration assay
Migration assays were performed using 8.0µm transwells (BD Biosciences, San Diego, CA) as previously described with minor modifications. Briefly, EA hy926 cells were grown to confluence on the upper chamber over night. THP-1 cells (10^4 per chamber) with different conditions were seeded on the upper chamber and co-incubated with EA hy926 cells and MCP-1 (100ng/ml) was added to the lower chamber. In 5 hours, cells migrating to the lower chamber were collected and quantified using the CyQUANT GR dye (Invitrogen, Carlsbad, CA). Fluorescence intensity, corresponding to cell number, was determined using a microplate reader (PerkinElmer, Waltham, MA) with excitation at 490 nm and detection at 520 nm.

Phagocytosis assay
Phagocytosis of THP-1 cells was measured by using a Phagocytosis Assay Kit following the manufacturer’s instructions (Cayman Chemical, Ann Arbor, MI).
Briefly, latex beads-rabbit IgG-FITC complex was added and incubated with cells for 4 hours. Florescent cells (phagocytised beads) were then determined by flow cytometry on the BD FACS Calibur (BD Biosciences, San Diego, CA).

**Measurement of the uptake of modified-LDL**

Dil-ac-LDL (Molecular Probes, Grand Island, NY) was used to measure LDL-uptaking capability of cells. Dil-ac-LDL (10µg/ml) was added to cells and incubated for 24 hr. Cells were washed and imaged under a fluorescence microscopy (Nikon ECLIPSE TE300). Intracellular ac-LDL was quantified by NIH FIJI software.

**Cellular lipid extraction and determination**

PMA (2nM) activated-mouse peritoneal macrophages were treated with vehicle or LDL (50µg/ml, Sigma-Aldrich, St Louis, MO) for 24 hours\(^3\), and lysed in whole cell lysis buffer. 20µl of protein sample was applied to lipids extraction as previously described\(^4\). The extracted lipids were measured for free cholesterol, cholesteryl ester and triglyceride by mass spectrometry, and their respective levels were normalized again input protein levels.

**Animal experiments**

Animals were bred and housed under standard conditions at the AMREP Animal Service Centre. Experimental procedures were approved by an institutional Animal Ethics Committee in accordance with the NIH guidelines. We used 8-10 week old male wild-type and caveolin-1 KO mice 10 times backcrossed in a pure C57BL6 genetic background. These mice were developed after backcrossing caveolin-1 KO mice originated\(^5\), originally backcrossed for four times with C57BL6 wild-type mice.
in T. Kurzchalia’s laboratory, for another additional 6 times with C57BL6 wild-type mice. To harvest peritoneal macrophage, 2.9% thioglycollate (BD Biosciences, San Diego, CA) was injected intraperitoneally (2ml/mouse). After 4 days, mice were euthanized by CO₂ and blood collected by cardiac puncture for isolation of PBMCs. Bone marrow cells were isolated from the femur and tibiae, and peritoneal cells extracted through intraperitoneal perfusion, as previously described⁶⁻⁸. Isolated cells were examined through flow cytometry, western blot and ChIP assay. Moreover, cells from the peritoneal cavity were seeded into culture plates. Medium was refreshed after 2 hours to remove non-adherent cells, and remaining adherent cells (macrophages) were using for further analysis.

**Statistical Analysis**

All data, from at least three independent experiments, are presented as the mean±SEM, with P<0.05 being considered as statistically significance. Two-group comparisons were analysed for statistical significance using two-tailed t-test. More than two groups were assessed by one-way ANOVA followed by Tukey post test, if P<0.05. All the statistical analyses were applied on GraphPad Prism 5.0 software (GraphPad Software Inc. La Jolla, CA).

**References:**


3. Kruth HS, Huang W, Ishii I, Zhang W-Y. Macrophage foam cell formation with


**Supplemental Figure Legends**

**Figure 1.** THP-1 cells were treated with PMA at increasing concentrations for 72 hours (A) or with PMA (100nM) for increasing incubation periods (B). Caveolin-1 and β-tubulin were detected by western blot analysis. Bar Graphs show the band density ratios of caveolin-1 to β-tubulin. Data are from 3 independent experiments. (*P<0.05 vs control group) (C) The protein levels of CD68 were detected by FITC-conjugated antibody through flow cytometry (blue curve: 100nM of PMA treatment for 72 hours; green curve: untreated THP-1 cells). FITC-isotype antibody (red curve) was used as the negative control. Bar graph demonstrates the group data of FITC geometric mean from 3 independent experiments. (*P<0.05) (D) Human primary monocytes were treated with human M-CSF (50ng/ml) for 7 days. The gene expression of CD68 was measured through real-time PCR in human primary monocytes. Graph shows the relative quantitative ratios of CD68 to 18s, and control group is set as 1. Data are from 3 independent experiments. (*P<0.05) (E) Mouse PBMCs were treated with mouse
GM-CSF (50ng/ml) for 7 days. The protein levels of ly6C, CD115 and F4/80 on CD11b+ PBMCs were detected by flow cytometry (red curve: control group; green curve: GM-CSF group). Bar graph is the group data of PE geo-means from 3 independent experiments. The fluorescence geo-means in control group are set as 100%. (* P<0.05 vs control group)

**Figure II. (A)** THP-1 cells were transfected with pcDNA3 (ctrl) or pcDNA3-caveolin-1 (CAV) plasmids. Caveolin-1 and β-tubulin were detected by western blot in transfected cells. **(B)** The transfected cells were treated with vehicle or PMA (100nM) for 72 hours. Then gene expression levels were measured by real-time PCR. Graphs show the relative quantitative ratios of target genes to 18s, and control groups are set as 1. Data are from 3 independent experiments. (* P<0.05 CAV vs control group; # P<0.05 PMA vs control group) **(C-D)** THP-1 cells were transfected with pcDNA6.2-GW/EmGFP-control-miR (ctrl) or pcDNA6.2-GW/EmGFP-caveolin-1-miR (CAV) respectively. Transfected cells were then treated with vehicle or PMA (100nM) for 72 hours. **(C)** Caveolin-1 and β-tubulin were detected by western blot analysis. Bar graph shows the band density ratio of caveolin-1 to β-tubulin. Data are from 3 independent experiments. (* P<0.05) **(D)** The gene expression levels were measured by real-time PCR under different treatments. Graphs show the relative quantitative ratios of target genes to 18s, and control groups are set as 1. Data are from 3-6 independent experiments. (* P<0.05)

**Figure III.** THP-1 cells were pretreated with 10μM of U0126, a MEK1/2 inhibitor, or vehicle for 1 hour, and then stimulated by PMA for 72 hours. Proteins in nuclear fraction (NP) and whole cell lysate (WCL) were detected by western blot. Bar graphs
show the ratios of EGR-1 in NP to WLC, phosphorylated ERK to total ERK, and EGR-1 or caveolin-1 to β-tubulin in WCL, respectively. (* P<0.05, n=4)

**Figure IV.** (A) THP-1 cells were transfected with control siRNA or caveolin-1 siRNA, and then treated with vehicle or PMA (100nM) for 72 hours. (B) Peritoneal macrophages were isolated from wild type (WT) and caveolin-1 KO (KO) mice. (A-B) DiI-ac-LDL (10µg/ml) was added to treated cells for 24 hours. Images display the cells taking up DiI-ac-LDL (red).

**Figure V.** PBMCs (A) and bone marrow cells (B) were isolated from wild type (WT) and caveolin-1 knockout (KO) mice with or without thioglycollate injection. PE-ly6C antibodies were applied with APC-CD11b antibodies to detect the isolated cells by flow cytometry. The dots gated by square frames in representative flow analysis are monocytes. The bar graphs group the percentage of gated cells. (* P<0.05, A. n=3; B. n=5-6)
**Table I:** Sequences of siRNAs and oligos. All of these sequences were designed and synthesized by Invitrogen.

**Control siRNA:**
Silencer Select Negative Control #1 siRNA (Invitrogen Product)

**Caveolin-1 siRNA:**
CCUUCACUGUGACGAAAUAtt
UAUUUCGUCAGGAATTg

**Caveolin-1 miR oligo:**
Bottom:
CCTGAATATTTCGTCAGTGAAGGGTCAGTCAGTGCCAAAACCACCTTCACCTGACGAAAATAC
Top:
TGCTGTATTTTCGTCACAGTGAAGGGTTTTGGCCACTGACTGACCCTTCACCTGACGAAAATATT

**Table II:** Sequences of primers

Primers were designed by Universal ProbeLibrary System ([https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=uplct_030000](https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=uplct_030000)) and synthesized by Geneworks (Hindmarsh, SA, Australia).

<table>
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<tr>
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<th>Name</th>
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**Table III:** The profile of lipids including cholesterol esters, free cholesterol, and triglycerides in mouse peritoneal macrophage from wild type and caveolin-1 knockout mice with or without 24-hour LDL treatment.

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<tr>
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<th>WT+LDL</th>
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Figure I

A

Caveolin-1

β-tubulin

<table>
<thead>
<tr>
<th>Ctrl</th>
<th>24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
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B

Caveolin-1

β-tubulin

<table>
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<tr>
<th>Ctrl</th>
<th>24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
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C

Cell Count (% of Max)

FITC Geo-mean (CD68)

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<tr>
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<tbody>
<tr>
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</table>
Figure III

EGR-1
β-tubulin
Caveolin-1

p-ERK
t-ERK

PMA:
U0126:

NP
WCL

0.0
0.5
1.0
1.5

EGR-1 NP / WCL

U0126: - - - +
PMA: - + + +

0
1
2
3

EGR-1 / β-tubulin (WCL)

U0126: - - - +
PMA: - + + +

0.0
0.5
1.0
1.5

p-ERK / t-ERK

U0126: - - - +
PMA: - + + +

0
1
2
3
4
5

Caveolin-1 / β-tubulin

U0126: - - + +
PMA: - + + +
Figure IV

A

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<td>PMA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMA</td>
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</table>

Control siRNA

CAV-1 siRNA

30 µm

B

<table>
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<th>Neg-control</th>
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<tbody>
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</tbody>
</table>

WT

CAV-1 KO

15 µm
Figure V

A

Control

Thioglycollate

WT

KO

WT

KO

Percentage of CD11b+ and ly6c+ cells (%)

Control

Thioglycollate

WT

KO

WT

KO

B

Control

Thioglycollate

WT

KO

WT

KO

Percentage of CD11b+ and ly6c+ cells (%)

Control

Thioglycollate