Deficiency of ROCK1 in bone marrow-derived cells protects against atherosclerosis in LDLR$^{-/-}$ mice

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ABSTRACT **Rho kinases (ROCKs) are serine-threonine protein kinases that regulate the actin cytoskeleton. Recent studies suggest that ROCKs also play an important role in cardiovascular disease. However, the isoform- and tissue-specific role of ROCKs in mediating this process is unknown. Using homologous recombination, we generated mutant mice harboring alleles with homozygous deletion of ROCK1 (ROCK1$^{-/-}$). Most ROCK1$^{-/-}$ mice die perinatally. However, a few ROCK1$^{-/-}$ mice survive to adulthood, are phenotypically normal, and have no apparent compensatory changes in ROCK2. Using these ROCK1$^{-/-}$ mice, we show that ROCK1 in bone marrow-derived macrophages is critical to the development of atherosclerosis, in part, by mediating foam cell formation and macrophage chemotaxis. Lipid accumulation and atherosclerotic lesions were reduced in atherosclerosis-prone LDLR$^{-/-}$ mice, whose bone marrows have been replaced with bone marrows derived from ROCK1$^{-/-}$ mice. Bone marrow-derived ROCK1-deficient macrophages exhibited impaired chemotaxis to monocyte chemotactic protein-1 and showed reduced ability to take up lipids and to develop into foam cells when exposed to modified low-density lipoprotein. These findings indicate that ROCK1 in bone marrow-derived cells is a critical mediator of atherogenesis and suggest that macrophage ROCK1 may be an important therapeutic target for vascular inflammation and atherosclerosis.—Wang, H.-W., Liu, P.-Y., Oyama, N., Rikitake, Y., Kitamoto, S., Gitlin, J., Liao, J. K., Boisvert, W. A. Deficiency of ROCK1 in bone marrow-derived cells protects against atherosclerosis in LDLR$^{-/-}$ mice. *FASEB J.* 22, 3561–3570 (2008)**

Key Words: Rho kinase · vascular · foam cell · cell migration

Atherosclerosis is a chronic inflammatory disorder of the vascular wall, and circulating immune cells, such as monocytes, lymphocytes, and platelets, contribute to the disease process (1, 2). In particular, infiltration of monocytes and their subsequent transformation into macrophage-derived foam cells is one of the most important features of atherosclerosis. Although the initial recruitment of monocytes to the vasculature is thought to be beneficial in removing potentially harmful lipid particles, progressive accumulation of these inflammatory cells ultimately leads to atherosclerotic lesion progression. Indeed, macrophages secrete cytokines and chemokines, which have been shown to mediate the disease process (3). Recent findings suggest that only particular subsets of monocytes, based on the surface expression of several different chemokine receptors, cause hypercholesterolemia-associated monocytosis by adhering to activated endothelium, infiltrating into the lesions, and becoming lesional macrophages (4).

The Rho kinase (ROCK) isoforms, ROCK1 and ROCK2, were initially discovered as downstream targets of the small GTP-binding protein Rho. ROCKs are serine/threonine protein kinases that phosphorylate many mediators that regulate the actin cytoskeleton (5, 6). Because ROCKs are critical for cellular function, embryonic and perinatal lethality occurs in both ROCK1$^{-/-}$ and ROCK2$^{-/-}$ mice (7, 8). ROCK1$^{-/-}$ mice develop large omphalocoeles caused by a defect in umbilical ring closure from impairment of filamentous actin accumulation (7). In contrast, ROCK2$^{-/-}$ mice die from dysfunction and intrauterine growth retardation caused by thrombus formation in the labyrinth layer of the placenta (8). These findings suggest distinct tissue distribution and downstream targets of ROCK1 and ROCK2. For example, ROCK1 is required for microfilament bundle and focal adhesion assembly, ROCK2 plays a necessary role in the establishment of a mature fibronectin fibrillar matrix (9).

There is increasing evidence that the RhoA/ROCK pathway plays an important pathophysiological role in cardiovascular diseases (10), such as hypertension (11, 12), myocardial hypertrophy (13), cerebral and coronary vasospasm (14), cerebral ischemia (15, 16), pulmonary hypertension (17), neointima formation (18), and atherosclerosis (19). Most of these studies used one of two well-known ROCK inhibitors, Fasudil or Y-27632. Because both of these inhibitors can inhibit the ATP-dependent kinase domain, which is highly homologous between ROCK1 and ROCK2, neither ROCK inhibitors

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Y-27632 nor Fasudil can distinguish between cellular processes mediated by ROCK1 and ROCK2. Furthermore, when given *in vivo* for prolonged periods and at higher concentrations, these ROCK inhibitors could also inhibit other serine-threonine kinases such as PKA and PKC (20, 21). Therefore, the purpose of this study was to determine the role of ROCK1 in development of atherosclerosis. Although our studies clearly show that ROCK1 in bone marrow derived cells plays a role in atherosclerosis, it is not known whether ROCK2 has a similar role in the disease process.

MATERIALS AND METHODS

Animals

ROCK$^{-/-}$ mice were obtained as described previously (22). LDLR$^{-/-}$ mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). All mice were on the C57BL/6 background. Twenty-four 6-wk-old female LDLR$^{-/-}$ mice were subjected to 1000 rad of total body irradiation, and received 2×10^6 marrow cells from ROCK1$^{+/+}$ or ROCK 1$^{-/-}$ mice *via* intravenous (i.v.) injection. After bone marrow transplantation (BMT), the mice were placed on a chow diet for 4 wk, after which an atherogenic diet containing 15.8% (wt/wt) fat and 1.25% cholesterol (no cholate, diet 94059; Harlan Teklad, Madison, WI, USA) was given for 12 wk. All of the animals survived except one, which was sacrificed at 8 wk post-BMT due to dermatitis. The mice were maintained in the Harvard Medical School animal facilities. The Standing Committee on Animals at Harvard Medical School approved all protocols pertaining to experimentation with animals.

Measurement of Rho kinase activity

Rho kinase activity was performed as described previously (23). Rho kinase activity was expressed as the ratio of phospho-Thr853–MBS in each sample per phosphoThr853–MBS and normalized by positive control.

Quantitation of atherosclerotic lesions in the aorta

Details of the preparation of aortas and aortic valves are as described previously (24). Optimal cutting temperature (OCT) compound-embedded hearts were sectioned in a cryostat until all three leaflets were visible within the aortic valve. The lesions were visualized by staining with Oil Red O followed by counterstaining with hematoxylin. The remaining aorta were cut longitudinally from the aortic root to the iliac bifurcations, pinned on the surface of black and stained with Oil Red O. The total and the atherosclerotic areas of each aorta were measured by NIH Image J. v1.33 (U.S. National Institutes of Health, Bethesda, MD, USA), and the percentage of the atherosclerotic lesion to total area was evaluated.

Plasma cholesterol determination

Blood was obtained at weeks 0, 6, and 12 by retro-orbital puncture after a 6-h fast; plasma cholesterol levels were measured enzymatically using a kit from Invitrogen (Carlsbad, CA, USA).

Macrophage chemotaxis assay

Bone marrow-derived macrophages (0.5×10^6 cells/ml) were placed in the upper wells of Boyden chamber, with the lower wells containing MCP-1 at the indicated concentrations. Following incubation at 37°C for 4 h, cells remaining on the upper surface of the filter were removed mechanically. Migrated cells were counted manually under the microscope (×400). For experiments using the ROCK inhibitor, Fasudil, bone marrow-derived macrophages (1×10^5 cells) were added to a modified Boyden chamber in which the synthetic basement membrane matrix, Matrigel, was added to the filter through which the cells had to invade to migrate to the other side. The bottom chamber contained MCP-1 (30 ng/ml). The migrated cells were collected and counted.

AcLDL internalization by bone marrow-derived macrophages

Following incubation of bone marrow-derived macrophages in serum-free Dulbecco modified Eagle medium (DMEM) for 2 h, fluorescently labeled AcLDL (10 μg/ml, Alexa Fluor 488-AcLDL, Molecular Probes/Invitrogen) was added to the media and incubated for 1 h in DMEM. After washing with ice-cold PBS 3 times, 2% Trypan blue was added to quench the extracellular fluorescence, and antifade mounting solution was added. Cells were examined under a fluorescent microscope and photographed. The fluorescence intensity was analyzed by NIH Image J. ver. 1.33 (NIH, Bethesda, MD, USA).

In vitro foam cell formation

Bone marrow-derived macrophages from ROCK1$^{+/+}$ and ROCK1$^{-/-}$ mice were incubated with 100 μg/ml OxLDL (Intracell, Frederick, MD, USA) in DMEM for 48 h. RAW264.7 cells (American Type Culture Collection, Manassas, VA, USA) treated or not treated with 10 μM Y-27632 (Calbiochem, San Diego, CA, USA) were also incubated with 100 μg/ml OxLDL for 48 h. After incubations, the cells were fixed in 4% paraformaldehyde and then stained with Oil Red O; the cells were counterstained with hematoxylin and mounted.

Phagocytic activity of macrophages

Bone marrow-derived macrophages from ROCK1$^{+/+}$ and ROCK1$^{-/-}$ mice were incubated for 10 min in the presence or absence of 10 μM cytochalasin D. Then bone marrow-derived macrophages were incubated with FITC-labeled yeast particles at 1×10^7 cells/ml at 37°C for 30 min. Nonphagocytosed yeast particles in the suspension were removed with ice-cold PBS washes, and 0.2% Trypan blue was added to quench extracellular fluorescence. Macrophages with phagocytosed yeast particles were examined under a fluorescence microscope.

Immunohistochemistry

Details of the immunohistochemical staining protocol can be found elsewhere (24). Mouse atherosclerotic lesions were stained with polyclonal rat antibody to the mouse monocyte-macrophage marker MOMA-2 (Serotec, Minneapolis, MN, USA), followed by streptavidin-HRP-conjugated Goat-anti-rat antibody (Invitrogen). T-cell infiltration was examined with rabbit anti-CD3 antibody (Sigma-Aldrich, St. Louis, MO, USA) and streptavidin-HRP-conjugated Goat anti-rabbit anti-

body (Invitrogen), and color reaction was developed with 9-amino-3-ethylene-carbazole (AEC, Sigma-Aldrich). In both cases, 3 aortic root sections per mouse were used for the quantitation of cells in the lesions.

Statistical analysis

Results are given as mean ± SE. Statistical analysis was performed by unpaired Student's t test in comparing the difference between two groups. Mann-Whitney U test was used to compare the Oil Red O-stained aortic valve lesion areas. A value of $P < 0.05$ was considered significant.

RESULTS

Characteristics of peripheral blood and bone marrow-derived macrophages in ROCK1 haploinsufficient and deficient mice

We have previously reported that the expression level of ROCK1 in ROCK1$^{+/-}$ heart was approximately half of that in ROCK1$^{+/+}$ heart (22). Here, we examined the ROCK1 expression in bone marrow derived-macrophages from ROCK1$^{+/+}$, ROCK1$^{+/-}$, and ROCK1$^{-/-}$ mice. Haploinsufficient ROCK1$^{+/-}$ mice had ~50% expression of ROCK1, and there was no detectable ROCK1 protein in ROCK1$^{-/-}$ bone marrow-derived macrophages. There were no compensatory changes in ROCK2 (**Fig. 1*A***). Furthermore, ROCK activities in the peripheral white blood cells (WBCs), expressed as a ratio of phosphorylated myosin binding subunit (pMBS)/total (t)MBS, showed that WBCs from ROCK1$^{+/-}$ and ROCK1$^{-/-}$ mice exhibited only 70 and 50% of ROCK activities, respectively, compared to cells from ROCK1$^{+/+}$ mice. (Fig. 1*B*).

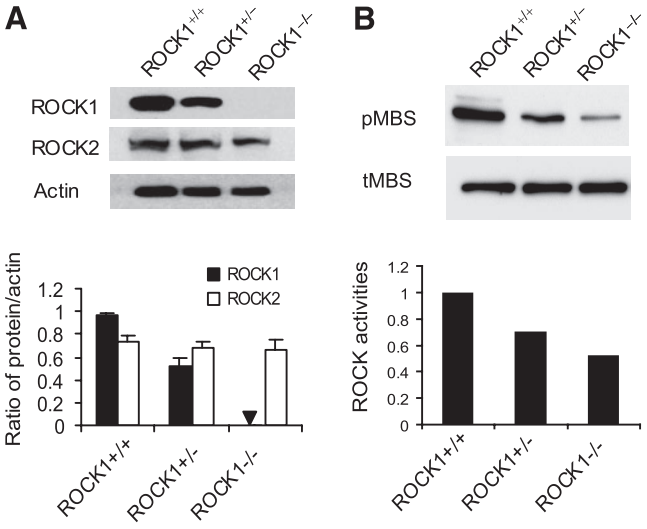

Figure 1. Characteristics of peripheral blood and bone marrow-derived macrophages in haploinsufficient ROCK1 and ROCK1$^{-/-}$ mice. *A*) Compared to ROCK1$^{+/+}$ mice, there was only 52% expression of ROCK1 protein in ROCK1$^{+/-}$ bone marrow-derived macrophages. ROCK1 was not detected (▼) in those macrophages from ROCK1$^{-/-}$ mice, with no compensatory changes in ROCK2 protein expression. *B*) Peripheral blood WBCs phosphorylated myosin binding subunit (pMBS) and total (t)MBS expression, with ROCK activity expressed as ratio of pMBS/tMBS. Compared with WBCs in ROCK1$^{+/+}$ mice, ROCK1$^{+/-}$ mice expressed 70% and ROCK1$^{-/-}$ mice expressed 50% of ROCK activities.

Analysis of peripheral blood in LDLR$^{-/-}$ mice after bone marrow transplantation

To determine whether ROCK1 expression in macrophages and other bone marrow-derived cells could influence atherogenesis, we performed wild-type and ROCK1$^{-/-}$ BMT into irradiated LDLR$^{-/-}$ mice. There were no discernible differences in total WBCs and differential counts between ROCK1$^{+/+}$ and ROCK1$^{-/-}$ BMT mice (**Fig. 2*A*, *B***). Western blot analysis of ROCK1 levels in macrophages from ROCK1$^{-/-}$ → LDLR$^{-/-}$ BMT mice revealed that there was no detectable ROCK1 and no compensatory changes in ROCK2 compared to that of macrophages from wild-type BMT mice (Fig. 2*C*). Plasma cholesterol levels following an atherogenic diet were similar between the wild-type → LDLR$^{-/-}$ and ROCK1$^{-/-}$ → LDLR$^{-/-}$BMT mice (Fig. 2*D*), indicating that ROCK1 expression in bone marrow-derived cells did not affect plasma cholesterol levels and that any differences in atherosclerosis between these mice were not due to differences in plasma cholesterol levels.

Atherosclerosis in LDLR$^{-/-}$ mice with bone marrow-specific ROCK1 deficiency

Atherosclerotic lesion area on the entire aortic surface was determined by *en face* staining and quantification. Most lesions were localized to the ascending aorta and aortic arch, with some additional lesions near small artery branching areas and the iliac bifurcation (**Fig. 3*A***). The percentage of the atherosclerotic lesion to the entire aorta in ROCK1$^{-/-}$ BMT mice was substantially smaller compared with that of wild-type BMT mice (2.58 ± 0.47 *vs.* $5.17\pm0.67\%$; $n=11$–12; $P<0.01$). Quantification of aortic root lesion areas using Oil Red O-stained cryosections showed that LDLR$^{-/-}$ mice receiving wild-type bone marrow developed much larger atherosclerotic lesions than those receiving ROCK1$^{-/-}$ bone marrow ($448.5\pm33.3\times10^3$ μm^2 *vs.* $181.5\pm15.6\times10^3$ μm^2; $n=11$–12; $P<0.05$) (Fig. 3*B*).

Quantification of macrophage accumulation in the atherosclerotic lesions

To evaluate whether the ROCK1 deficiency affects macrophage accumulation in atherosclerotic lesions, we performed immunohistochemical staining of the aortic root tissue sections with an antibody that recognizes murine macrophages, MOMA-2. The MOMA-2-positive areas were analyzed with NIH Image J. There were more MOMA-2-positive cells in the aortic lesions in LDLR$^{-/-}$ mice receiving wild-type bone marrow cells (**Fig. 4*A***) compared to those receiving ROCK1$^{-/-}$ bone

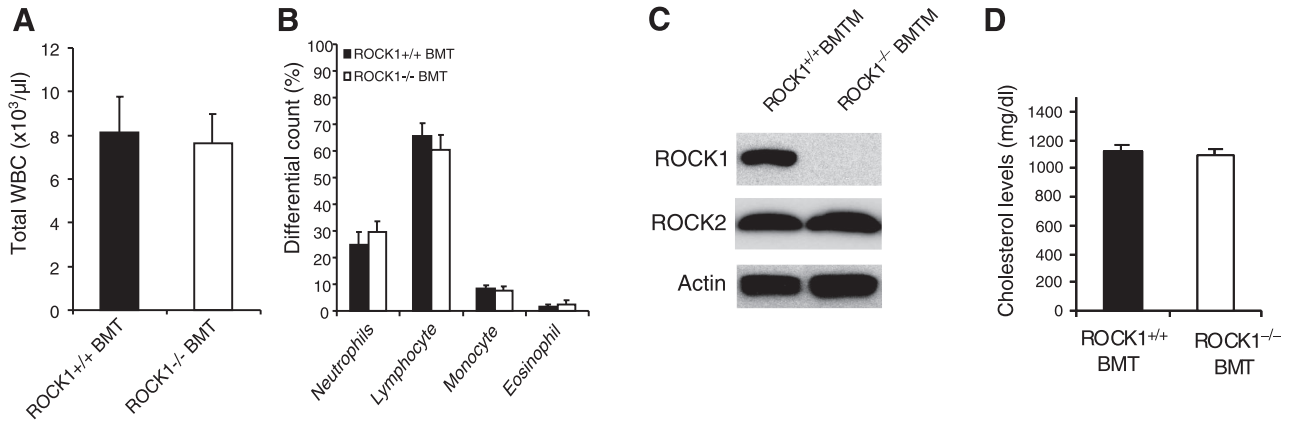

Figure 2. Analysis of peripheral blood cell in LDLR$^{-/-}$ after BMT. *A*) Total peripheral white blood cell counts show no differences between ROCK1$^{+/+}$ BMT (n=12) and ROCK1$^{-/-}$ BMT mice (n=11). *B*) Differential counts are comparable in these two groups. *C*) Western blot analysis of bone marrow-derived macrophages from LDLR$^{-/-}$ recipients for ROCK1 and ROCK2 expression. There is no detectable ROCK1 in macrophages from ROCK1$^{-/-}$ BMT mice and no compensatory changes in ROCK2 compared to that of macrophages from ROCK1$^{+/+}$ BMT mice. *D*) Similar cholesterol levels in ROCK1$^{+/+}$ BMT (n=12) and ROCK1$^{-/-}$ BMT (n=11) mice after a 12-wk atherogenic diet.

marrow cells (Fig. 4*B*), MOMA-2-positive area: 45.1 ± 7.8 *vs.* 36.6 ± 6.9%; $P < 0.05$ (Fig. 4*C*).

Quantification of T cell accumulation in the atherosclerotic lesions

To assess T lymphocyte infiltration into the lesion, we performed immunohistochemical analysis of atherosclerotic vessels to examine the presence of CD3$^+$ cells. We found that CD3$^+$ cells were located mainly near the peripheral regions of the aortic lesions in both ROCK1$^{+/+}$ BMT (**Fig. 5*A***) and ROCK1$^{-/-}$ BMT mice (Fig. 5*B*). Quantification of T cell infiltration showed that more T cells were present in the lesions of ROCK1$^{+/+}$ BMT mice (106.12±9.35 /mm^2) compared to that in ROCK1$^{-/-}$ BMT mice (78.39±5.21/mm^2; P<0.05) (Fig. 5*C*).

Lipid uptake and phagocytosis activities in ROCK1$^{-/-}$ macrophages

The fluorescently labeled AcLDL has been shown to be a useful tool in assessing macrophage uptake of lipids (25). To determine whether ROCK1 has a functional role in regulating lipid uptake by macrophages, we used Alexa Fluor 488-AcLDL for incubation with bone marrow-derived macrophages from ROCK1$^{-/-}$ mice to compare with those from wild-type mice. After 1-h incubation at 37°C, marked intracellular granular patterns of fluorescent signals were observed in the wild-type bone marrow-derived macrophages (**Fig. 6*A***). The bone marrow-derived macrophages from ROCK1$^{-/-}$ mice, however, showed much less internalizing of the fluorescently labeled AcLDL. Quantification of the fluorescence intensity revealed significant differences between the wild-type and ROCK1$^{-/-}$ macrophages.

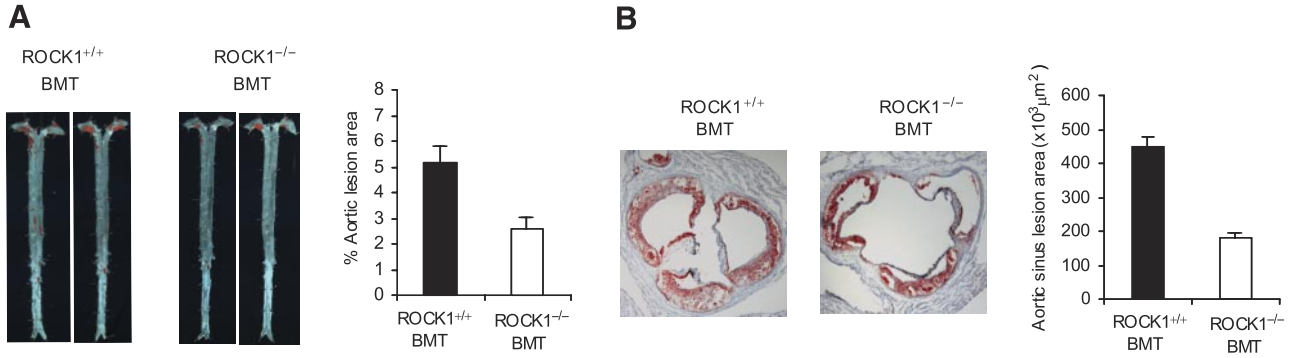

Figure 3. Atherosclerosis in LDLR$^{-/-}$ mice with ROCK1$^{-/-}$ BMT. *A*) Oil Red O-stained, longitudinally opened aortas from female LDLR$^{-/-}$ recipient of ROCK1$^{+/+}$ bone marrow cells (left 2 aortas) and ROCK1$^{-/-}$ bone marrow cells (right 2 aortas) after 12 wk on the atherogenic diet. Percent lesion area in *en face* preparations of aortas from ROCK1$^{+/+}$ BMT mice (5.17±0.67%; n=12) and ROCK1$^{-/-}$ BMT mice (2.58±0.47%; n=11; P<0.01). *B*) Representative Oil Red O-stained aortic valve sections from LDLR$^{-/-}$ mice that received bone marrow cells from ROCK1$^{+/+}$ mice (left), and ROCK1$^{-/-}$ mice (right). The difference in lesion area between ROCK1$^{+/+}$ BMT (448.46±33.31×10^3 μm^2; n=12) and ROCK1$^{-/-}$ BMT mice (181.52±15.63×10^3 μm^2; n=11) was statistically significant (P<0.01).

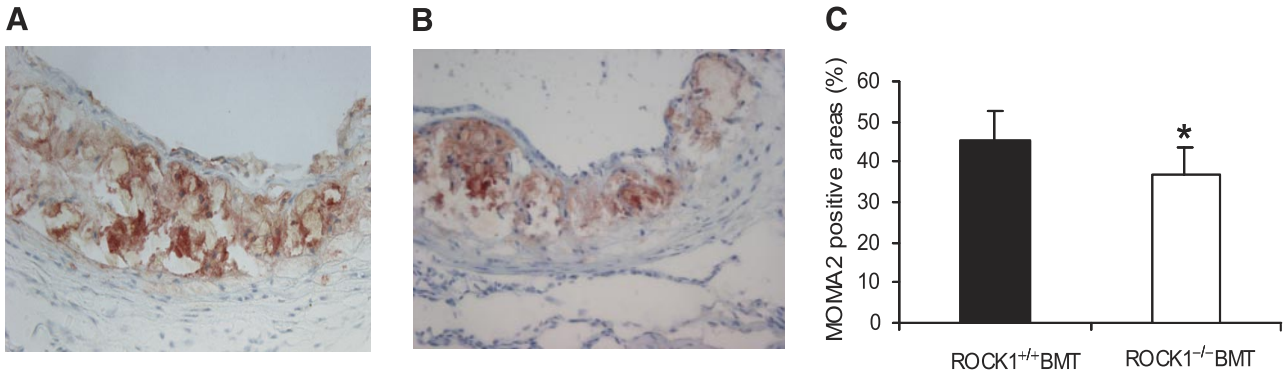

Figure 4. Quantification of macrophage infiltration in the aortic sinus lesions. *A*, *B*) MOMA2 staining of macrophages in LDLR$^{-/-}$ mouse ROCK1$^{+/+}$ (*A*) and ROCK1$^{-/-}$ (*B*) bone marrow cells after 12 wk on a high-cholesterol diet. *C*) Comparison of the percentage of MOMA2-positive areas in the aortic sinus lesion from ROCK1$^{+/+}$ BMT (45.05±2.49%; n=10) and ROCK1$^{-/-}$ BMT mice (36.59±2.31%; n=9; *P<0.05).

Similar findings were observed using a mouse macrophage cell line, RAW264.7 cells, when treated with ROCK inhibitor, Y-27632 (Fig. 6*B*).

To determine whether there is any interaction between ROCK1 and scavenger receptors during lipid uptake, we added polyinosinic acid and polycytidylic acid to bone marrow-derived macrophages from both wild-type and ROCK1$^{-/-}$ mice prior to subjecting the macrophages to AcLDL exposure. Whereas ROCK1$^{-/-}$ macrophages consistently showed reduced AcLDL uptake compared to wild-type macrophages using various doses of the ineffective inhibitor, polycytidylic acid, these differences were abolished in a concentration-dependent manner with the effective inhibitor of scavenger receptor, polyinosinic acid (Supplemental Fig. 1). These results indicate that ROCK1-mediated uptake of AcLDL is dependent on scavenger receptor.

Because ROCKs are involved in actin cytoskeletal reorganization, it is important to determine whether this defective ability of ROCK1$^{-/-}$ macrophages to take up modified LDL is due to a general defect in phagocytic activity. Accordingly, we incubated bone marrow-derived macrophages from wild-type and ROCK1$^{-/-}$ mice with fluorescently labeled nonspecific yeast particles. Bone marrow-derived macrophages from both wild-type and ROCK1$^{-/-}$ mice showed comparable yeast phagocytosis within 30 min of incubation (Fig. 6*C*). The yeast phagocytosis was almost completely inhibited by pretreatment with cytochalasin D (data not shown). These findings suggest that ROCK1 in macrophages specifically mediates uptake of cholesterol from modified lipoprotein particles but is not involved in phagocytosis of foreign material.

To examine the consequence of decreased cholesterol uptake on foam cell formation, we incubated bone marrow-derived macrophages with oxidized (Ox)LDL (100 μg/ml) for 48 h and determined foam cell development by Oil Red O staining. OxLDL incubation resulted in marked enhancement of foam cell formation in the wild-type bone marrow-derived macrophages compared to ROCK1$^{-/-}$ macrophages (Fig. 6*D*). Because primary mouse macrophages do not take up OxLDL very efficiently, we also used RAW264.7 cells as a foam cell formation model. These cells were incubated with OxLDL in the presence or absence of the ROCK inhibitor, Y-27632. Compared to untreated cells, cells treated with Y-27632 had markedly less Oil Red O staining. However, the level of CD36 expression in resting as well as oxLDL-stimulated bone marrow derived macrophages was not different between the wild-type and

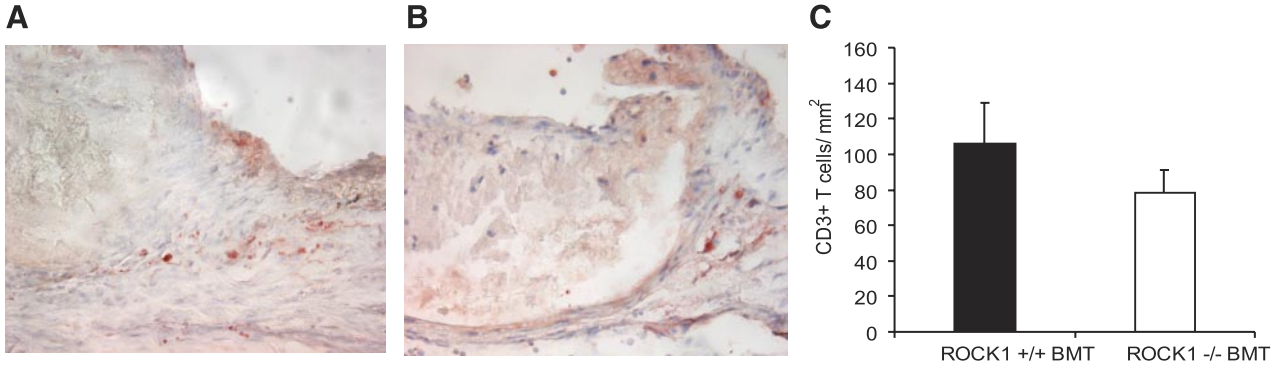

Figure 5. Quantification of T lymphocytes infiltration in the aortic sinus lesions. *A*, *B*) CD3-positive cell in aortic sinus lesion from ROCK1$^{+/+}$ BMT mice (*A*) and ROCK1$^{-/-}$ BMT mice (*B*) after a 12-wk high-cholesterol diet. *C*) Comparison of the number of CD3-positive cells in the aortic sinus lesion from ROCK1$^{+/+}$ BMT (106.12±9.35 /mm^2; n=6) and ROCK1$^{-/-}$ BMT mice (78.39±5.21/mm^2; n=6; P<0.05).

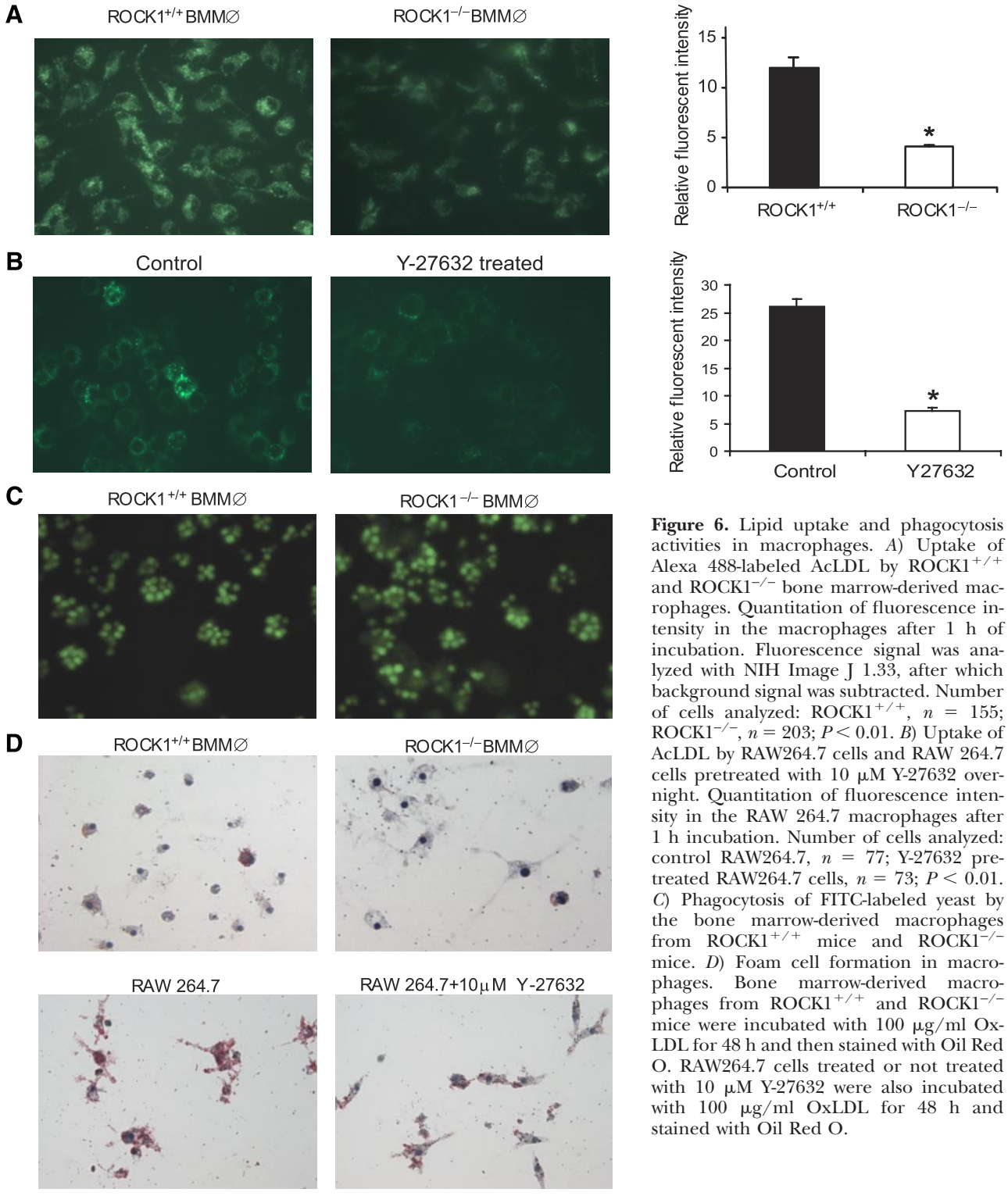

Figure 6. Lipid uptake and phagocytosis activities in macrophages. *A*) Uptake of Alexa 488-labeled AcLDL by ROCK1$^{+/+}$ and ROCK1$^{-/-}$ bone marrow-derived macrophages. Quantitation of fluorescence intensity in the macrophages after 1 h of incubation. Fluorescence signal was analyzed with NIH Image J 1.33, after which background signal was subtracted. Number of cells analyzed: ROCK1$^{+/+}$, $n = 155$; ROCK1$^{-/-}$, $n = 203$; $P < 0.01$. *B*) Uptake of AcLDL by RAW264.7 cells and RAW 264.7 cells pretreated with 10 μM Y-27632 overnight. Quantitation of fluorescence intensity in the RAW 264.7 macrophages after 1 h incubation. Number of cells analyzed: control RAW264.7, $n = 77$; Y-27632 pretreated RAW264.7 cells, $n = 73$; $P < 0.01$. *C*) Phagocytosis of FITC-labeled yeast by the bone marrow-derived macrophages from ROCK1$^{+/+}$ mice and ROCK1$^{-/-}$ mice. *D*) Foam cell formation in macrophages. Bone marrow-derived macrophages from ROCK1$^{+/+}$ and ROCK1$^{-/-}$ mice were incubated with 100 μg/ml OxLDL for 48 h and then stained with Oil Red O. RAW264.7 cells treated or not treated with 10 μM Y-27632 were also incubated with 100 μg/ml OxLDL for 48 h and stained with Oil Red O.

ROCK1$^{-/-}$ mice (data not shown). These results indicated that ROCK plays an important role in cholesterol uptake by macrophages and formation of foam cells.

Decreased chemotactic response in bone marrow-derived macrophages from ROCK1$^{-/-}$ mice

In an attempt to determine if ROCK1 plays a role in the mobility of macrophages, we first tested the ability of bone marrow-derived macrophages to adhere to fibronectin. Both wild-type and ROCK1$^{-/-}$ macrophages showed comparable adhesion abilities (Supplemental Fig. 2). To determine the function of ROCK1 in mediating macrophage chemotaxis, we next examined the migration of bone marrow-derived macrophages in response to monocyte chemotactic protein (MCP)-1. Compared to wild-type macrophages, the chemotactic response of ROCK1$^{-/-}$ macrophages to MCP-1 was

reduced, indicating that ROCK1 mediate macrophage chemotaxis in response to MCP-1 (**Fig. 7*A***). Similarly, the ROCK inhibitor, Y-27632, also inhibited RAW264.7 macrophage migratory response to MCP-1 (Fig. 7*B*). We also examined the ability of bone marrow-derived macrophages to infiltrate through Matrigel, an artificial basement membrane meant to mimic the extracellular matrix, in response to MCP-1. The addition of MCP-1 significantly enhanced the migration of macrophages through Matrigel, which was abrogated by the ROCK inhibitor, Fasudil (Fig. 7*C*).

DISCUSSION

Although whether ROCK2 participates in atherogenesis is still to be determined, our findings indicate that ROCK1 deficiency leads to a reduction of atherosclerosis by inhibiting macrophage chemotaxis, decreasing uptake of modified LDL, and preventing foam cell formation. The decrease in aortic fatty deposits and atherosclerotic lesions in ROCK1$^{-/-}$ BMT was not due to alteration in plasma cholesterol levels since the plasma cholesterol levels in LDLR$^{-/-}$ chimeric mice (ROCK1$^{+/+}$ BMT and ROCK1$^{-/-}$ BMT) were similar. These results indicate that the reduced lesion size in ROCK1$^{-/-}$ BMT mice was not due to changes in circulating cholesterol levels brought on by ROCK1 deficiency, but by nonlipid effects of ROCK1 deficiency on inflammatory cells.

Our results are in general agreement with recent studies showing that ROCK inhibitors reduce various vascular diseases (19, 26, 27). These studies, however, could not determine which ROCK isoforms mediates this process or which target tissue was responsible. Although the kinase domains of ROCK1 and ROCK2 are 92% identical (28), ROCK1 and ROCK2 have different subcellular localization and downstream targets. For example, ROCK1 is found to be important for stress fiber formation, whereas ROCK2 activity is involved in phagocytosis of matrix-coated beads, a function not sensitive to ROCK1 (29). More recently, ROCK1 is shown to have a high degree of substrate specificity toward native Zipper-interacting protein kinase, which consequently regulates Ca^{2+}-independent phosphorylation of both smooth muscle and nonmuscle myosin (30). A previous study using ROCK inhibitor Y-27632 suggests that ROCK activity contributes to the development of early atherosclerosis by regulating T cell proliferation (19). By using a genetic approach, we were able to investigate the specific pathophysiological roles of ROCK1 in atherogenesis. Indeed, we observed less T cell infiltration in the atherosclerotic lesions of ROCK1$^{-/-}$ BMT mice compared to those in ROCK1$^{+/+}$ BMT mice. Furthermore, those T cells were located mainly in the peripheral region of the lesion, suggesting that T cells may be more important in the early part of disease development. Because macrophages play an important role in the initiation and progression of atherosclerosis, we utilized BMT scheme to study the function of ROCK1 in bone marrow-derived cells, in particular, macrophages. Our findings indicate that ROCK1 in bone marrow-derived macrophages, indeed, contributes to the advanced development of atherosclerosis.

One of the earliest events in atherogenesis is the recruitment of monocyte/macrophages to the vessel wall. Activation of RhoA has been shown to promote

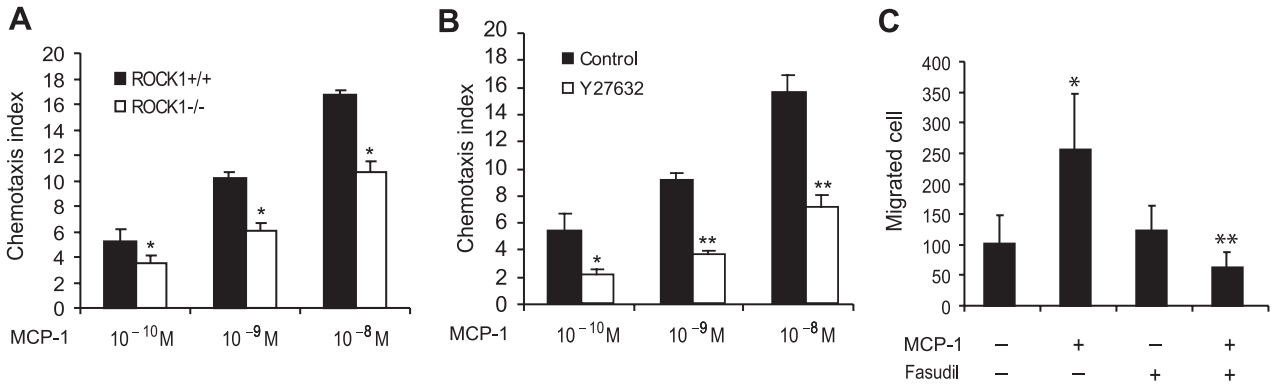

Figure 7. Macrophages chemotaxis toward MCP-1. *A*) Chemotactic response of bone marrow-derived macrophages from ROCK1$^{+/+}$ and ROCK1$^{-/-}$ mice was evaluated using MCP-1 at concentrations ranging from 10^{-10} M to 10^{-8} M. Results are shown as chemotactic index (migrated cells/background migration in the absence of chemotactic stimulus, average of 5 random high-power fields for each well). Migration in ROCK1$^{-/-}$ bone marrow-derived macrophages is significantly reduced; *$P < 0.05$. *B*) RAW264.7 macrophages were pretreated with ROCK inhibitor, Y-27632 (10 μM). Chemotactic response of RAW264.7 cells was evaluated using MCP-1 at concentrations ranging from 10^{-10} M to 10^{-8} M. Results are shown as chemotactic index (migrated cells/background migration in the absence of chemotactic stimulus, average of 5 random high-power fields each well). Migration in Y-27632-treated RAW264.7 cells is significantly reduced (*P<0.05, **P<0.01). *C)* The effect of ROCK inhibition on migration of wild-type macrophages through Matrigel. Bone marrow-derived macrophages were added to a modified Boyden chamber in which Matrigel was added to the filter. The cells had to invade through the Matrigel in the presence (10 μM) or absence of the ROCK inhibitor, Fasudil. The bottom chamber contained MCP-1 as the macrophage chemoattractant (10^{-8} M). Whereas cell migration is significantly increased with the addition of MCP-1 (*P<0.05), cells migrating toward MCP-1 are significantly reduced with ROCK inhibition (**P<0.05 *vs.* MCP-1-treated cells).

monocyte adhesion and transendothelial migration, and this enhanced migration can be abolished by ROCK inhibition (31). Indeed, similar to our findings, ROCK inhibitors have been shown to inhibit monocyte/macrophage chemotaxis (32, 33). An important finding in this study is that ROCK1 specifically is critically important for macrophage chemotaxis. This likely explains why we observed less macrophage infiltration in the atherosclerotic lesions of ROCK1$^{-/-}$ BMT mice. Myosin light-chain kinase (MLCK) and ROCK are two major kinases that phosphorylate MLC. It has been reported that in T cells, MLCK is concentrated at the leading edge, whereas ROCK is present in the trailing edge, indicating that ROCK activity is required for the detachment of the trailing edge (34). In this study, we demonstrated that migration of macrophages toward MCP-1 is reduced with ROCK inhibitor in Matrigel, a β1 integrin-mediated process (33). The density of activated β1 integrin is associated with the number of polymerizing actin ends during cell migration (35). A plausible explanation is that ROCK1 and integrin cytoplasmic domain-associated protein-1 colocalize in the cell membrane, together with β1 integrins, in both the leading edge and the trailing edge, where ROCK affects cell migration (36). In addition, RhoA/ROCK pathway is important in regulating monocyte tail retraction during transendothelial migration *via* β2 integrin-mediated process (37). Furthermore, ROCK signaling is involved in integrin recycling, which influences cell migration (38). Comparable ability of bone marrow-derived macrophages from wild-type and ROCK1$^{-/-}$ mice to adhere to fibronectin indicates that migratory differences occur after the cell adhesion. Corroborating our results, ROCK1 expression rather than ROCK2 is up-regulated on macrophage adhesion (39). Migration of the elongated cells in Matrigel is ROCK independent, whereas that of the rounded cells using bleb-like extensions for motility do require Rho/ROCK signaling for movement (40). Additionally, there is evidence that ROCK preferentially regulates phosphorylation of regulatory light chain associated with myosin IIA during cell rounding and migration (41).

The fact that ROCK activities were decreased in peripheral blood leukocyte from ROCK1$^{-/-}$ mice provides a functional basis of less monocyte/macrophage accumulation in the atherosclerotic lesions in these animals. The importance of ROCK in this process is illustrated by the fact that MLC phosphatase activity is dependent on simultaneous presence of ATP-γ-S and ROCK, and that activated ROCK phosphorylates MBS, which results in an increased contraction of smooth muscle or interaction of actin and myosin leading to stress fiber formation in nonmuscle cells (42).

Accumulation of foam cells in the subendothelium is a key contributing factor in atherogenesis. Foam cells are predominantly derived from monocyte/macrophages, although some smooth muscle cells may also become foam cells. We found that ROCK1 in bone marrow-derived macrophages mediates cholesterol uptake and foam cell formation. Although the precise mechanisms by which ROCK1 regulates modified lipid uptake by macrophages is not known, OxLDL has been shown to induce or enhance endothelial and smooth muscle cell contraction *via* activation of Rho/ROCK pathway (43, 44). Specifically, ROCK1's ability to regulate actin filament reorganization may be one of the mechanisms by which ROCK1 contributes to lipid uptake in macrophages (32, 45). Inhibition of AcLDL uptake by polycytidylic acid, but not by polyinositic acid, suggests that ROCK1 influences lipid uptake *via* scavenger receptors. Furthermore, OxLDL is found to increase Ca^{2+} sensitivity of myofilaments in arteriolar vascular smooth muscle and enhance their constriction, which may be through stimulation of Rho and ROCKs (44). Inhibition of ROCK with Y-27632 also decreases OxLDL-induced endothelial cell proliferation (46).

Another way by which ROCK may regulate cholesterol uptake by the macrophages is through membrane lipid transport mechanism. The intracellular transport of cholesterol from the plasma membrane to the site of its esterification involves a combination of vesicular and nonvesicular pathways. Vesicular pathways involve scavenger receptors class A-I/II and CD36 for the preponderance of modified LDL uptake in macrophages leading to lipid loading in macrophages (47). The uptake of DiI-LDL is also dependent on clathrin, the components of which occur randomly within a large number of active domains throughout the plasma membrane and surrounded by regions of low activity, presumably constrained by the underlying cytoskeleton (48). On the other hand, when macrophages are preincubated with AcLDL and subsequently labeled with dehydroergosterol, the fluorescent sterol can be delivered to lipid droplets in the cytoplasm from the plasma membrane by a nonvesicular mechanism (49). Indeed, there is evidence that macrophage foam cells can be generated by macropinocytosis of LDL without receptor-mediated binding of LDL (50). The fact that a recent study showed that human urotensin II-induced up-regulation of acyl-coenzyme A: cholesterol acyltransferase, which is critical for cholesteryl ester formation, can be abolished with ROCK inhibition, suggests that ROCK pathways are important for cholesterol storage in macrophages (51).

In our study, ROCK1 does not appear to be involved in general phagocytic activity of macrophages. This is in agreement with a previous study using siRNA-treated cells to show phagocytic uptake of fibronectin-coated beads was down-regulated in ROCK2-depleted cells but not in those lacking ROCK1 (29). In accordance with this, complement receptor-mediated phagocytosis is myosin II dependent *via* ROCK activation, but FcγR-mediated phagocytosis is independent of Rho or ROCK activity (52).

In summary, our results indicate that ROCK1 plays a key role in macrophage chemotaxis, cholesterol uptake, and foam cell formation, all of which are hallmark events in the pathogenesis of atherosclerosis. Indeed, deficiency of ROCK1 in bone marrow-derived cells led

to an attenuation of atherosclerosis. These findings suggest that selective ROCK1 inhibitor may have therapeutic benefits in patients with atherosclerotic and vascular inflammatory diseases. However, it is important to note that it is not yet known if ROCK2 also participates in atherogenesis and that the role of ROCK2 in the development of atherosclerosis remains to be determined. FJ

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