Targeting GGTase-I Activates RHOA, Increases Macrophage Reverse Cholesterol Transport, and Reduces Atherosclerosis in Mice

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**Background**—Statins have antiinflammatory and antiatherogenic effects that have been attributed to inhibition of RHO protein geranylgeranylation in inflammatory cells. The activity of protein geranylgeranyltransferase type I (GGTase-I) is widely believed to promote membrane association and activation of RHO family proteins. However, we recently showed that knockout of GGTase-I in macrophages activates RHO proteins and proinflammatory signaling pathways, leading to increased cytokine production and rheumatoid arthritis. In this study, we asked whether the increased inflammatory signaling of GGTase-I–deficient macrophages would influence the development of atherosclerosis in low-density lipoprotein receptor–deficient mice.

**Methods and Results**—Aortic lesions in mice lacking GGTase-I in macrophages (Pgg1b−/−) contained significantly more T lymphocytes than the lesions in controls. Surprisingly, however, mean atherosclerotic lesion area in Pgg1b−/− mice was reduced by ≈60%. GGTase-I deficiency reduced the accumulation of cholesterol esters and phospholipids in macrophages incubated with minimally modified and acetylated low-density lipoprotein. Analyses of GGTase-I–deficient macrophages revealed upregulation of the cyclooxygenase 2–peroxisome proliferator-activated-γ pathway and increased scavenger receptor class B type I– and CD36-mediated basal and high-density lipoprotein–stimulated cholesterol efflux. Lentivirus-mediated knockdown of RHOA, but not RAC1 or CDC42, normalized cholesterol efflux. The increased cholesterol efflux in cultured cells was accompanied by high levels of macrophage reverse cholesterol transport and slightly reduced plasma lipid levels in vivo.

**Conclusions**—Targeting GGTase-I activates RHOA and leads to increased macrophage reverse cholesterol transport and reduced atherosclerosis development despite a significant increase in inflammation. *(Circulation. 2013;127:782-790.)*

**Key Words:** atherosclerosis ■ cholesterol ■ hydroxymethylglutaryl-CoA reductase inhibitors ■ macrophages ■ prenylation ■ statins

The RHO family proteins contain a carboxyl-terminal CAAX motif and undergo posttranslational modification with a 20-carbon geranylgeranyl lipid. The reaction is catalyzed by protein geranylgeranyltransferase type I (GGTase-I), a cytosolic enzyme composed of a unique β subunit encoded by Pgg1b and an α subunit that is shared with protein farnesyltransferase. The geranylgeranylation and farnesylation reactions, which are conserved from yeast to humans, render the carboxyl terminus of CAAX proteins more hydrophobic and promote their interactions with membranes and other proteins within cells. The most well-studied protein substrates for GGTase-I are RHOA, RAC1, and CDC42.

The RHO proteins control the actin cytoskeleton during cell movements such as extravasation, migration, and phagocytosis, and they participate directly in intracellular signaling pathways. These activities are important for the proper function of macrophages and other inflammatory cells. Geranylgeranylation is considered essential for membrane targeting and activation of the RHO proteins. Therefore, inhibiting GGTase-I to block RHO protein activity has been proposed as a strategy to reduce inflammation and to treat arthritis, atherosclerosis, and other inflammatory disorders. Reduced geranylgeranylation and inhibition of RHO proteins have also been proposed to explain the antiinflammatory and some antiatherogenic properties of statins. Statins reduce plasma cholesterol levels but also interfere with the production
of geranylgeranyl lipids, which in turn reduces RHO protein geranylgeranylation.11,12 There has been considerable support for the notion that blocking geranylgeranylation inactivates RHO proteins.9,13–16

However, a recent study showed that knockout of GGTase-I in macrophages blocked CAAX protein geranylgeranylation and led to accumulation of GTP-bound active RHOA, RAC1, and CDC42.17 The increased RHO protein activity led to increased RAC1, p38, and nuclear factor-κB signaling; increased reactive oxygen species; and increased proinflammatory cytokines, leading to rheumatoid arthritis in vivo. These results suggested that geranylgeranylation serves to inhibit, rather than activate, RHO proteins in macrophages and further suggested a need to reevaluate the importance of RHO protein geranylgeranylation and GGTase-I activity in other pathways and specific disease processes.

RHO proteins are involved in signaling pathways that regulate macrophage foam cell formation and cholesterol efflux, 2 processes relevant to the pathogenesis and treatment of atherosclerosis.18–20 For example, several studies have suggested that activation of RHOA and CDC42 inhibits peroxisome proliferator-activated-γ (PPARγ) activity and cholesterol efflux in macrophages.5,20–22 However, most of those studies were performed by expressing dominant-negative RHO constructs or by treating cells with compounds that alter the activity of the RHO proteins or interfere with protein geranylgeranylation. Thus far, no one has used a genetic strategy to block GGTase-I activity and then define the impact of this intervention on the behavior of macrophages in vivo.

In this study, we investigated how inactivation of GGTase-I in macrophages affects the development of atherosclerosis in low-density lipoprotein (LDL) receptor–deficient mice. We hypothesized that knockout of GGTase-I would accelerate atherosclerosis. This hypothesis was based on 3 observations. First, macrophages lacking GGTase-I mount a robust inflammatory response that would likely promote lesion development.17,21 Second, GGTase-I–deficient mice develop rheumatoid arthritis,17 an inflammatory disorder associated with a high risk of atherosclerosis in humans.24 Third, activation of RHO proteins should inhibit cholesterol efflux and stimulate foam cell formation.5,20,25

Methods

Mouse Breeding

Mice homozygous for a conditional knockout allele of the GGTase-I β subunit and heterozygous for the lysozyme M-Cre knock-in allele (Pgt1b<sup>fl/fl</sup>LC) have been described.26 Pgt1b<sup>fl/fl</sup>LC mice were bred with LDL receptor knockout mice (Ldlr<sup>−/−</sup>; Jackson Laboratories) and backcrossed 6 times to a C57BL/6 background. Mice were genotyped as described.26,27 Pgt1b<sup>fl/fl</sup>LC:Ldlr<sup>−/−</sup> mice were designated Pgt1b<sup>fl/−</sup>; littermate control Pgt1b<sup>+/+</sup>LC:Ldlr<sup>−/−</sup> and Pgt1b<sup>+/+</sup>LC:Ldlr<sup>−/−</sup> mice were indistinguishable in phenotype and designated Pgt1b<sup>+/+</sup>. Six- to 7-week-old male mice were fed a Western-type diet containing 1.25% cholesterol (Harlan AB, Sweden) for 12 and 24 weeks. Mouse experiments were approved by the local research animal ethics committee.

Preparation of En Face Aortas and Proximal Aortic Sections

Aortas were dissected to the iliac bifurcation, fixed in 4% formaldehyde, pinned out under an inverted microscope, and stained with Sudan IV as described.28 Aortic roots were frozen in optimal cutting temperature freezing medium, and 10-µm-thick sections were stained with Oil Red O and hematoxylin.29,30

Quantification of Aortic Lesions and Immunohistochemistry

En face aortas were photographed with a Sony DSC25 digital camera, and lesion areas were quantified with ImageJ software.31 Aortic root sections were scanned in a Mirax Scanner (Zeiss, Germany), and lesions were quantified with BioPix iQ 2.1.8 software. Macrophages, smooth muscle cells, and lymphocytes in lesions were analyzed immunohistochemically with antibodies against monocyte/macrophage antibody-2 (MC519G, Accurate, NY), α-smooth muscle actin (ab5694, Abcam), CD4, and CD8 (553043 and 553027, BD Pharmingen). Apoptotic cells in aortic root sections were analyzed with the In Situ Cell Death Detection Kit (11684809910, Roche).

Plasma Lipid and Cytokine Analyses

Plasma cholesterol and triglycerides were determined with Konelab/T Series kits and a Konelab 20 Autoanalyzer (Thermo-Fisher). Cholesterol was also measured in lipoprotein fractions of pooled plasma after fast performance liquid chromatography as described.32 Plasma cytokines were quantified with the Mouse Prolinflammatory 7-plex Ultrasensitive Kit in a Sector 2400 Imager (Meso Scale Discovery).

Generating Bone Marrow and Peritoneal Macrophages

Bone marrow cells were cultured in high-glucose Dulbecco modified Eagle medium supplemented with 10% FCS, 1% HEFES, 1% glucose, 1% gentamycin, 0.01% β-mercaptoethanol, and 10% whole supernatant of cell line CMG14-12 as a source of monocyte colony-stimulating factor.33 Experiments were done on differentiated macrophages 7 to 10 days after plating. Intraperitoneal macrophages were collected after rinsing of the mouse peritoneal cavity with 5 mL PBS. Experiments were done 1 to 3 days after plating.

Cytokine Array

Macrophages were incubated with minimally modified LDL for 24 hours, and cytokines in the supernatant were quantified with the Mouse Cytokine Antibody Array Panel A (ARY006, R&D Biosystems).

Foam Cell Formation Assay and Quantification of Cholesterol Esters

Macrophages were seeded on glass slides in 24-well plates, incubated with 50 µg/mL acetylated LDL (acLDL) or minimally modified LDL for 24 hours, fixed with ethanol, and stained with Oil Red O as described.11 The cells were imaged in the Mirax Scanner, and Oil Red O staining and cell area were determined with BioPix iQ 2.1.8 software. Lipid uptake was also analyzed by fluorescence-activated cell sorter analysis after cells were incubated with Alexa Fluor 488–labeled acLDL (I23380, Invitrogen) for 30 minutes at 4C and 16 hours at 37C. Cholesterol ester content in macrophages was measured 36 hours after incubation with acLDL. Neutral lipids were extracted in 2:3 n-hexane/isopropanol for 20 minutes, dried, and dissolved in 10% Triton X-100 in isopropanol. Total cholesterol and free cholesterol were determined (Wako Chemicals), and cholesterol ester content was calculated by subtracting free from total cholesterol and normalized to total cellular protein levels.

Lipidomics Analyses

Lipids were extracted from bone marrow macrophages with the procedure of Folch et al.34 Cholesterol esters were quantified with straight-phase high-performance liquid chromatography with evaporative light-scattering detection; phospholipids were analyzed with a QTRAP 5500 mass spectrometer equipped with a NanoMate HD
robocylindrical nanoflow ion source (Advion Biosciences); and ceramide and glucosylceramide were analyzed by high-performance liquid chromatography coupled to a Quattro Premier XE triple-quadrupole mass spectrometer.39

**Cholesterol Efflux Assay, Apoptosis, and Cell Death Analyses**

Macrophages were incubated with 1 to 2 μCi [3H]cholesterol (preincubated with 50 μg/mL acLDL) in medium containing endotoxin-and free fatty acid-free 0.2% BSA for 24 hours. Cholesterol pools were equilibrated overnight in medium containing 0.1% BSA. Basal and lipid acceptor–stimulated efflux was measured 6 hours after the addition of vehicle, apolipoprotein AI (10 μg/mL), or high-density lipoprotein (HDL; 100 μg/mL). Radioactivity was measured in the medium and cell lysate, and efflux was calculated as percent radioactivity in the medium relative to total radioactivity in cells and medium and cell lysate, and efflux was calculated as percent radioactivity in the medium.

**Western Blotting**

Cells were lysed as described.36 GTP-bound RAC1 and CDC42 were affinity precipitated with PAK1-GST (EZ Detect Rac Activation Kit, Pierce), and GTP-bound RHOA was precipitated with RhoTag GST (RhoA Activation Biochem Kit, Cytoskeleton) as described.37 Total lysates or affinity-precipitated proteins were resolved on 12% and 4%–15% sodium dodecyl sulfate (SDS)–polyacrylamide gels (Criterion and Protein, BioRad), transferred to nitrocellulose or polyvinylidene difluoride membranes, and incubated with antibodies to RAC1 (05-389, Millipore), RHOA (ARH03-B, Cytoskeleton), CDC42 (sc-2462), and actin (A2066, Sigma-Aldrich). Protein bands were visualized with horseradish peroxidase–conjugated secondary antibodies (sc-2354 from Santa Cruz Biotechnology), liver X receptor-α (LXRα; ab19402, Abcam), cyclooxygenase 2 (COX2; NB110-1498), CDC42 (NB400-144), scavenger receptor class B type I (SR-B1; NB400-104), ATP-binding cassette (ABC) family members A1 (NB400-105) and G1 (ABCG1; NB400-132, Novus Biologicals), and actin (A2066, Sigma-Aldrich). Protein bands were visualized with horseradish peroxidase–conjugated secondary antibodies (sc-2354 from Santa Cruz Biotechnology and NA931 and NA934 from GE Healthcare) and the enhanced chemiluminescence Western blotting system (GE Healthcare). Band densities were analyzed with Quantity One (version 4.4.0, Bio-Rad).

**PPARγ Activity Assay**

Macrophages were cultured to 75% confluence on 150-mm 2 plates and incubated without monocyte colony-stimulating factor for 12 hours. Basal PPARγ activity in nuclear extracts was determined with a PPARγ activity assay kit (10006855, Cayman), and values were normalized to total nuclear protein content.

**Gene Expression Analyses**

RNA was isolated with the RNeasy Mini Kit (Qiagen); cDNA was synthesized with the iScript cDNA Kit (170–889, BioRad); and gene expression was analyzed by TaqMan reverse transcription–quantitative polymerase chain reaction using mouse and human probe sets for *Abca1* (Mm00442646_m1, Hs01059181_m1), *Abcg1* (Mm00437390_m1, Hs00245154_m1), *Cox2* (m00478374_m1, Hs00153133_m1), *Cd36* (Mm00432403_m1, Hs01567185_m1), *Scarb1* (Mm00450234_m1, Hs00869821_m1), *Pparg* (Mm01184322_m1, Hs01115513_m1), and *Lxrα* (Mm00443451_m1). Values were normalized to Gapdh (Mm01205631_m1, Hs00468791_s1).

**Lentivirus Experiments and Inhibitors**

Lentiviruses expressing short hairpin (sh) RNAs targeting mouse *Abca1* (TRCN0000271812-60), *Abcg1* (TRCN0000105286–87), *Cd36* (TRCN000066518–22), *Rhoa* (TRCN0000055192), *Rac1* (TRCN0000030490), and *Sr-Bi* (TRCN000066573-75) were from Sigma-Aldrich; shCDC42 lentiviruses were from Santa Cruz Biotechnology (SC-29257-V). Macrophages were incubated with lentiviruses at a multiplicity of infection of 10 to 20 for 72 to 96 hours before experiments. Lentiviral construct expressing human *Pggt1b* gene was from SBI. Inhibitors of GGTase-I (*Pggt1b*-298), famesyltransferase (FTI-276), COX2 (Celecoxib; PZ20008), RHO-associated protein kinase (Y-27632), and etoposide (E1383) were from Sigma; the inhibitor of P21-activated kinase (PAK18) was from Merck.

**THP-1 Cells**

The human acute monocytic leukemia cell line THP-1 was differentiated into macrophage-like cells with phorbol 12-myristate 13-acetate.38 For cholesterol efflux, gene expression analyses, and Western blotting, THP-1 macrophages were incubated with a GGT1 (1, 5, and 10 μM/L) for 48 hours before experiments.

**Reverse Cholesterol Transport**

Bone marrow macrophages were loaded with 25 μg/mL acLDL and 5 μCi/mL [3H]cholesterol for 30 hours, washed twice with PBS, scraped into RPMI-1640/0.2% BSA, spun (1000 rpm, 5 minutes, 4°C), and resuspended in RPMI-1640/0.2% BSA. Cell viability and radioactivity were counted, and 1.3x10^6 counts per minute (CPM) was injected intraperitoneally into *Pggt1b*/− recipient mice. Plasma samples were obtained at baseline and after 24 and 48 hours, and 5-μl aliquots were counted in a scintillation counter. Feces were collected at 0, 24, and 48 hours; soaked in distilled water (100 mg/mL) overnight at 4°C; and homogenized in an equal volume of ethanol. Then, 500-μl aliquots of the homogenate were counted in a scintillation counter. CPM/mL in the plasma and CPM/100 mg feces were expressed as percent of injected CPMs. This protocol was a modification of those used in previous studies.38,39

**Statistics**

Values are mean±SEM unless stated otherwise. Differences between groups were assessed with the t test or Mann-Whitney test and considered significant at *P*<0.05.

**Results**

Inactivating Macrophage GGTase-I Reduces Atherosclerosis but Increases Inflammation

To define the role of macrophage GGTase-I in atherogenesis, we bred mice lacking *Pggt1b* expression in macrophages26 onto an *Ldr*–deficient background. These mice, designated *Pggt1b*/−, were fed a Western-type diet for 12 or 24 weeks. Atherosclerotic lesions in *Pggt1b*/− aortas were 50% smaller than in littermate controls at 12 weeks and 60% smaller at 24 weeks, as judged by en face analyses of the aorta (Figure 1A). Lesions were also reduced in aortic root sections (Figure 1B and IC in the online-only Data Supplement). Areas of necrosis in aortic root sections were less frequent in *Pggt1b*/− compared to controls, whereas levels of apoptotic cells did not differ (Figure 1B and IC in the online-only Data Supplement). Body weight curves in *Pggt1b*/− and control *Pggt1b*/+ mice were similar, but *Pggt1b*/+ mice had slightly lower plasma cholesterol and triglyceride levels (Figure 1B and IC). The lower plasma lipid levels were also evident when the plasma lipoproteins were fractionated by fast protein liquid chromatography (Figure 1D and Figure ID in the online-only Data Supplement).

The macrophage lesion area of aortic root sections was reduced in *Pggt1b*/− mice compared with controls, whereas the area of smooth muscle cells was similar in both groups of mice, as judged by immunostaining for monocyte/macrophage marker.
cholesterol efflux in \( \text{Pgtg}t \text{t}b^{+/+} \) macrophages (Figure 4C–4F and Figure IVA and IVB in the online-only Data Supplement).

We asked if apoptosis of \( \text{Pgtg}t \text{t}b^{\Delta/\Delta} \) macrophages accounts for the increased release of \(^{3}H\)cholesterol to the media in the efflux assays. Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining of \( \text{Pgtg}t \text{t}b^{+/+} \) and \( \text{Pgtg}t \text{t}b^{\Delta/\Delta} \) macrophages after incubation with acLDL revealed no differences in levels of apoptosis. Cell death was slightly reduced in \( \text{Pgtg}t \text{t}b^{\Delta/\Delta} \) macrophages, as judged by lactate dehydrogenase cytotoxicity assay (Figure VA and VB in the online-only Data Supplement). Moreover, apoptosis induction with etoposide did not affect levels of cholesterol efflux in \( \text{Pgtg}t \text{t}b^{\Delta/\Delta} \) macrophages (Figure VC in the online-only Data Supplement).

To identify the pathways that mediate increased cholesterol efflux in \( \text{Pgtg}t \text{t}b^{\Delta/\Delta} \) macrophages, we quantified levels of PPAR\( \gamma \) in nuclear extracts with an ELISA; we also assessed levels of \( \text{Pparg} \) transcripts. Basal PPAR\( \gamma \) activity was 2-fold higher in \( \text{Pgtg}t \text{t}b^{\Delta/\Delta} \) macrophages than in \( \text{Pgtg}t \text{t}b^{+/+} \) macrophages (Figure 5A and 5B). Basal expression levels of COX2 and LXR\( \alpha \) were also higher, as judged by mRNA levels and Western blots (Figure 5B and 5C). When COX2 activity in \( \text{Pgtg}t \text{t}b^{\Delta/\Delta} \) macrophages was inhibited with Celecoxib, cholesterol efflux returned to normal levels (Figure 5D).

Cholesterol efflux can be mediated by PPAR\( \gamma \)-LXR\( \alpha \)-induced upregulation of ABCA1 and ABCG1 and by COX2-PPAR\( \gamma \)-mediated upregulation of CD36 and SR-B1.\(^{40–42} \) ABCA1 and ABCG1 expression in \( \text{Pgtg}t \text{t}b^{\Delta/\Delta} \) macrophages was not different from that in \( \text{Pgtg}t \text{t}b^{+/+} \) macrophages; however, levels of SR-B1 and CD36 proteins were significantly increased (Figure 5B and 5C). SR-B1 levels were also higher in aortic sections from \( \text{Pgtg}t \text{t}b^{\Delta/\Delta} \) mice, as judged by immunohistochemical studies (Figure VIA and VIB in the online-only Data Supplement).

To assess the importance of SR-B1 and CD36 in efflux of \( \text{Pgtg}t \text{t}b^{\Delta/\Delta} \) macrophages, we suppressed their expression with lentiviral shRNAs. We also suppressed expression of ABCA1 and ABCG1 in macrophages after incubation with acLDL revealed no differences in levels of apoptosis. Cell death was slightly reduced in \( \text{Pgtg}t \text{t}b^{\Delta/\Delta} \) macrophages, as judged by lactate dehydrogenase cytotoxicity assay (Figure VA and VB in the online-only Data Supplement). Moreover, apoptosis induction with etoposide did not affect levels of cholesterol efflux in \( \text{Pgtg}t \text{t}b^{\Delta/\Delta} \) macrophages (Figure VC in the online-only Data Supplement).
A cholesterol efflux increased, as did mRNA and protein expression of Pgg1b+/+ expressed as percent of that in Pgg1b−/−, Quantification of staining divided by cell number and expressed as percent of that in Pgg1b+/+ macrophages, we suppressed their expression with lentiviral shRNA. The shRNAs reduced expression of Rhoa, Rac1, and Cdc42 transcripts by 50% to 75% (Figure IXB in the online-only Data Supplement). Knockdown of Rhoa, but not Rac1 and Cdc42, reduced cholesterol efflux in Pgg1b+/+ macrophages to the levels observed in Pgg1b−/− macrophages (Figure 5E). Rhoa knockdown also reduced the expression of Cox2, Pparg, Lxra, Cd36, and Scarb1 (Figure 5F). Consistent with these findings, cholesterol efflux in Pgg1b+/+ macrophages was reduced when Rhoa signaling was inhibited with a Rho kinase inhibitor. An inhibitor of RAC signaling had no effect (Figure IXC in the online-only Data Supplement). Overall, the data suggest that RHOA activation, acting upstream of COX2, increases cholesterol efflux in Pgg1b−/− macrophages.

GGTase-I Deficiency Increases Macrophage-Stimulated Reverse Cholesterol Transport In Vivo

To determine whether the increased cholesterol efflux in Pgg1b+/+ macrophages results in higher levels of reverse cholesterol transport in vivo, we loaded Pgg1b+/+ and Pgg1b−/− macrophages with [3H]cholesterol:acLDL, injected the cells into the peritoneal cavity of Pgg1b+/+ mice, and measured radioactivity in plasma and feces after 24 and 48 hours. Levels of [3H]cholesterol in plasma and feces were 1.5- to 2-fold higher in mice injected with Pgg1b+/+ macrophages than in those injected with Pgg1b−/− macrophages (Figure 6A and 6B).

Figure 3. Geranylgeranyltransferase type I (GGTase-I) inactivation reduces macrophage foam cell formation. A and B, Oil Red O staining of intraperitoneal macrophages incubated with (A) 50 μg/mL acetylated low-density lipoprotein (acLDL; n=5–7 per genotype) and (B) 50 μg/mL minimally modified LDL (mmLDL; n=3–4 per genotype) for 24 hours. Left, Representative photographs. Right, Quantification of staining divided by cell number and expressed as percent of that in Pgg1b+/+ macrophages. C, Fluorescence-activated cell sorter analyses of the uptake of Alexa Fluor-conjugated acLDL by bone marrow macrophages (n=4 per genotype). D, Macrophage cholesterol ester level after a 36-hour incubation with acLDL normalized to total cell protein content and expressed as percent of that in Pgg1b+/+ macrophages (n=7 per genotype). **P<0.01; ***P<0.001.

Figure 4. Increased cholesterol efflux in macrophages from Pgg1b+/+ mice. A and B, Basal and (A) apolipoprotein A1 (apoA1) and (B) high-density lipoprotein (HDL)-stimulated cholesterol efflux in bone marrow (BM) macrophages (n=4 per genotype). C, Western blots demonstrating high levels of nonprenylated (np) RAP1A in Pgg1b+/+ BM macrophages and in Pgg1b−/− macrophages incubated with a geranylgeranyltransferase type I (GGTase-I; 10 μmol/L) and low levels in Pgg1b−/− cells and in Pgg1b+/− cells incubated with a lentivirus expressing human PGGT1B. D, Control Western blots demonstrating reduced electrophoretic mobility of HDJ2 in Pgg1b−/− cells incubated with a farnesyltransferase inhibitor (FTI; 10 μmol/L). E and F, Basal cholesterol efflux in BM macrophages incubated with dimethyl sulfoxide (DMSO), FTI, or GGTI (n=3–4 per treatment; E) or with lentivirus-GGT1B at a multiplicity of infection of 20 (n=3; F). *P<0.05; **P<0.01; ***P<0.001.
Discussion

Levels of atherosclerosis in mice are generally worsened by proinflammatory factors. In this study, we found that GGTagase-I deficiency in macrophages markedly reduces atherosclerosis despite higher levels of inflammation. GGTagase-I–deficient macrophages produced high levels of proinflammatory cytokines in response to minimally modified LDL, and T-cell recruitment to subendothelial lesions was increased. The Pgg1tb+/− mice also develop rheumatoid arthritis, which is associated with more atherosclerotic disease in humans. Thus, the lower levels of atherosclerosis in Pgg1tb−/− mice were surprising. Uncoupling of inflammation and lipid accumulation in atherogenesis has been observed previously, although not to the same degree as in Pgg1tb−/− mice.

The findings of reduced atherosclerosis in Pgg1tb−/− mice is likely explained by 3 factors. First, foam cell formation in Pgg1tb−/− macrophages was reduced as a consequence of increased cholesterol efflux, triggered by RHOA and signaling through the COX2–PPARγ–scavenger receptor pathway. Second, the increased cholesterol efflux of Pgg1tb−/− macrophages resulted in increased levels of reverse cholesterol transport in Pgg1tb−/− mice. Third, plasma lipid levels were slightly lower in Pgg1tb−/− mice, likely a consequence of the increased reverse cholesterol transport. Each of these factors—more unloading of cholesterol by cultured macrophages, increased macrophage reverse cholesterol transport in vivo, and lower plasma lipid levels—would be expected, on the basis of previous studies, to reduce atherosogenesis.

The signaling pathway that led to increased cholesterol efflux of Pgg1tb−/− macrophages was triggered by activated RHOA, was dependent on COX2 activity, and was associated with increased expression of COX2, PPARγ, CD36, and SR-B1. RHOA was upstream in this pathway because RHOA inhibition normalized both the gene-expression changes and cholesterol efflux. Treatment with a COX2 inhibitor also normalized efflux. Knockdown experiments demonstrated that SR-B1 and CD36 were functionally involved in the increased basal and HDL-stimulated cholesterol efflux in Pgg1tb−/− macrophages.

LXRα expression was increased in Pgg1tb−/− macrophages, but its targets, ABCA1 and ABCG1, were probably not involved in the increased efflux because their expression was unaffected by Pgg1tb deficiency and because knockdown of those genes had no impact on cholesterol efflux. One potential explanation for why the high levels of LXRα in Pgg1tb−/− macrophages did not affect ABCA1 and ABCG1 expression
could be an accumulation of geranylgeranyl pyrophosphate, one of the substrates for GGTase-I. Geranylgeranyl pyrophosphate and its metabolite, GGOH, block the transcriptional activity of LXRα by interfering with the interaction between LXRα and nuclear coactivators.25–53

The finding that ABCA1 and ABCG1 were not involved in the increased cholesterol efflux of \( P_{gggt1b}^{-/-} \) macrophages was surprising because those transporters are considered crucial for the unloading of cholesterol from macrophages.46,49 However, SR-B1 and CD36 contributed to macrophage cholesterol efflux in previous studies.40–42 and our present experiments provide strong evidence for their contribution to efflux of \( P_{gggt1b}^{-/-} \) macrophages.

Blocking GGTase-I activity in other tissues can be associated with significant pathology;44; thus, our results should not prompt unbridled enthusiasm for using GGTIs in the treatment of atherosclerosis. However, they demonstrate that blocking GGTase-I activity in macrophages is atheroprotective despite local and systemic inflammation and despite the presence of severe rheumatoid arthritis. The results also shed light on mechanisms underlying pleiotropic effects of statins. Many studies, including several that assessed the impact of statins on cholesterol efflux,9,13–16,21,22,55,56 concluded that statins and GGTIs inhibit the geranylgeranylation of RHO proteins, interfering with membrane attachment and thereby inactivating the proteins. Most of those studies were performed on the assumption that nonprenylated RHO proteins are GDP bound and inactive. But, with few exceptions, these studies did not assess the activation status of RHO proteins in statin- and GGTI-treated cells. Our genetic experiments clearly demonstrate that nonprenylated RHO proteins are GTP bound and active in macrophages and should refine our understanding of the cellular and in vivo importance of CAAX protein geranylgeranylation.

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Disclosures

None.

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Targeting GGTase-I activates RHOA, increases macrophage reverse cholesterol transport, and reduces atherosclerosis in mice

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Supplementary Figure 1

(A) Quantification of Oil Red O staining of lipid lesions in aortic root sections expressed as percent of total aortic root area. Right panel, representative photographs of Oil Red O–stained aortic root sections. Scale bar, 50 μm. The mice were fed a high-fat diet for 12 weeks.

(B) Quantification of necrosis in aortic root sections. The mice were fed a high-fat diet for 24 weeks. Right panel, photographs of Oil Red O–stained aortic root lesions; areas of necrosis are indicated. Scale bar, 20 μm.

(C) Quantification of TUNEL positive cells in aortic root lesions from mice shown in panel B. Right panel, representative immunofluorescence images. Thymus was used as a positive control. Scale bar, 20 μm.

(D) Cholesterol and triglycerides levels in FPLC-fractionated plasma pooled from 10 mice per genotype. The mice were fed a high-fat diet for 24 weeks.
Supplementary Figure 2

(A) Average diameter of BM and IP macrophages in suspension. (B) Average circularity of BM and IP macrophages in suspension. Values were obtained in a Vi-Cell XR cell counter (Beckman Coulter).
Supplementary Figure 3 HPLC- and mass spectrometry–based lipidomic analyses of cholesterol esters (A); ceramide (CER) and glucosylceramide (gluCER) (B); phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin (SM) (C) in BM macrophages before and after a 36-h incubation with acLDL (50 μg/ml). * P < 0.05 and ** P < 0.01.
Supplementary Figure 4

(A) ApoA1-mediated cholesterol efflux in BM macrophages incubated with DMSO, FTI (10 μM), and GGTI (10 μM) (n = 3–4/treatment). (B) HDL-mediated cholesterol efflux in BM macrophages incubated with DMSO, FTI, and GGTI (n = 2/treatment).
Supplementary Figure 5

(A) TUNEL staining of BM macrophages incubated for 24 h with 50 μg acLDL. Etoposide (25 μM) was used as a positive control. Right panel, representative immunofluorescence images. Scale bar, 10 μm. (B) Levels of lactate dehydrogenase (LDH; cytotoxicity assay) in cell culture media of BM macrophages from the efflux phase of the cholesterol efflux assay (n = 3/genotype). (C) Basal cholesterol efflux of BM macrophages incubated with etoposide (25 μM) or DMSO during the equilibration and efflux phases of the cholesterol efflux assay (n = 3 Pggt1bΔ/Δ and 4 Pggt1b+/+ cell lines).
Supplementary Figure 6 (A) Immunohistochemical staining of SR-BI in aortic root sections of mice fed a high-fat diet for 12 weeks. Scale bar, 10 μm (B) Quantification of SR-BI staining in aortic root lesions (n = 10/genotype).
Supplementary Figure 7

(A) Taqman analyses showing gene expression in BM macrophages incubated with lentiviruses expressing shRNAs for Abca1, Abcg1, Cd36, and Scarb1, or containing a scrambled (SCR) sequence (n = 2/treatment).

(B, C) Basal (B) and HDL-mediated (C) cholesterol efflux in BM macrophages incubated with lentiviruses described in A (n = 6–9/treatment). * P < 0.05.
Supplementary Figure 8 (A) Basal cholesterol efflux in THP-1 human macrophages incubated with DMSO or GGTI (10 μM) for 48 h. Values are the mean of two independent experiments performed in triplicate. (B) TaqMan analysis showing gene expression in THP-1 macrophages incubated with DMSO or GGTI (10 μM) for 48 h (n = 4/treatment). (C) Western blots of lysates from THP-1 macrophages incubated with DMSO or GGTI for 48 h. The experiment was repeated three times with similar results. * P < 0.05 and ** P < 0.01.
Supplementary Figure 9

(A) Western blots showing levels of GTP-bound and total RHOA, RAC1, and CDC42 in lysates of BM macrophages. (B) Taqman analyses showing gene expression in Pggt1b Δ/Δ BM macrophages incubated with lentiviruses expressing shRNAs targeting RHOA, RAC1, and CDC42, or containing a scrambled (SCR) sequence (n = 3/treatment). (C) Basal cholesterol efflux in BM macrophages incubated with DMSO, ROCK inhibitor, and PAK kinase inhibitor (n = 6–8/genotype).

* P < 0.05, ** P < 0.01, and *** P < 0.001.