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 (Pggt1b−/−) contained significantly more T lymphocytes than the lesions in controls. Surprisingly, however, mean atherosclerotic lesion area in Pggt1b−/− 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 Pggt1b 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 proinflammatory cytokines. The increased production of proinflammatory cytokines may contribute to atherosclerosis; therefore, a reduction in proinflammatory cytokines may reduce the development of atherosclerosis.
of geranylgeranyl lipids, which in turn reduces RHO protein geranylgeranylation. There has been considerable support for the notion that blocking geranylgeranylation inactivates RHO proteins.

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. 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. For example, several studies have suggested that activation of RHOA and CDC42 inhibits pneumosome proliferate-activated-γ (PPARγ) activity and cholesterol efflux in macrophages. 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. Second, GGTase-I–deficient mice develop rheumatoid arthritis, an inflammatory disorder associated with a high risk of atherosclerosis in humans. Third, activation of RHO proteins should inhibit cholesterol efflux and stimulate foam cell formation.

**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 (Pggt1bfl/flLC) have been described. PGgt1bfl/flLC mice were bred with LDL receptor knockout mice (Ldl−/−; Jackson Laboratories) and backcrossed 6 times to a C57BL/6 background. Mice were genotyped as described. PGgt1bfl/flLC;Ldl−/− mice were designated PGgt1bfl/flLC;Ldl−/−; littermate control PGgt1b+/+LC;Ldl−/− and PGgt1b−/−LC;Ldl−/− mice were indistinguishable in phenotype and designated PGgt1b−/−. 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. Aortic roots were frozen in optimal cutting temperature freezing medium, and 10-µm-thick sections were stained with Oil Red O and hematoxylin.

**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. 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 (MCA519G, 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. Plasma cytokines were quantified with the Mouse Proinflammatory 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% HEPES, 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. Experiments were done on differentiated macrophages 7 to 10 days after plating. Intraportal 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. 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 4°C and 16 hours at 37°C. 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. 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
robic 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.\(^9\)

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

Macrophages were incubated with 1 to 2 \(\mu\) Ci \([^{3}H]\)cholesterol (pre-incubated with 50 \(\mu\)g/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 \(\mu\)g/mL), or high-density lipoprotein (HDL; 100 \(\mu\)g/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 relative to total radioactivity in cells and medium, normalized to total protein content. Macrophage apoptosis was detected with the ApopTag Fluorescein In Situ Apoptosis Detection Kit (S7110, Millipore), and lactate dehydrogenase in media was detected with the Cytotoxicity Detection Kit (11644793001, Roche).

**Western Blotting**

Cells were lysed as described.\(^9\) GTP-bound RAC1 and CDC42 were affinity precipitated with PAK-1-GST (EZ Detect Rac Activation Kit, Pierce), and GTP-bound RHOA was precipitated with Rhotakin-GST (RhoA Activation Biochem Kit, Cytoskeleton) as described.\(^9\) Total lysates or affinity-precipitated proteins were resolved on 12% and 7.5% SDS-PAGE gels (Cytoskeleton, CDC42 (sc-2462), nPRAP1A (sc-1482, Santa Cruz Biotechnology), liver X receptor-\(\alpha\) (LXR\(\alpha\); ab41902, Abcam), cyclodextrin (COX2; NB110-1948), 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).

**PPAR\(\gamma\) Activity Assay**

Macrophages were cultured to 75% confluence on 150-mm 2 plates and differentiated into macrophage-like cells with phorbol 12-myristate 13-acetate.\(^9\) For cholesterol efflux, gene expression analyses, and Western blotting, THP-1 macrophages were incubated with a GGTA1 (1, 5, and 10 \(\mu\)mol/L) for 48 hours before experiments.

**Reverse Cholesterol Transport**

Bone marrow macrophages were loaded with 25 \(\mu\)g/mL acLDL and 5 \(\mu\)Ci/mL \([^{3}H]\)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 1.3×10⁶ counts per minute (CPM) was injected intraperitoneally into Pgg1b+/− recipient mice. Plasma samples were obtained at baseline and after 24 and 48 hours, and 5-\(\mu\)L 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-\(\mu\)L 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.\(^9,39\)

**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 GGTA1 Reduces Atherosclerosis but Increases Inflammation**

To define the role of macrophage GGTA1 in atherosclerosis, we bred mice lacking Pgg1b expression in macrophages\(^26\) onto an Ldlr-deficient background. These mice, designated Pgg1b+/−, were fed a Western-type diet for 12 or 24 weeks. Atherosclerotic lesions in Pgg1b+/− 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 1A in the online-only Data Supplement). Areas of necrosis in aortic root sections were less frequent in Pgg1b+/− than in control Pgg1b+/+ mice; however, levels of apoptotic cells did not differ (Figure 1B and IC in the online-only Data Supplement). Body weight curves in Pgg1b+/− and control Pgg1b+/+ mice were similar, but Pgg1b+/− mice had slightly lower plasma cholesterol and triglyceride levels (Figure 1B and 1C). The lower plasma lipid levels were also evident when the plasma lipoproteins were fractionated by fast protein liquid chromatography (Figure 1D and Figure 1E in the online-only Data Supplement).

The macrophage lesion area of aortic root sections was reduced in Pgg1b+/− 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
antibody-2 and α-smooth muscle actin (Figure 2A and 2B). However, the number of T cells in lesions was 2-fold higher in \( \text{Pggt1b}\)–/– mice than in controls, as judged by immunostaining for CD4 (Figure 2C) and CD8 (not shown). Quantification of inflammatory markers in plasma revealed increased levels of the proinflammatory cytokine CXCL1 and reduced levels of the antiinflammatory cytokine interleukin-10 in \( \text{Pggt1b}\)–/– mice (Figure 2D). Consistent with the increased inflammatory response in vivo, cytokine production was markedly increased in \( \text{Pggt1b}\)–/– macrophages (Figure 2E). Similar results were observed in bone marrow–derived macrophages (not shown). Thus, GGTase-I–deficient macrophages are capable of robust inflammatory responses, but the \( \text{Pggt1b}\)–/– mice exhibited smaller atherosclerotic lesions.

**Knockout of GGTase-I Impairs Macrophage Foam Cell Formation**

To determine whether GGTase-I deficiency affects lipid accumulation, we quantified Oil Red O staining in macrophages incubated for 24 hours with acLDL and minimally modified LDL. \( \text{Pggt1b}\)–/– macrophages had lower levels of staining compared with \( \text{Pggt1b}\)+/+ cells (Figure 3A and 3B). To exclude the possibility that the spreading defect of \( \text{Pggt1b}\)–/– macrophages confounds 2-dimensional analysis of lipid accumulation, we quantified uptake of Alexa Fluor–conjugated acLDL by fluorescence-activated cell sorter analysis. (\( \text{Pggt1b}\)+/+ and \( \text{Pggt1b}\)–/– macrophages in suspension are similar in size and shape; Figure II in the online-only Data Supplement.) The fluorescence-activated cell sorter analyses also revealed reduced levels of lipid accumulation by \( \text{Pggt1b}\)–/– cells (Figure 3C). Consistent with those observations, after a 36-hour incubation with acLDL, cholesterol ester levels were 45% lower in \( \text{Pggt1b}\)–/– macrophages than in \( \text{Pggt1b}\)+/+ macrophages, as judged by enzymatic assays and lipidomics analyses (Figure 3D and Figure IIIA in the online-only Data Supplement). Moreover, ceramide and phospholipid levels were 37% to 55% lower in \( \text{Pggt1b}\)–/– macrophages (Figure IIIB and IIIC in the online-only Data Supplement). Basal lipid levels were similar in \( \text{Pggt1b}\)–/– and \( \text{Pggt1b}\)+/+ macrophages (Figure IIIA–IIIC in the online-only Data Supplement).

**GGTase-I Deficiency Increases Cholesterol Efflux by COX2- and PPARγ-Mediated Increase in SR-B1 and CD36**

\( \text{Pggt1b}\)–/– macrophages have normal phagocytic activity. Thus, we asked whether increased lipid export might account for the reduced lipid accumulation in \( \text{Pggt1b}\)–/– macrophages. For this, we loaded cells with \(^{1}H\)cholesterol and measured cholesterol efflux at baseline and after adding apolipoprotein AI or HDL. Basal levels of efflux in \( \text{Pggt1b}\)–/– macrophages were 4-fold higher than in \( \text{Pggt1b}\)+/+ macrophages (Figure 4A and 4B). Efflux was increased 2-fold by apolipoprotein AI and 3-fold by HDL (Figure 4A and 4B). In keeping with these findings, cholesterol efflux was higher in \( \text{Pggt1b}\)–/– macrophages incubated with a protein GGTI. In addition, the high levels of cholesterol efflux in \( \text{Pggt1b}\)–/– macrophages could be reduced with a lentivirus expressing human PGGT1B. A protein farnesyltransferase inhibitor had no impact on...
cholesterol efflux in Pggt1b+/+ macrophages (Figure 4C–4F and Figure IVA and IVB in the online-only Data Supplement).

We asked if apoptosis of Pggt1b−/− macrophages accounts for the increased release of [3H]cholesterol to the media in the efflux assays. Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining of Pggt1b−/− and Pggt1b+/+ macrophages after incubation with acLDL revealed no differences in levels of apoptosis. Cell death was slightly reduced in Pggt1b−/− 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 Pggt1b−/− macrophages (Figure VC in the online-only Data Supplement).

To identify the pathways that mediate increased cholesterol efflux in Pggt1b−/− macrophages, we quantified levels of PPARγ using nuclear extracts with an ELISA; we also assessed levels of Pparg transcripts. Basal PPARγ activity was 2-fold higher and Pparg mRNA levels were 4-fold higher in Pggt1b−/− macrophages than in Pggt1b+/+ macrophages (Figure 5A and 5B). Basal expression levels of COX2 and LXRα were also higher, as judged by mRNA levels and Western blots (Figure 5B and 5C). When COX2 activity in Pggt1b−/− macrophages was inhibited with Celecoxib, cholesterol efflux returned to normal levels (Figure 5D).

Cholesterol efflux can be mediated by PPARγ-LXRα−induced upregulation of ABCA1 and ABCG1 and by COX2-PPARγ−mediated upregulation of CD36 and SR-B1.40–42 ABCA1 and ABCG1 expression in Pggt1b−/− macrophages was not different from that in Pggt1b+/+ 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 Pggt1b−/− 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 Pggt1b−/− macrophages, we suppressed their expression with lentiviral shRNAs. We also suppressed expression of ABCA1 and ABCG1 in our studies, Abca1, Abcg1, Cd36, and Scarb1 transcripts were reduced by 60% to 80% (Figure VIA in the online-only Data Supplement). Knockdown of SR-B1 reduced basal cholesterol efflux in Pggt1b+/+ macrophages, and knockdown of CD36 reduced HDL-stimulated efflux (Figure VIIIB and VIIC in the online-only Data Supplement). Knockdown of ABCA1 and ABCG1 had no discernible effect on cholesterol efflux in Pggt1b+/+ macrophages.
the expression of ABCA1 or ABCG1. Thus, mouse and human macrophages respond similarly to GGTase-I inhibition.

**RHOA Activation Mediates the Increased Cholesterol Efflux in GGTase-I–Deficient Macrophages**

We previously showed that GGTase-I–deficient macrophages accumulate high levels of active GTP-bound RHOA, RAC1, and CDC42, and we confirmed those results in the present model (Figure IXA in the online-only Data Supplement). To determine whether activated RHO proteins contribute to cholesterol efflux in Pgggt1b−/− 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 Pgggt1b−/− macrophages to the levels observed in Pgggt1b+/- macrophages (Figure 5E). RHOA knockdown also reduced the expression of Cox2, Pparg, Lxra, Cdx36, and Scarb1 (Figure 5F). Consistent with these findings, cholesterol efflux in Pgggt1b−/− 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 Pgggt1b−/− macrophages.

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

To determine whether the increased cholesterol efflux in Pgggt1b−/− macrophages results in higher levels of reverse cholesterol transport in vivo, we loaded Pgggt1b+/- and Pgggt1b−/− macrophages with [3H]cholesterol:acLDL, injected the cells into the peritoneal cavity of Pgggt1b+/- 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 Pgggt1b−/− macrophages than in those injected with Pgggt1b+/- 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 Pgggt1b+/- 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 Pgggt1b−/− macrophages (n=7 per genotype). **P<0.01; ***P<0.001.

To assess the effects of GGTase-I inhibition on human macrophages, we incubated THP1 cells with a GGTI. Basal cholesterol efflux increased, as did mRNA and protein expression of COX2, PPARG, CD36, and SCARB1 (Figure VIII–VIIIIC in the online-only Data Supplement). The GGTI did not affect the expression of ABCA1 or ABCG1. Thus, mouse and human macrophages respond similarly to GGTase-I inhibition.

**Figure 4.** Increased cholesterol efflux in macrophages from Pgggt1b−/− 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 Pgggt1b−/− BM macrophages and in Pgggt1b+/- macrophages incubated with a geranylgeranyltransferase type I (GGTase-I; 10 µmol/L) and low levels in Pgggt1b−/− cells and in Pgggt1b+/- 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 lentiv-PGGT1B 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 GGTase-I deficiency in macrophages markedly reduces atherosclerosis despite higher levels of inflammation. GGTase-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 Pggt1b−/− mice also develop rheumatoid arthritis, which is associated with more atherosclerotic disease in humans. Thus, the lower levels of atherosclerosis in Pggt1b−/− mice were surprising. Uncoupling of inflammation and lipid accumulation in atherogenesis has been observed previously, although not to the same degree as in Pggt1b−/− mice.

The finding of reduced atherosclerosis in Pggt1b−/− mice is likely explained by 3 factors. First, foam cell formation in Pggt1b−/− 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 Pggt1b−/− macrophages resulted in increased levels of reverse cholesterol transport in Pggt1b−/− mice. Third, plasma lipid levels were slightly lower in Pggt1b−/− 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 atherogenesis.

The signaling pathway that led to increased cholesterol efflux of Pggt1b−/− 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 Pggt1b−/− macrophages.

LXRα expression was increased in Pggt1b−/− macrophages, but its targets, ABCA1 and ABCG1, were probably not involved in the increased efflux because their expression was unaffected by Pggt1b deficiency and because knockdown of those genes had no impact on cholesterol efflux. One potential explanation for why the high levels of LXRs in Pggt1b−/− macrophages did not affect ABCA1 and ABCG1 expression...
could be an accumulation of geranylgeranyl pyrophosphate, one of the substrates for GGTLase-I. Geranylgeranyl pyrophosphate and its metabolite, GGOH, block the transcriptional activity of LXRα by interfering with the interaction between LXRα and nuclear coactivators.\textsuperscript{25–53}

The finding that ABCA1 and ABCG1 were not involved in the increased cholesterol efflux of \textit{Pggt1b}\textsuperscript{-/-} macrophages was surprising because those transporters are considered crucial for the unloading of cholesterol from macrophages.\textsuperscript{48,49} However, SR-B1 and CD36 contributed to macrophage cholesterol efflux in previous studies.\textsuperscript{40–42} and our present experiments provide strong evidence for their contribution to efflux of \textit{Pggt1b}\textsuperscript{-/-} macrophages.

Blocking GGTLase-I activity in other tissues can be associated with significant pathology;\textsuperscript{54} thus, our results should not prompt unbridled enthusiasm for using GGTLases in the treatment of atherosclerosis. However, they demonstrate that blocking GGTLase-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,\textsuperscript{9,13–16,21,22,55,56} concluded that statins and GGTLIs 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 GGTLI-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.

References


24. Symmons DP, Gabriel SE. Epidemiology of CVD in rheumatic disease, with a focus on RA and SLE. \textit{Nat Rev Rheumatol}. 2011;7:399–408.


Moreover, reduced prenylation of RHO proteins is frequently cited as a mechanism for the antiatherogenic properties of statins. Prenylation in the face of inflammation and rheumatoid arthritis, diseases that normally carry increased risks of atherosclerosis.

γ-sterase 2–peroxisome proliferator-activated receptor-γ (PGC-1γ) signaling, which resulted in increased expression of the scavenger receptors SR-B1 and CD36. The results are clinically relevant in that they demonstrate a potent antithromogenic effect of blocking prenylation in the face of inflammation and rheumatoid arthritis, diseases that normally carry increased risks of atherosclerosis. Moreover, reduced prenylation of RHO proteins is frequently cited as a mechanism for the antithrombogenic properties of statins.

**Clinical Perspective**

The RHO family proteins undergo posttranslational lipidation by protein geranylgeranyltransferase (GGTase-I). It is widely believed that geranylgeranylation is essential for membrane targeting and activity of the RHO proteins; this notion is supported by numerous studies using statins to reduce prenylation in cells. However, we recently demonstrated that knockdown of GGTase-I in macrophages hyperactivates RHOA, RAC1, and CDC42; increases cytokine production; and induces rheumatoid arthritis in mice. In this study, we tested whether the increased inflammatory signaling of GGTase-I–deficient macrophages and the systemic inflammation and rheumatoid arthritis would accelerate atherosclerosis development in low-density lipoprotein receptor–deficient mice. As expected, aortic lesions in mice lacking GGTase-I in macrophages contained significantly more foam cells when compared with control. But surprisingly, aortic lesions were markedly reduced. Analyses of GGTase-I–deficient macrophages revealed reduced foam cell formation and a striking increase in basal and high-density lipoprotein–stimulated cholesterol efflux, which resulted in increased levels of macrophage-stimulated reverse cholesterol transport in vivo. The increased cholesterol efflux of GGTase-I–deficient macrophages was caused by RHOA-mediated upregulation of cyclooxygenase 2–peroxisome proliferator-activated receptor-γ signaling, which resulted in increased expression of the scavenger receptors SR-B1 and CD36. The results are clinically relevant in that they demonstrate a potent antithromogenic effect of blocking prenylation in the face of inflammation and rheumatoid arthritis, diseases that normally carry increased risks of atherosclerosis.
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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 Pgtt1bΔ/Δ and 4 Pgtt1b+/+ 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 \( Pggt1b^{+/}\) 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 *** \( P < 0.001 \).