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

Running title: Khan et al; The role of macrophage GGTase-I in atherosclerosis

Omar M. Khan, PhD1; Murali K. Akula, MS1; Kristina Skålen, PhD2; Christin Karlsson, PhD1; Marcus Ståhlman, PhD2; Stephen G. Young, MD3; Jan Borén, MD, PhD2; Martin O. Bergo, PhD1

1Sahlgrenska Cancer Center; 2Wallenberg Laboratory, Dept of Molecular and Clinical Medicine, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden; 3Depts of Medicine and Human Genetics, David Geffen School of Medicine, University of California, Los Angeles, CA

Address for Correspondence:
Martin Bergo, PhD
Sahlgrenska Cancer Center
Medicinaregatan 1G, Box 425
SE-413 90 Gothenburg, Sweden
Tel: +46317866731
Fax: +46-31-7866731
E-mail: martin.bergo@gu.se

Abstract:

Background—Statins have anti-inflammatory 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 LDL receptor–deficient mice.

Methods and Results—Aortic lesions in mice lacking GGTase-I in macrophages (Pggt1bΔ/Δ) contained significantly more T lymphocytes than the lesions in controls. Surprisingly, however, mean atherosclerotic lesion area in Pggt1bΔ/Δ mice was reduced by ~60%. GGTase-I deficiency reduced the accumulation of cholesterol esters and phospholipids in macrophages incubated with minimally modified and acetylated LDL. Analyses of GGTase-I-deficient macrophages revealed upregulation of the COX2-PPARγ pathway and increased SR-B1- and CD36-mediated basal and HDL-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.

Key words: atherosclerosis, prenylation, macrophage, cholesterol efflux
Introduction

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 geranylglyceranyltransferase 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 (FTase). 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 treat arthritis, atherosclerosis, and other inflammatory disorders. Reduced geranylgeranylation and inhibition of RHO proteins have also been proposed to explain the anti-inflammatory and some of the anti-atherogenic properties of statins. Statins reduce plasma cholesterol levels but also interfere with the production 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 NFκB signaling, increased reactive oxygen species and 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, two processes relevant to the pathogenesis and treatment of atherosclerosis. For example, several studies have suggested that activation of RHOA and CDC42 inhibits PPAR 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 LDL receptor–deficient mice. We hypothesized that knockout of GGTase-I would accelerate atherosclerosis. This hypothesis was based on three 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.26 Pggt1bfl/flLC mice were bred with LDL receptor knockout mice (Ldlr−/−; Jackson Laboratories) and backcrossed six times to a C57BL/6 background. Mice were genotyped as described.26,27 Pggt1bfl/flLC;Ldlr−/− mice were designated Pggt1bΔ/Δ; littermate control Pggt1b+/+LC;Ldlr−/− and Pggt1b+/+LC;Ldlr−/− mice were indistinguishable in phenotype and designated Pggt1b+/+. Six- to seven-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 OCT, 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 MOMA2 (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-plexUltrasensitive Kit in a Sector 2400 Imager (Meso Scale Discovery).

Generating BM and peritoneal macrophages

BM cells were cultured in high-glucose DMEM supplemented with 10% fetal calf serum, 1% HEPES, 1% glutamine, 1% gentamycin, 0.01% β-mercaptoethanol, and 10% whole supernatant of cell line CMG14-12 as a source of M-CSF. Experiments were done on differentiated macrophages 7–10 days after plating. Intraperitoneal macrophages were collected after rinsing of the mouse peritoneal cavity with 5 ml PBS. Experiments were done 1–3 days after plating.

Cytokine array

Macrophages were incubated with mmLDL for 24 h, 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 acLDL or mmLDL for 24 h, 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 FACS after cells were incubated with Alexa Fluor 488–labeled acLDL (I23380, Invitrogen) for 30 min at 4°C and 16 h at 37°C. Cholesterol ester content in macrophages was measured 36 h after incubation with acLDL. Neutral lipids were extracted in 2:3 n-hexane/isopropanol for 20 min, dried, and dissolved in
10% Triton X-100 in isopropanol. Total 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 BM macrophages using the Folch procedure. Cholesterol esters were quantified using straight-phase high-performance liquid chromatography (HPLC) with evaporative light-scattering detection; phospholipids were analyzed with a QTRAP 5500 mass spectrometer equipped with a NanoMate HD robotic nanoflow ion source (Advion Biosciences); and ceramide and glucosylceramide were analyzed by HPLC coupled to a Quattro Premier XE triple quadropole mass spectrometer.

**Cholesterol efflux assay, apoptosis, and cell death analyses**

Macrophages were incubated with 1–2 μCi [3H]cholesterol (preincubated with 50 μg/ml acLDL) in medium containing endotoxin- and free fatty acid–free 0.2% BSA for 24 h. Cholesterol pools were equilibrated overnight in medium containing 0.1% BSA. Basal and lipid acceptor–stimulated efflux was measured 6 h after addition of vehicle, apoAI (10 μg/ml), or 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, normalized to total protein content. Macrophage apoptosis was detected with the ApopTag Fluorescein In Situ Apoptosis Detection Kit (S7110, Millipore) and lactate dehydrogenase (LDH) in media was detected with the Cytotoxicity Detection Kit (11644793001, Roche).

**Western blotting**

Cells were lysed as described. GTP-bound RAC1 and CDC42 were affinity precipitated with PAK1-GST (EZ Detect Rac1 Activation Kit, Pierce), and GTP-bound RHOA was precipitated
with Rhotekin-GST (RhoA Activation Biochem Kit, Cytoskeleton) as described. Total lysates or affinity-precipitated proteins were resolved on 12% and 7.5% SDS-PAGE gels (Criterion and Protean, BioRad), transferred to nitrocellulose or PVDF membranes, and incubated with antibodies to RAC1 (05-389, Millipore), RHOA (ARH03-B, Cytoskeleton), CDC42 (sc-2462), np-RAP1A (sc-1482, Santa Cruz Biotechnology), LXRα (ab41902, Abcam), COX2 (NB110-1948), CD36 (NB400-144), SR-B1 (NB400-104), ABCA1 (NB400-105), 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 ECL 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% confluency on 150-mm² plates and incubated without M-CSF for 12 h. 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, Hs01059118_m1), *Abcg1* (Mm00437390_m1, Hs00245154_m1), *Cox2* (m00478374_m1, Hs00153133_m1), *Cd36* (Mm00432403_m1, Hs01567185_m1), *Scarb1* (Mm00450234_m1, Hs00969821_m1), *Pparg* (Mm01184322_m1, Hs01115513_m1), and *Lxra* (Mm00443451_m1). Values were normalized to *Gapdh* (Mm4352932E, Hs402869).
Lentivirus experiments and inhibitors

Lentiviruses expressing short hairpin (sh) RNAs targeting mouse ABCA1 (TRCN0000271812-60), ABCG1 (TRCN0000105286–87), CD36 (TRCN000066518–22), RHOA (TRCN00000055192), RAC1 (TRCN0000304690), 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 an MOI of 10^{20} for 72–96 h before experiments. Lentiviral construct expressing human PGGT1B gene was from SBI. Inhibitors of GGTase-I (GGTI-298), FTase (FTI-276), COX2 (Celecoxib, PZ0008), and RHO-associated protein kinase (ROCK) (Y-27632), and etoposide (E1383) were from Sigma; the inhibitor of PAK kinase (PAK18) was from Merck.

THP-1 cells

Human acute monocytic leukemia cell line THP-1 was differentiated into macrophage-like cells with phorbol 12-myristate 13-acetate. For cholesterol efflux, gene expression analyses, and western blotting, THP-1 macrophages were incubated with a GGTI (1, 5, and 10 μM) for 48 h before experiments.

Reverse cholesterol transport

BM macrophages were loaded with 25 μg/ml acLDL and 5 μCi/ml [3H]cholesterol for 30 h, washed twice with PBS, scraped into RPMI-1640/0.2% BSA, spun (1000 rpm, 5 min, 4 °C), and resuspended in RPMI-1640/0.2% BSA. Cell viability and radioactivity were counted and 1.3 × 10^6 CPM was injected intraperitoneally into Pgg1bΔ/Δ recipient mice. Plasma samples were obtained at baseline and after 24 and 48 h, and 5-μl aliquots were counted in a scintillation counter. Feces were collected at 0, 24 and 48 h, soaked in distilled water (100 mg/ml) overnight at 4 °C, and homogenized in an equal volume of ethanol; 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 when 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 Ldlr-deficient background. These mice, designated Pggt1bΔ/Δ, were fed a Western-type diet for 12 or 24 weeks. Atherosclerotic lesions in Pggt1bΔ/Δ 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 (Supplementary Figure 1A). Areas of necrosis in aortic root sections were less frequent in Pggt1bΔ/Δ than in control Pggt1b+/+ mice; however, levels of apoptotic cells did not differ (Supplementary Figure 1B and 1C). Body weight curves in Pggt1bΔ/Δ and control Pggt1b+/+ mice were similar, but Pggt1bΔ/Δ 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 FPLC (Figure 1D and Supplementary Figure 1D).

The macrophage lesion area of aortic root sections was reduced in Pggt1bΔ/Δ 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 (MOMA-2) and SMα (Figure 2A and 2B). However, the number of T-cells in lesions was twofold higher in

\(Pggt1b^{Δ/Δ}\) 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 anti-inflammatory cytokine IL-10 in \(Pggt1b^{Δ/Δ}\) mice (Figure 2D). Consistent with the increased inflammatory response in vivo, cytokine production was markedly increased in \(Pggt1b^{Δ/Δ}\) intraperitoneal macrophages incubated with minimally modified (mm) LDL (Figure 2E). Similar results were observed in bone marrow–derived (BM) macrophages (not shown). Thus, GGTase-I-deficient macrophages are capable of robust inflammatory responses but the \(Pggt1b^{Δ/Δ}\) 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 h with acetylated (ac) LDL and mmLDL. \(Pggt1b^{Δ/Δ}\) macrophages had lower levels of staining compared to \(Pggt1b^{+/+}\) cells (Figure 3A and 3B). To exclude the possibility that the spreading defect of \(Pggt1b^{Δ/Δ}\) macrophages 17 confounds two-dimensional analysis of lipid accumulation, we quantified uptake of Alexa Fluor–conjugated acLDL by FACS. \(Pggt1b^{+/+}\) and \(Pggt1b^{Δ/Δ}\) macrophages in suspension are similar in size and shape; Supplementary Figure 2.) The FACS analyses also revealed reduced levels of lipid accumulation by \(Pggt1b^{Δ/Δ}\) cells (Figure 3C). Consistent with those observations, after a 36-h incubation with acLDL, cholesterol ester levels were 45% lower in \(Pggt1b^{Δ/Δ}\) macrophages than
in \( \text{Pggt1b}^{+/+} \) macrophages, as judged by enzymatic assays and lipidomics analyses (Figure 3D and Supplementary Figure 3A). Moreover, ceramide and phospholipid levels were 37–55% lower in \( \text{Pggt1b}^{\Delta/\Delta} \) macrophages (Supplementary Figure 3B and 3C). Basal lipid levels were similar in \( \text{Pggt1b}^{\Delta/\Delta} \) and \( \text{Pggt1b}^{+/+} \) macrophages (Supplementary Figure 3A–C).

GGTase-I deficiency increases cholesterol efflux by COX2- and PPAR\( \gamma \)-mediated increase in SR-B1 and CD36

\( \text{Pggt1b}^{\Delta/\Delta} \) macrophages have normal phagocytic activity.\(^{17} \) Thus, we asked whether increased lipid export might account for the reduced lipid accumulation in \( \text{Pggt1b}^{\Delta/\Delta} \) macrophages. For this, we loaded cells with \([3H]\)cholesterol and measured cholesterol efflux at baseline and after adding apoAI or HDL. Basal levels of efflux in \( \text{Pggt1b}^{\Delta/\Delta} \) macrophages were fourfold higher than in \( \text{Pggt1b}^{+/+} \) macrophages (Figure 4A and 4B). Efflux was increased twofold by apoAI and threefold by HDL (Figure 4A and 4B). In keeping with these findings, cholesterol efflux was higher in \( \text{Pggt1b}^{+/+} \) macrophages incubated with a protein geranylgeranyltransferase inhibitor (GGTI). Also, the high levels of cholesterol efflux in \( \text{Pggt1b}^{\Delta/\Delta} \) macrophages could be reduced with a lentivirus expressing human \( \text{PGGT1B} \). A protein farnesyltransferase inhibitor (FTI) had no impact on cholesterol efflux in \( \text{Pggt1b}^{+/+} \) macrophages (Figure 4C–F, and Supplementary Figure 4A and 4B).

We asked if apoptosis of \( \text{Pggt1b}^{\Delta/\Delta} \) macrophages accounts for the increased release of \([3H]\)cholesterol to the media in the efflux assays. TUNEL staining of \( \text{Pggt1b}^{\Delta/\Delta} \) and \( \text{Pggt1b}^{+/+} \) macrophages following incubation with acLDL revealed no differences in levels of apoptosis; levels of cell death was slightly reduced in \( \text{Pggt1b}^{\Delta/\Delta} \) macrophages, as judged by LDH
cytotoxicity assay (Supplementary Figure 5A and 5B). Moreover, apoptosis induction with etoposide did not affect levels of cholesterol efflux in *Pggt1b*Δ/Δ macrophages (Supplementary Figure 5C).

To identify the pathways that mediate increased cholesterol efflux in *Pggt1b*Δ/Δ macrophages, we quantified levels of PPARγ in nuclear extracts with an ELISA; we also assessed levels of *Pparg* transcripts. Basal PPARγ activity was twofold higher, and *Pparg* mRNA levels were fourfold higher, in *Pggt1b*Δ/Δ macrophages than in *Pggt1b*Δ/+ macrophages (Figure 5A and 5B). Basal expression levels of cyclo-oxygenase 2 (COX2) and liver X receptor alpha (LXRα) were also higher, as judged by mRNA levels and western blots (Figure 5B and 5C). When COX2 activity in *Pggt1b*Δ/Δ macrophages was inhibited with Celecoxib, cholesterol efflux returned to normal levels (Figure 5D).

Cholesterol efflux can be mediated by PPARγ-LXRα-induced upregulation of ATP-binding cassette (ABC) family members A1 and G1 and also by COX2-PRARγ-mediated upregulation of CD36 and scavenger receptor class B, type I (SR-B1).40–42 ABCA1 and ABCG1 expression in *Pggt1b*Δ/Δ macrophages was not different from that in *Pggt1b*Δ/+ 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*Δ/Δ mice, as judged by immunohistochemical studies (Supplementary Figure 6A and 6B).

To assess the importance of SR-B1 and CD36 in efflux of *Pggt1b*Δ/Δ macrophages, we suppressed their expression with lentiviral short hairpin (sh) RNAs. We also suppressed expression of ABCA1 and ABCG1. In our studies, *Abca1, Abcg1, Cd36,* and *Scarb1* transcripts were reduced by 60–80% (Supplementary Figure 7A). Knockdown of SR-B1 reduced basal
cholesterol efflux in \textit{Pggt1b}\textsuperscript{Δ/Δ} macrophages, and knockdown of CD36 reduced HDL-stimulated efflux (\textbf{Supplementary Figure 7B} and 7C). Knockdown of ABCA1 and ABCG1 had no discernible effect on cholesterol efflux in \textit{Pggt1b}\textsuperscript{Δ/Δ} macrophages.

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 \textit{COX2, PPARG, CD36}, and \textit{SCARBL} (\textbf{Supplementary Figure 8A–C}). The GGTI did not affect the expression of ABCA1 or ABCG1. Thus, mouse and human macrophages respond similarly to GGTase-I inhibition.

\textbf{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 \textsuperscript{17}, and we confirmed those results in the current model (\textbf{Supplemental Figure 9A}). To determine whether activated RHO proteins contribute to cholesterol efflux in \textit{Pggt1b}\textsuperscript{Δ/Δ} macrophages, we suppressed their expression with lentiviral shRNA. The shRNAs reduced expression of \textit{Rhoa, Rac1}, and \textit{Cdc42} transcripts by 50–75\% (\textbf{Supplementary Figure 9B}). Knockdown of RHOA, but not RAC1 and CDC42, reduced cholesterol efflux in \textit{Pggt1b}\textsuperscript{Δ/Δ} macrophages to the levels observed in \textit{Pggt1b}\textsuperscript{+/-} macrophages (\textbf{Figure 5E}). RHOA knockdown also reduced the expression of \textit{Cox2, Pparg, Lxra, Cd36}, and \textit{Scarb1} (\textbf{Figure 5F}). Consistent with these findings, cholesterol efflux in \textit{Pggt1b}\textsuperscript{Δ/Δ} macrophages was reduced when RHOA signaling was inhibited with a RHO kinase (ROCK) inhibitor. An inhibitor of RAC signaling had no effect (\textbf{Supplementary Figure 9C}). Overall, the data suggest that RHOA activation, acting upstream of COX2, increases cholesterol efflux in
GGTase-I deficiency increases macrophage-stimulated reverse cholesterol transport in vivo

To determine if the increased cholesterol efflux in *Pggt1bΔΔ* macrophages results in higher levels of reverse cholesterol transport in vivo, we loaded *Pggt1b*+/+ and *Pggt1bΔΔ* macrophages with [3H]cholesterol:acLDL, injected the cells into the peritoneal cavity of *Pggt1bΔΔ* mice, and measured radioactivity in plasma and feces after 24 and 48 h. Levels of [3H]cholesterol in plasma and feces were 1.5–2-fold higher in mice injected with *Pggt1bΔΔ* macrophages than in those injected with *Pggt1b*+/+ macrophages (Figure 6A and 6B).

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 mmLDL and T-cell recruitment to subendothelial lesions was increased. The *Pggt1bΔΔ* mice also develop rheumatoid arthritis, which is associated with more atherosclerotic disease in humans. Thus, the lower levels of atherosclerosis in *Pggt1bΔΔ* mice were surprising. Uncoupling of inflammation and lipid accumulation in atherogenesis has been observed previously, although not to the same degree as in *Pggt1bΔΔ* mice.

The finding of reduced atherosclerosis in *Pggt1bΔΔ* mice is likely explained by three factors. First, foam cell formation in *Pggt1bΔΔ* 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 Pggt1bΔ/Δ macrophages resulted in increased levels of reverse cholesterol transport in Pggt1bΔ/Δ mice. Third, plasma lipid levels were slightly lower in Pggt1bΔ/Δ 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, based on previous studies, to reduce atherogenesis.47–52

The signaling pathway that led to increased cholesterol efflux of Pggt1bΔ/Δ 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 as 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 Pggt1bΔ/Δ macrophages.

LXRα expression was increased in Pggt1bΔ/Δ 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 LXRα in Pggt1bΔ/Δ macrophages did not affect ABCA1 and ABCG1 expression could be an accumulation of GGPP, one of the substrates for GGTase-I. GGPP 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 $Pggf1b^{Δ/Δ}$ macrophages was surprising as 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 current experiments provide strong evidence for their contribution to efflux of $Pggf1b^{Δ/Δ}$ macrophages.

Blocking GGTase-I activity in other tissues can be associated with significant pathology;\textsuperscript{54} 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,\textsuperscript{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.

\textbf{Acknowledgements:} We thank Stephen Ordway for editing the manuscript and Maria Heyden and Drs. Pernilla Jirholt, Galia Nyström, and Lillemor Mattsson Hultén for advice and technical assistance.

\textbf{Funding Sources:} This study was supported by a Starting Investigator Grant from the European
Conflict of Interest Disclosures: None.

References:


10. Jain MK, Ridker PM. Anti-inflammatory effects of statins: Clinical evidence and basic


33. Takeshita S, Kaji K, Kudo A. Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts. J


54. Yang SH, Chang SY, Tu YP, Lawson GW, Bergo MO, Fong LG, Young SG. Severe hepatocellular disease in mice lacking one or both caax prenyltransferases. *J Lipid Res.* 2012;53:77-86.


Figure legends:

**Figure 1.** Knockout of macrophage GGTase-I reduces atherosclerosis development in Ldlr-deficient mice. (A) Quantification of lipid lesions in aortas of Pggt1b+/+ and Pggt1bΔ/Δ mice fed a high-fat diet for 12 and 24 weeks. Right panel, representative photographs of aortas. (B) Body weight curves of Pggt1b+/+ mice (n = 11) and Pggt1bΔ/Δ mice (n = 13) fed a high-fat diet. (C) Plasma cholesterol and triglyceride levels. Numbers in bars indicate number of mice. (D) Cholesterol and triglyceride levels in FPLC-fractionated plasma pooled from 10 mice per genotype. The mice were fed a high-fat diet for 12 weeks.

**Figure 2.** GGTase-I deficiency in macrophages increases inflammation. (A–C) Immunohistochemical analyses of the cellular composition in aortic root lesions of mice fed a high-fat diet for 12 weeks. Quantification (left) and representative photographs (right) of sections stained with MOMA-2 (macrophages) (A), SMα (smooth muscle cells) (B), and CD4 (T lymphocytes) (C). Numbers in bars indicate number of mice. (D) Cytokine levels in serum of mice fed a high-fat diet for 24 weeks (n = 10/genotype). (E) Cytokine levels in medium of Pggt1b+/+ and Pggt1bΔ/Δ intraperitoneal macrophages (n = 5/genotype) incubated with mmLDL for 24 h.

**Figure 3.** GGTase-I inactivation reduces macrophage foam cell formation. (A, B) Oil Red O staining of intraperitoneal macrophages incubated with (A) 50 µg/ml acLDL (n = 5–7/genotype) and (B) 50 µg/ml mmLDL (n = 3–4/genotype) for 24 h. Left panels, representative photographs. Right panels, quantification of staining divided by cell number and expressed as percent of that
in *Pggt1b*+/+ macrophages. (C) FACS analyses of the uptake of Alexa Fluor–conjugated acLDL by BM macrophages (*n = 4/genotype*). (D) Macrophage cholesterol ester level after a 36-h incubation with acLDL normalized to total cell protein content and expressed as percent of that in *Pggt1b*+/+ macrophages (*n = 7/genotype*). ** *P < 0.01 and *** *P < 0.001.

**Figure 4.** Increased cholesterol efflux in macrophages from *Pggt1b*Δ/Δ mice. (A, B) Basal and (A) apoA1- and (B) HDL-stimulated cholesterol efflux in BM macrophages (*n = 4/genotype*). (C) Western blots demonstrating high levels of nonprenylated (np) RAP1A in *Pggt1b*Δ/Δ BM macrophages and in *Pggt1b*+/+ macrophages incubated with a GGTI (10 µM) and low levels in *Pggt1b*+/+ cells and in *Pggt1b*Δ/Δ cells incubated with a lentivirus expressing human *PGGT1B*. (D) Control western blots demonstrating reduced electrophoretic mobility of HDJ2 in *Pggt1b*+/+ cells incubated with an FTI (10 µM). (E, F) Basal cholesterol efflux in BM macrophages incubated with DMSO, FTI, or GGTI (*n = 3–4/treatment*) (E) or with lenti-*PGGT1B* at an MOI of 20 (*n = 3*) (F). * *P < 0.05, ** *P < 0.01, and *** *P < 0.001.

**Figure 5.** Increased PPARγ activation and scavenger receptor expression in GGTase-I-deficient macrophages. (A) PPARγ activity in nuclear extracts of BM macrophages (*n = 6–7/genotype*). (B) TaqMan analyses showing gene expression in intraperitoneal macrophages (*n = 6/genotype*). (C) Western blot showing levels of proteins analyzed in B. Left panel, representative blots; right panel, quantification of band density in cell preparations from 3–4 mice/genotype. (D, E) Basal cholesterol efflux in BM macrophages incubated with DMSO or COX2 inhibitor (Celecoxib, 12.5 µM; *n = 3–6/genotype and treatment*) (D) or with lentiviruses expressing shRNA targeting RHOA, RAC1, and CDC42, or containing a scrambled (SCR) sequence (*n = 4–6*) (E). (F)
TaqMan analysis of genes involved in cholesterol efflux in intraperitoneal macrophages incubated with RHOA, RAC1, and SCR shRNA lentiviruses (n = 3). * P < 0.05 and ** P < 0.01.

Figure 6. GGTase-I deficiency in macrophages increases reverse cholesterol transport in vivo. Transport of [3H]cholesterol into plasma (A) and feces (B) following an intraperitoneal injection of [3H]cholesterol:acLDL–loaded BM macrophages into Pgtl1bΔ/Δ recipient mice. Data are from three independent experiments (n = 6–8 mice/group). * P < 0.05 and ** P < 0.01.
Figure 1
Figure 3
Figure 4
Figure 5
Targeting GGTase-I Activates RHOA, Increases Macrophage Reverse Cholesterol Transport, and Reduces Atherosclerosis in Mice

Omar M. Khan, Murali K. Akula, Kristina Skålen, Christin Karlsson, Marcus Ståhlman, Stephen G. Young, Jan Borén and Martin O. Bergo

Circulation. published online January 18, 2013;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/early/2013/01/18/CIRCULATIONAHA.112.000588

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2013/01/18/CIRCULATIONAHA.112.000588.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/
Supplemental Material

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

Omar M. Khan,¹ Murali K. Akula,¹ Kristina Skålen,² Christin Karlsson,¹ Marcus Ståhlman,² Stephen G. Young,³ Jan Borén,² and Martin O. Bergo¹
**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 Pgtt1bΔΔ 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*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.