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## ATHEROSCLEROSIS

## Cyclodextrin promotes atherosclerosis regression via macrophage reprogramming

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Atherosclerosis is an inflammatory disease linked to elevated blood cholesterol concentrations. Despite ongoing advances in the prevention and treatment of atherosclerosis, cardiovascular disease remains the leading cause of death worldwide. Continuous retention of apolipoprotein B–containing lipoproteins in the subendothelial space causes a local overabundance of free cholesterol. Because cholesterol accumulation and deposition of cholesterol crystals (CCs) trigger a complex inflammatory response, we tested the efficacy of the cyclic oligosaccharide 2-hydroxypropyl- $\beta$ -cyclodextrin (CD), a compound that increases cholesterol solubility in preventing and reversing atherosclerosis. We showed that CD treatment of murine atherosclerosis reduced atherosclerotic plaque size and CC load and promoted plaque regression even with a continued cholesterol-rich diet. Mechanistically, CD increased oxysterol production in both macrophages and human atherosclerotic plaques and promoted liver X receptor (LXR)–mediated transcriptional reprogramming to improve cholesterol efflux and exert anti-inflammatory effects. In vivo, this CD-mediated LXR agonism was required for the antiatherosclerotic and anti-inflammatory effects of CD as well as for augmented reverse cholesterol transport. Because CD treatment in humans is safe and CD beneficially affects key mechanisms of atherogenesis, it may therefore be used clinically to prevent or treat human atherosclerosis.

## INTRODUCTION

Atherosclerosis is the underlying pathology that causes heart attacks, stroke, and peripheral vascular disease. Collectively, these conditions represent a common health problem, and current treatments are insufficient to adequately reduce the risk of disease development. Pharmacologic reduction (1–3) of high-cholesterol concentrations is among the most successful therapeutic approaches to reduce the risk of developing cardiovascular disease and stroke, but adequate reduction of low-density lipoprotein (LDL) cholesterol is not possible in all patients.

Atherosclerosis is characterized by arterial wall remodeling, which is initiated by the retention and accumulation of different classes of lipids in the subendothelial layer. Lipid deposition and the appearance of cholesterol crystals (CCs) have been associated with the induction of an inflammatory reaction in the vessel wall, which contributes to the pathogenesis (4, 5). Patients with increased systemic inflammation have increased risk of cardiovascular death, and studies are under way to test whether anti-inflammatory treatment can reduce cardiovascular event rates (6).

CCs, which can result from excessive cholesterol deposition in atherosclerotic lesions, are among the proinflammatory triggers that contribute to the inflammatory response during atherogenesis (7). CCs can trigger complement activation and neutrophil extracellular trap (NET) formation, as well as induction of innate immune pathways (4, 5, 8–10). Hence, therapeutic strategies aimed at the prevention of cholesterol phase transition or the removal of CCs could reduce tissue inflammation and disease progression.

Genetic approaches to increase the capacity of macrophages to remove free cholesterol from atherosclerotic lesions have proven to be highly successful in preclinical trials (11). This prompted us to test whether pharmacologically increasing cholesterol solubility, clearance, and catabolism can be exploited for the prevention or treatment of atherosclerosis. 2-Hydroxypropyl- $\beta$ -cyclodextrin (CD) is a U.S. Food and Drug Administration (FDA)–approved substance used to solubilize and entrap numerous lipophilic pharmaceutical agents for therapeutic delivery in humans (12, 13). Although it has previously been shown that CD increases cholesterol solubility, promotes the removal of cholesterol from foam cells in vitro, and initiates anti-inflammatory mechanisms (14–16), it remains unknown whether CD can exert antiatherogenic effects in vivo.

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Here, we found that subcutaneous administration of CD profoundly reduced atherogenesis and induced regression of established atherosclerosis in mouse models. CD augmented dissolution of CCs, reducing their appearance in lesions. Furthermore, CD increased cholesterol metabolism and liver X receptor (LXR)-dependent cellular reprogramming, which resulted in more efficient reverse cholesterol transport (RCT) as well as reduced proinflammatory gene expression. The atheroprotective effect of CD was dependent on LXR expression in myeloid cells transplanted into LDL receptor (LDLR)-deficient mice. These studies suggest that CD mediates atheroprotection by increasing production of oxysterols and LXR-dependent cellular reprogramming and provide preclinical evidence that CD could be developed into an effective therapy for atherosclerosis in humans.

## RESULTS

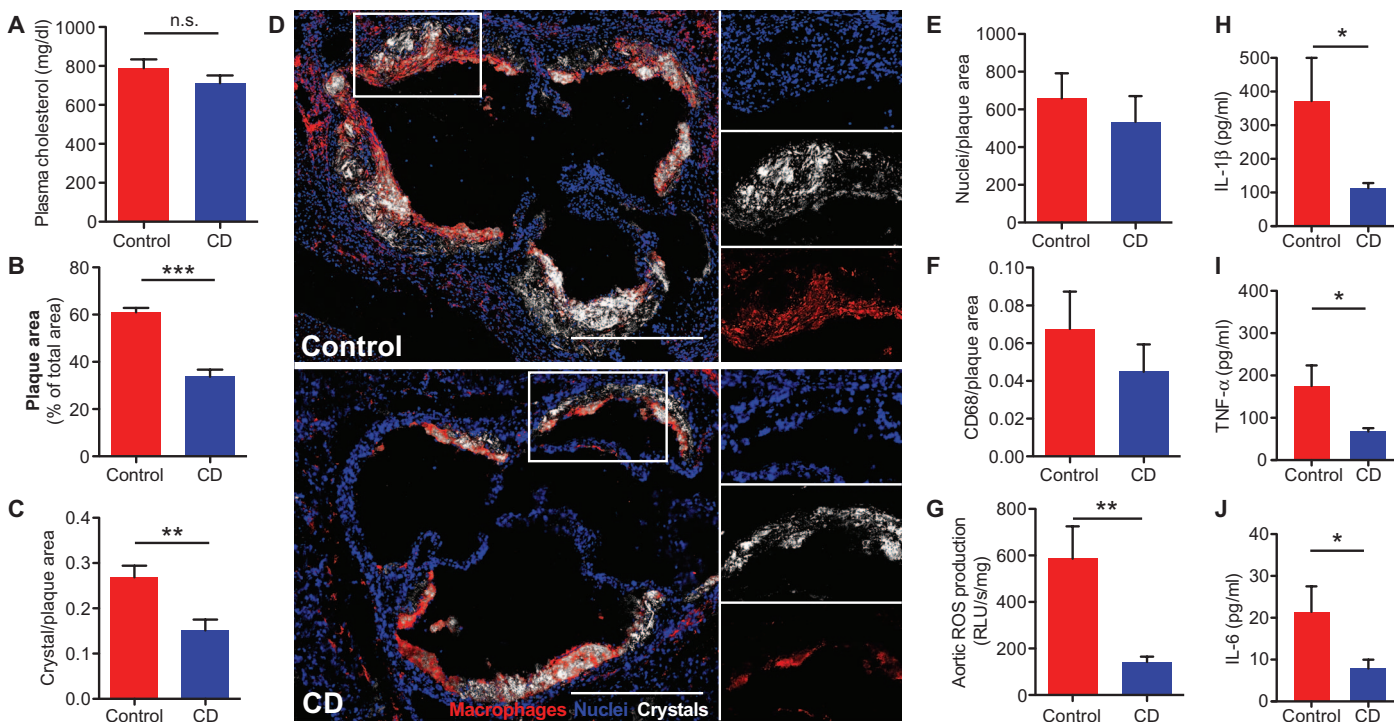
### CD treatment impairs atherogenesis

To investigate the efficacy of CD treatment in murine atherosclerosis, apolipoprotein E (ApoE<sup>-/-</sup>) deficient mice were fed a cholesterol-rich diet and concomitantly treated subcutaneously with CD or vehicle control for 8 weeks. Although plasma cholesterol, the main driver of atherosclerosis, remained unaffected (Fig. 1A), CD treatment profoundly reduced atherosclerotic lesions within the aortic root (Fig. 1B). Furthermore, we found

reduced amounts of CCs in atherosclerotic plaques of CD-treated mice as assessed by laser reflection microscopy (Fig. 1, C and D). CD did not influence weight gain, blood pressure, heart rate, or the number of bone marrow-derived or circulating sca1/flk1-positive cells (fig. S1, A to E). Moreover, plasma concentrations of phytosterols, cholestanol, and cholesterol precursors were not influenced by CD treatment, indirectly showing that CD did not alter enteric cholesterol uptake or overall endogenous biosynthesis (fig. S1F) (17). CD also did not change the relative plaque composition, including cellularity and macrophage content (Fig. 1, E and F). However, the production of aortic reactive oxygen species (Fig. 1G) and plasma concentrations of proinflammatory cytokines were reduced by CD treatment (Fig. 1, H to J), suggesting that CD may reduce the inflammatory response during atherogenesis.

### CD treatment mediates regression of atherosclerotic plaques

Although continuous drug administration in parallel to Western diet feeding of mice is a standard protocol to investigate potential atheroprotective substances (18), patients are generally not treated in early stages of atherogenesis. Therefore, we tested the effect of CD treatment on atherosclerosis regression. ApoE<sup>-/-</sup> mice are hypercholesterolemic even on normal or lipid-reduced chow, and thus, most murine atherosclerotic regression models rely on interventional strategies that normalize plasma lipids, such as viral gene transfer, transplantation,



**Fig. 1. CD treatment impairs murine atherogenesis.** ApoE<sup>-/-</sup> mice were fed a cholesterol-rich diet for 8 weeks and concomitantly treated with CD (2 g/kg) or vehicle control by subcutaneous injection twice a week ( $n = 7$  to 8 per group). (A) Plasma cholesterol concentrations. (B) Atherosclerotic plaque area relative to total arterial wall area. (C) Plaque CC load shown as the ratio of crystal reflection area to plaque area. (D) Representative images of the aortic plaques obtained by confocal laser reflection microscopy. Red, macrophages stained with anti-CD68 antibodies; white, reflection signal of

CCs; blue, nuclei stained with Hoechst. Enlarged images are the boxed areas in the left images. Scale bars, 500  $\mu$ m. (E) Plaque cellularity shown as the ratio of nuclei to plaque area. (F) Plaque macrophage load shown as the ratio of CD68 fluorescence area to total plaque area. (G) Aortic superoxide production determined by L-012 chemiluminescence. ROS, reactive oxygen species; RLU, relative light units. (H to J) Plasma IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 concentrations. Data are shown as means + SEM. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , and \* $P < 0.05$ , control versus CD (unpaired two-tailed Student's  $t$  test); n.s., not significant.

or infusion of high-density lipoprotein (HDL) particles (19). We adapted a less invasive regression protocol (20) in which ApoE<sup>-/-</sup> mice were first fed a cholesterol-rich diet for 8 weeks to induce advanced atherosclerotic lesions and then switched to a normal chow diet for another 4 weeks during which CD or vehicle control was administered (Fig. 2A). As expected, plasma cholesterol concentrations were decreased in both groups compared to baseline, but no difference between control and CD treatment was observed (Fig. 2B and fig. S2A). Although switching to a normal chow diet had no effect on atherosclerotic lesion size in vehicle-treated mice, CD treatment resulted in a regression of atherosclerotic plaques by about 45% (Fig. 2C). Although CC load in lesions was already decreased in vehicle-treated animals compared to the load before treatment, CC amounts were further reduced by CD treatment (Fig. 2D). Because patients with cardiovascular disease often do not adhere to the recommended lifestyle changes, which include dietary modifications, we next investigated whether CD treatment can affect atherosclerosis regression during continuous enteric cholesterol challenge. CD or vehicle treatment was started after 8 weeks of cholesterol-rich diet, which was continued for the entire 12 weeks (Fig. 2E). Although plasma cholesterol and general cholesterol

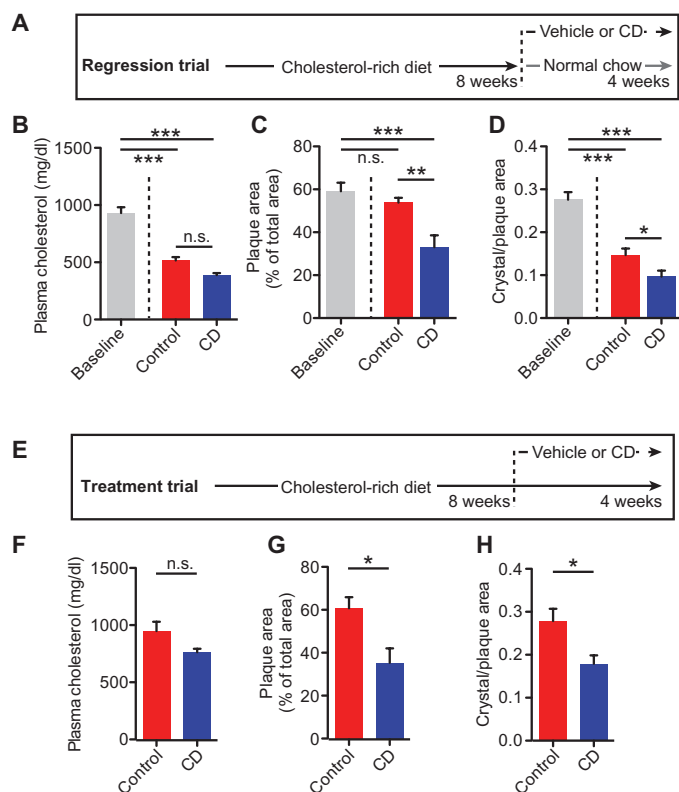
metabolism were not altered (Fig. 2F and fig. S2B), atherosclerotic plaque size and CC load were decreased in CD-treated mice on continuous cholesterol-rich diet (Fig. 2, G and H). These data demonstrate that CD treatment is effective in reducing established plaques.

### CD dissolves extra- and intracellular CCs

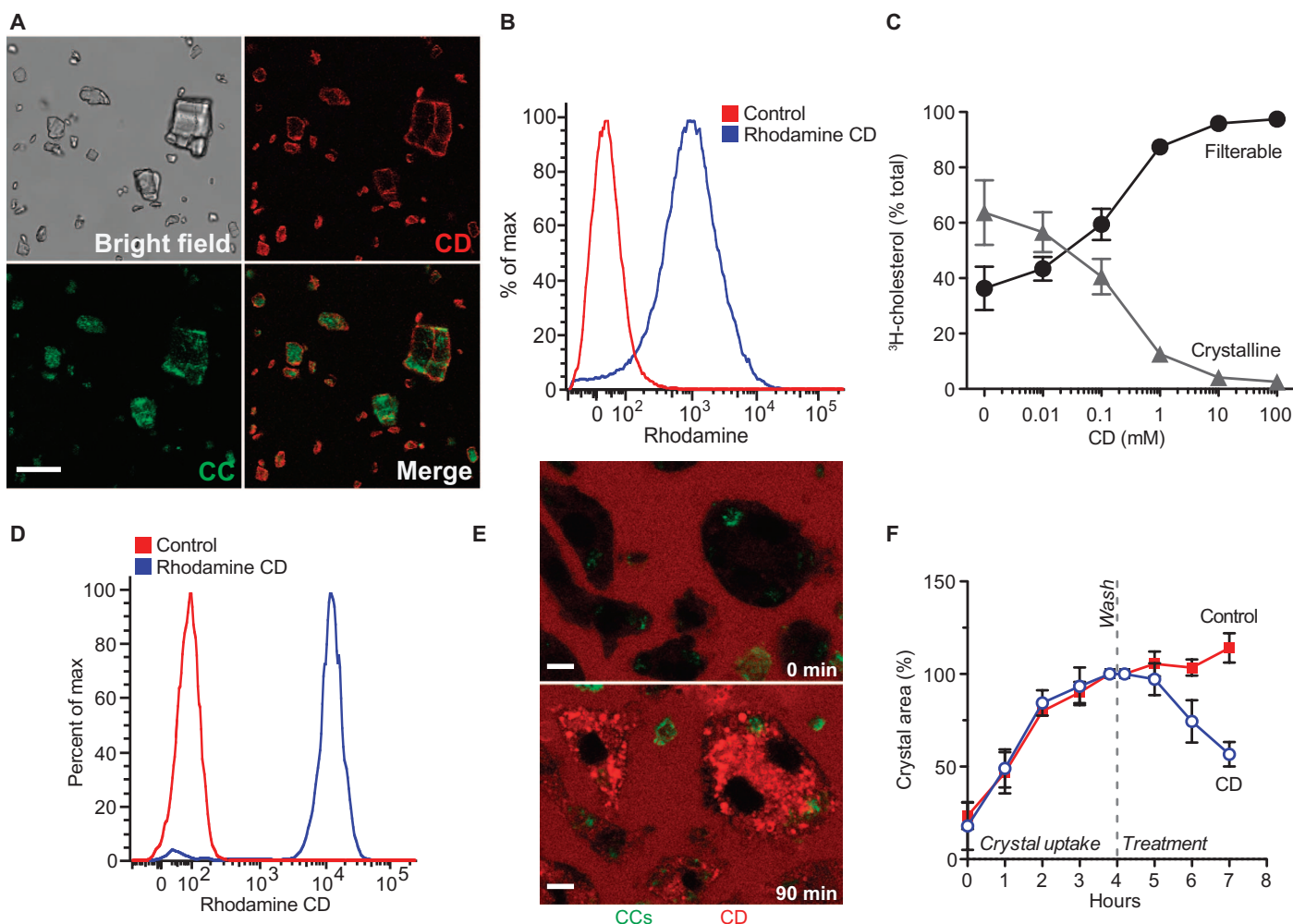
There are several possibilities to explain the protective effects of CD treatment on both atherogenesis and established atherosclerosis. Because CD is known to form soluble inclusion complexes with cholesterol, thereby enhancing its solubility in aqueous solutions by about 150,000-fold, we tested whether CD increases the solubility of CCs. Fluorescent CD bound to the surface of CCs (Fig. 3, A and B) and CD mediated the solubilization of CCs in a dose-dependent manner (Fig. 3C). To be effective in atherosclerotic plaques, CD must also act on intracellular CCs. Macrophages rapidly internalized fluorescent CD (Fig. 3D) and concentrated it in intracellular compartments (Fig. 3E). Furthermore, incubation with 10 mM CD, a subtoxic dose (fig. S3), enhanced the dissolution of intracellular CCs over time (Fig. 3F and fig. S4).

### Metabolism of crystal-derived cholesterol is increased by CD

Macrophages within the arterial wall take up excessive amounts of cholesterol and transform into foam cells, a process that can impair macrophage function and promote atherogenesis (21). This can be mimicked *in vitro* by loading macrophages with CCs (fig. S5). After uptake of CCs into phagosomes, cholesterol is moved from the lysosome via the Niemann-Pick type C1 (NPC1) transporter to the endoplasmic reticulum, where acetyl-coenzyme A (CoA) acetyltransferase catalyzes the formation of cholesteryl esters. This mechanism turns excess free cholesterol, which forms crystals and is cytotoxic, into cholesteryl esters that can be stored in lipid droplets. A second pathway to metabolize free cholesterol is the formation of water-soluble oxysterols. Oxysterols can diffuse across cell membranes and are known to reprogram macrophages through activation of LXR, which in turn modulates the inflammatory response and supports RCT to HDL (22–24). To study how CD influences the ability of macrophages to reduce the amount of cholesterol derived from CCs, we incubated macrophages with CCs prepared from D<sub>6</sub>-cholesterol (D<sub>6</sub>-CCs) and followed D<sub>6</sub>-cholesterol metabolism products in cells and cellular supernatants by gas chromatography–mass spectrometry selective ion monitoring (GC-MS-SIM) (Fig. 4A). This analysis revealed that CD treatment promoted esterification of crystal-derived D<sub>6</sub>-cholesterol (Fig. 4B). Furthermore, CD amplified D<sub>6</sub>-cholesterol concentrations in supernatants while reducing the overall cellular pool of D<sub>6</sub>-cholesterol (Fig. 4C). Hence, CD treatment increased the cholesterol efflux capacity of macrophages, which represents an important protective factor in patients with coronary artery disease (25, 26). Active cholesterol transport is mediated primarily by the adenosine 5'-triphosphate-binding cassette transporters A1 and G1 (ABCA1 and ABCG1), which transfer free cholesterol to ApoA1 and mature HDL particles, respectively (27). In line with the observed increase in cholesterol efflux capacity, macrophages incubated with CCs had increased expression of both ABCA1 and ABCG1, which was even further enhanced by CD treatment (Fig. 4, D to F). Genes involved in driving cholesterol efflux, including *Abca1* and *Abcg1*, are under the control of the LXR/retinoid X receptor (LXR/RXR) transcription apparatus (22, 28). Because the transcriptional activities of LXRs are positively regulated by oxysterols, we next analyzed whether CD can potentiate cholesterol oxidation. We found that CD treatment of D<sub>6</sub>-CC-loaded macrophages resulted in a marked 15-fold increase in



**Fig. 2. CD treatment facilitates regression of murine atherosclerosis.** ApoE<sup>-/-</sup> mice were fed a cholesterol-rich diet for 8 weeks to induce advanced atherosclerotic lesions. Then, the diet was either changed to a normal chow (A to D) or the cholesterol-rich diet was continued for another 4 weeks (E to H). Mice were simultaneously treated with CD (2 g/kg) or vehicle control twice a week ( $n = 6$  to 8 per group). (A and E) Diet and treatment schemes. (B and F) Plasma cholesterol concentrations. (C and G) Atherosclerotic plaque area relative to total arterial wall area. (D and H) Plaque CC load shown as the ratio of crystal reflection area to plaque area. Data are shown as means + SEM. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , and \* $P < 0.05$ , control versus CD (unpaired two-tailed Student's *t* test).



**Fig. 3. CD interacts with and dissolves extra- and intracellular CCs.** (A and B) CCs (1 mg) were incubated with 0.5 mM rhodamine-labeled CD or phosphate-buffered saline as control. (A) Representative images obtained by confocal laser reflection microscopy. Scale bar, 20  $\mu$ m. (B) Quantification of rhodamine fluorescence on CCs by flow cytometry. (C)  $^3$ H-CCs were incubated with CD solutions of the indicated concentrations overnight with shaking at 37°C. Upon filtration through 0.22- $\mu$ m filter plates, radioactivity was determined in the filtrate (filterable/solubilized) and the retentate (crystalline). (D and

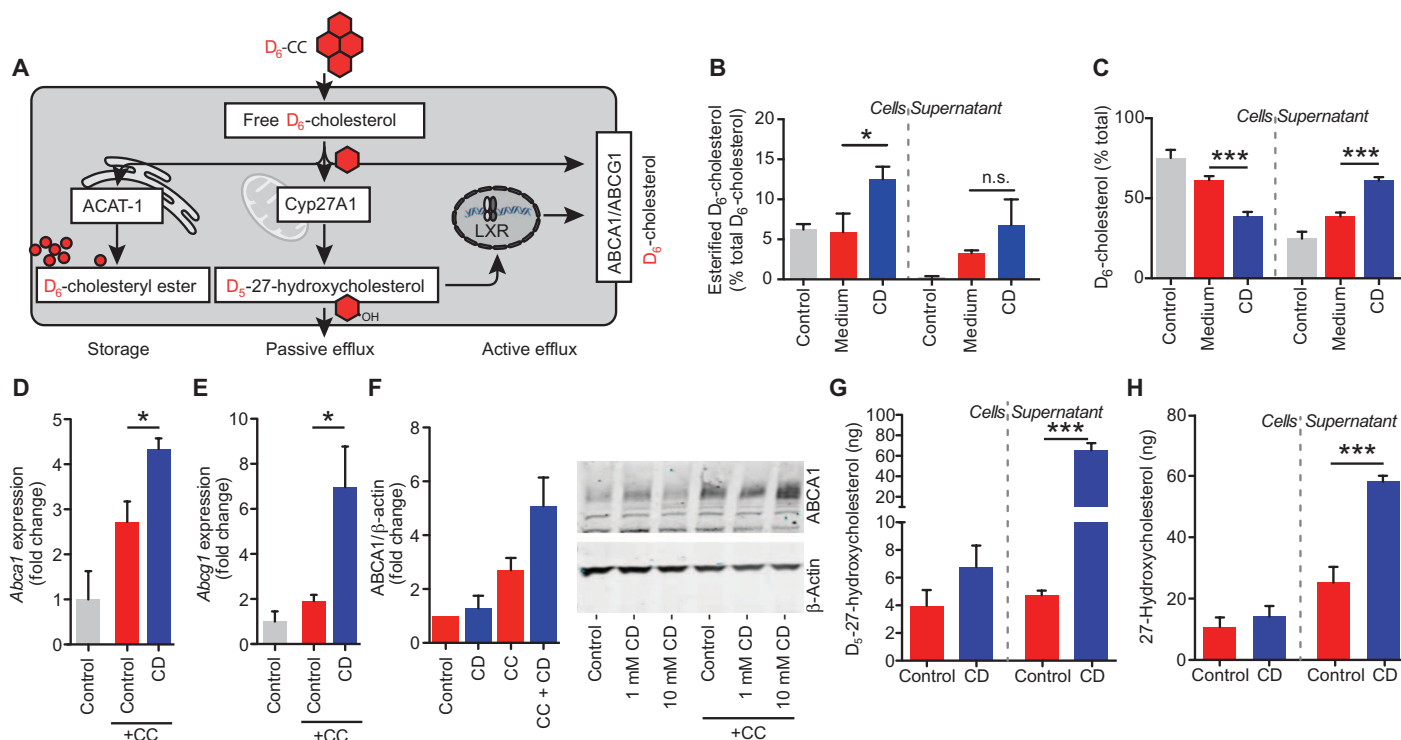
E) iMac (immortalized macrophages) were loaded with 200  $\mu$ g of CC per  $1 \times 10^6$  cells for 3 hours before incubation with 1 mM rhodamine-labeled CD. (D) Quantification of rhodamine fluorescence by flow cytometry. (E) Representative images obtained by confocal microscopy. Red, rhodamine-labeled CD; green, laser reflection signal. Scale bars, 5  $\mu$ m. (F) Intracellular CC dissolution in BMDMs treated with 10 mM CD or control for the indicated times determined by polarization microscopy. Data are shown as means  $\pm$  SEM of at least three independent experiments.

$D_6$ -cholesterol-derived 27-hydroxycholesterol ( $D_5$ -27-hydroxycholesterol) (Fig. 4G), although the expression of *Cyp27a1* was not altered (fig. S6). Unexpectedly, CD also increased 27-hydroxycholesterol production and secretion from macrophages under normocholesterolemic conditions, meaning macrophages not treated with  $D_6$ -CCs (Fig. 4H). Hence, CD increases the metabolism of free cholesterol and could thereby lower the potential for its phase transition into crystals.

### CD induces LXR target gene expression in macrophages

The drastic CD-mediated increase in oxysterol production upon  $D_6$ -CC loading and the unanticipated finding that CD can increase oxysterols in normocholesterolemic macrophages prompted us to comprehensively investigate whether CD influences the expression profiles of LXR-regulated genes. Wild-type or *LXR $\alpha$ <sup>-/-</sup> $\beta$ <sup>-/-</sup>* macrophages were exposed to CD, CC, or CC and CD, and gene expression was assessed

by genome-wide mRNA profiling. To investigate whether CD changes LXR target gene expression in macrophages, we performed gene set enrichment analysis (GSEA) (29) with a set of 533 of previously identified LXR target genes (30) (Fig. 5A and table S1). Enrichment of LXR target gene sets was identified when wild-type macrophages were incubated with CCs (Fig. 5B), presumably because of cholesterol overloading of macrophages. Consistent with the strong induction of CC-derived 27-hydroxycholesterol and the observed increase in cholesterol efflux by CD, LXR target gene sets were enriched when CD was added together with CCs (Fig. 5B). CD treatment alone also resulted in LXR gene set enrichment under normocholesterolemic conditions, which correlates with the observed induction of cellular 27-hydroxycholesterol (Fig. 4H). In *LXR $\alpha$ <sup>-/-</sup> $\beta$ <sup>-/-</sup>* macrophages, none of the conditions resulted in significant enrichments of LXR target gene sets (Fig. 5C). Furthermore, these findings could be confirmed for the key LXR target genes



**Fig. 4. CD mediates metabolism and efflux of crystal-derived cholesterol.**

(A) Macrophages loaded with CCs prepared from  $D_6$ -cholesterol ( $D_6$ -CC) can reduce the amount of free, crystal-derived  $D_6$ -cholesterol by three main mechanisms. First, acetyl-CoA acetyltransferase (ACAT-1) can catalyze the formation of  $D_6$ -cholesteryl esters, the storage form of cholesterol, which are deposited in lipid droplets. Second, the mitochondrial enzyme 27-hydroxylase (Cyp27A1) can catalyze the formation of  $D_5$ -27-hydroxycholesterol, which can passively diffuse across cell membranes. Third,  $D_5$ -27-hydroxycholesterol is a potent activator of LXR transcription factors, which in turn mediate the up-regulation of the cholesterol efflux transporters ABCA1 and ABCG1. (B and C) iMac3 loaded with 200  $\mu$ g of  $D_6$ -CC per  $1 \times 10^6$  cells for 3 hours were treated with 10 mM CD or vehicle control before GC-MS-SIM analysis of crystal-derived cholesterol. (B) Percentage of esterified  $D_6$ -cholesterol in cell and supernatant fractions before CD treatment (control bar) and after 48 hours of CD treatment. (C) Efflux of  $D_6$ -cholesterol into supernatants of  $D_6$ -CC-loaded

macrophages before CD treatment (control bar) and upon 24 hours of CD treatment. (D to F) Gene expression of *Abca1* and *Abcg1* and protein expression of ABCA1 in BMDMs loaded with 100  $\mu$ g of CC per  $1 \times 10^6$  cells for 3 hours and then incubated with 10 mM CD or medium control for (D and E) 4 or (F) 24 hours. Immunoblot in (F) is representative of three independent experiments, and densitometric analysis of all three experiments is provided for 10 mM CD and presented as ABCA1 expression relative to the loading control  $\beta$ -actin. Data are shown as means  $\pm$  SEM of at least three independent experiments. (G)  $D_5$ -27-hydroxycholesterol in cell and supernatant fractions of iMac3 loaded with 200  $\mu$ g of  $D_6$ -CC per  $1 \times 10^6$  cells for 3 hours before 48 hours of treatment with 10 mM CD or medium control, determined by GC-MS-SIM. (H) 27-Hydroxycholesterol in cell and supernatant fractions of iMac3 after 48 hours of treatment with 10 mM CD or medium control. \*\*\* $P < 0.001$  and \* $P < 0.05$ , medium versus CD (B to C); CC + control versus CC + CD (D to F); control versus CD (G and H) (unpaired two-tailed Student's *t* test).

ABCA1 and ABCG1 in wild-type and  $LXR\alpha^{-/-}\beta^{-/-}$  macrophages on the mRNA and protein levels (Fig. 5, D to F) (31).

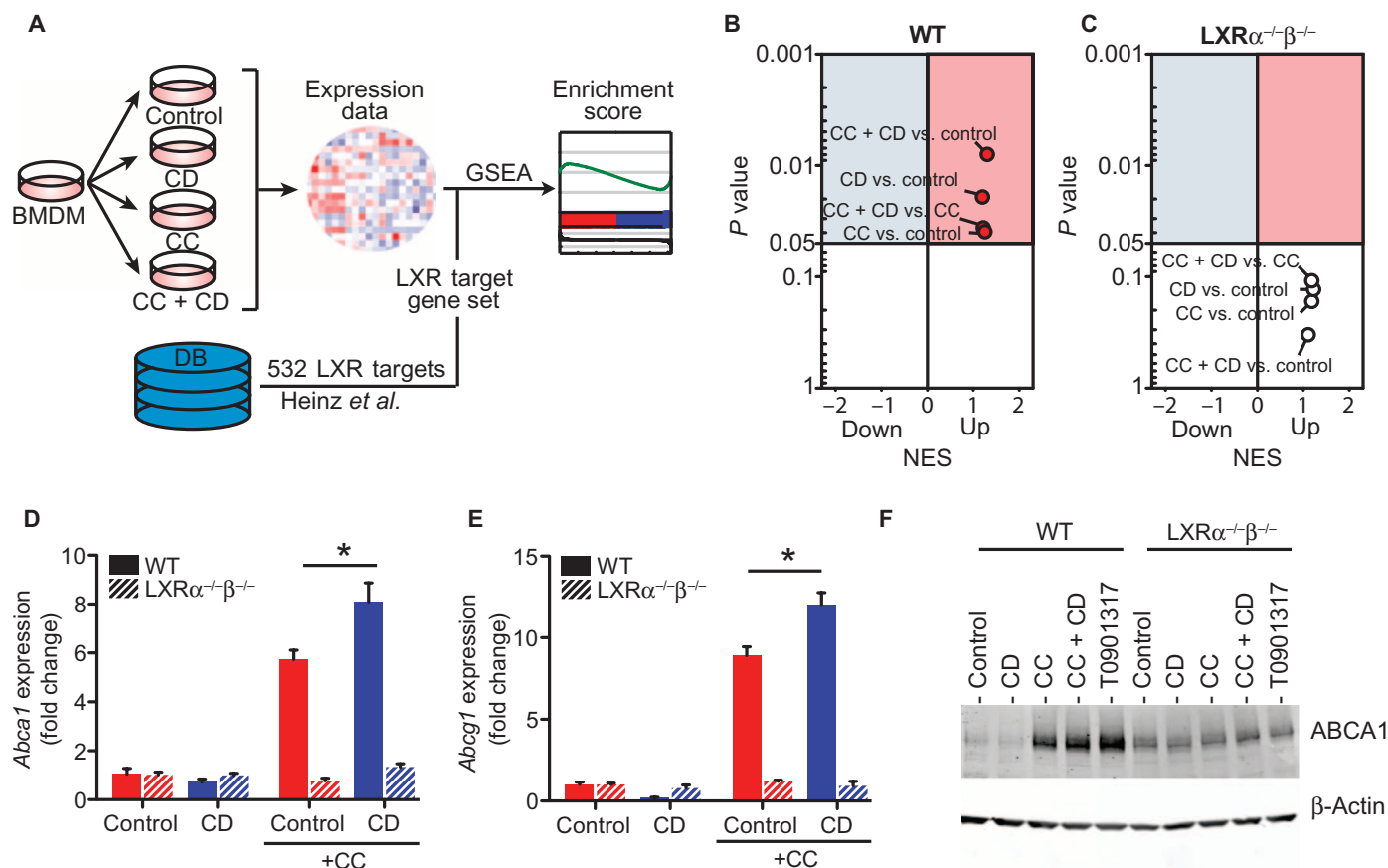
### CD increases in vivo RCT

To test whether CD-induced LXR reprogramming of macrophages improves macrophage cholesterol efflux in vivo, bone marrow-derived macrophages (BMDMs) from wild-type or  $LXR\alpha^{-/-}\beta^{-/-}$  mice were loaded with  $D_6$ -CCs ex vivo and injected into the peritoneum of wild-type mice. The mice carrying crystal-loaded macrophages were then treated with CD or vehicle control, and  $D_6$ -cholesterol excretion into the feces and urine was monitored by GC-MS-SIM (Fig. 6A). CD increased RCT of crystal-derived  $D_6$ -cholesterol from wild-type and, to a lower extent,  $LXR\alpha^{-/-}\beta^{-/-}$  macrophages (Fig. 6B). Of note, CD treatment not only induced  $D_6$ -cholesterol excretion into the feces but also promoted urinary  $D_6$ -cholesterol elimination (Fig. 6C), a process that is normally not observed during RCT. Prior work on NPC disease, a rare genetic disorder in which cholesterol cannot escape the lysosome, has shown that CD can mobilize lysosomal cholesterol

and activate LXR-dependent gene expression (32, 33). NPC1-deficient patients receive weekly injections of CD with the aim of overcoming this cholesterol transport defect. To investigate whether CD can also stimulate urinary cholesterol excretion in humans, we monitored urinary cholesterol excretion of patients with NPC1 mutations after CD infusion over time. CD, which is primarily excreted through the urinary tract, resulted in a time-dependent cholesterol excretion into the urine (Fig. 6D). These data suggest that CD enhances in vivo RCT from macrophages, partially in an LXR-dependent manner, but can also directly extract and transport cholesterol for excretion.

### CD modifies human plaque cholesterol metabolism and gene expression

To test whether the protective functions of CD on murine macrophages are also exerted in human atherosclerotic plaques, we next performed lipid and genomic analyses on biopsy specimens obtained from carotid endarterectomies (Fig. 7A). Comparable to our findings in murine

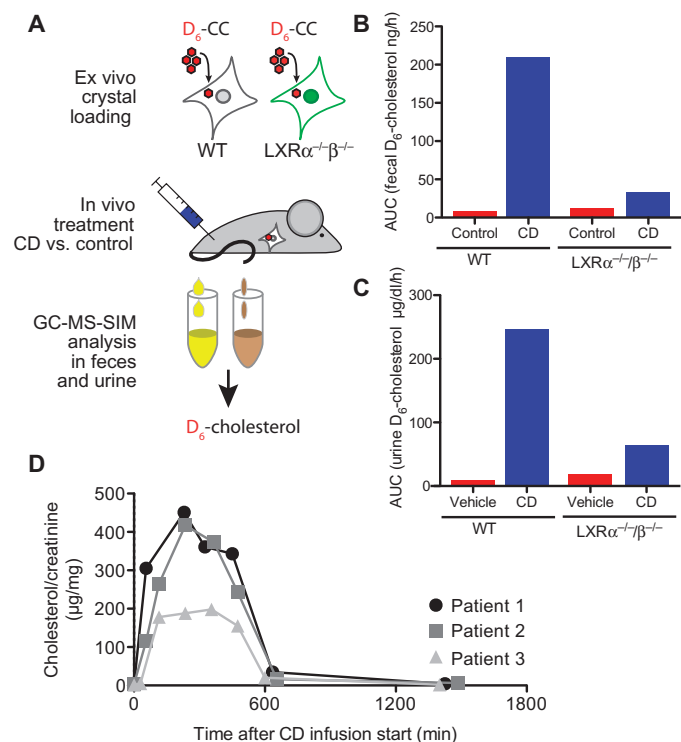


**Fig. 5. CD induces LXR target gene expression in wild-type macrophages.** (A) BMDMs from wild-type (WT) and  $LXR\alpha^{-/-}\beta^{-/-}$  mice were loaded with 100  $\mu\text{g}$  of CC per  $1 \times 10^6$  cells for 3 hours and incubated with 10 mM CD for 4 hours for microarray analysis. GSEA for the LXR target gene sets described by Heinz *et al.* (30) (table S1) was performed on gene expression data. DB, database. (B and C) GSEA results for (B) WT and (C)  $LXR\alpha^{-/-}\beta^{-/-}$  BMDMs presented as volcano plots of normalized enrichment score (NES) and enrichment  $P$  values. Red circles show positively and significantly enriched gene

sets (NES > 1,  $P < 0.05$ ). (D to F) Gene expression of (D) *Abca1* and (E) *Abcg1*, and (F) protein expression of ABCA1 in BMDMs from WT and  $LXR\alpha^{-/-}\beta^{-/-}$  mice loaded with 100  $\mu\text{g}$  of CC per  $1 \times 10^6$  cells for 3 hours and then incubated with 10 mM CD for (D and E) 4 or (F) 24 hours. The synthetic LXR agonist T0901317 (10  $\mu\text{M}$ ) was used as a positive control for ABCA1 protein induction. Immunoblot in (F) is representative of two independent experiments. Data are shown as means + SEM of two independent experiments. \* $P < 0.05$ , CC + control versus CC + CD (unpaired two-tailed Student's  $t$  test).

macrophages, incubation of human atherosclerotic plaques with CD resulted in a transfer of cholesterol from plaques to supernatants (Fig. 7B). Moreover, we observed an increase in the production of 27-hydroxycholesterol, which was mainly released into the supernatants of the CD-treated plaques (Fig. 7C). Gene expression profiling of a large panel of human immunology-related genes and selected LXR target genes (table S3) was performed in resting or treated plaque tissue. These gene expression data were analyzed by several bioinformatics approaches. First, we performed gene ontology enrichment analysis (GOEA) using the genes differentially expressed (DE) after treatment with CD or vehicle control. Consistent with our lipid results, we found that genes involved in lipid transport, storage, metabolism, and efflux were up-regulated upon CD exposure. Conversely, genes known to regulate immune responses, represented by terms such as “regulation of immune responses in lymphocytes,” “regulation of leukocyte-mediated immunity,” or “interleukin response, T cell, and natural killer cell regulation,” were down-regulated after CD treatment (Fig. 7D). Further interrogation of the GOEA revealed that CD treatment of human plaques affected many key genes in the GO

term “regulation of inflammatory response” (GO:0050727). These included innate immune receptors, such as Toll-like receptors (TLRs) 2, 3, 4, 7, and 9; the TLR adapter MyD88; the inflammasome sensor NLRP3; and the inflammasome-dependent proinflammatory cytokines interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-18 (Fig. 7E). Because we observed that CD increased the endogenous LXR agonist 27-hydroxycholesterol, we next analyzed whether CD regulates the expression of LXR target genes in human atherosclerotic plaques. GSEA revealed an enrichment of LXR target genes after CD treatment when compared to control-treated plaques (Fig. 7F and table S2). Additionally, many LXR target genes were found among the most DE genes (Fig. 7G, red or blue gene labels). Of note, the inflammasome sensor *NLRP3* and the inflammasome inhibitor *HSP90* (34) are both LXR target genes (24) and CD treatment resulted in *NLRP3* down-regulation and an up-regulation of *HSP90* when compared to control (Fig. 7H). Together, these data show that CD activates LXR-dependent transcriptional programs in human plaques, influencing both cholesterol transport and several inflammatory processes, which are relevant to the pathogenesis of atherosclerosis.



**Fig. 6. CD facilitates RCT in vivo and promotes urinary cholesterol excretion.**

(A) BMDMs from WT or LXR $\alpha^{-/-}\beta^{-/-}$  mice were loaded with 100  $\mu\text{g}$  of D<sub>6</sub>-CC per  $1 \times 10^6$  cells and injected into the peritoneum of WT mice. Subsequently, mice were treated subcutaneously with CD (2 g/kg) or vehicle control ( $n = 4$  per group). (B and C) D<sub>6</sub>-cholesterol content in feces and urine collected every 3 hours over 30 hours after CD injection. Data are shown as total area under the curve (AUC) of excreted D<sub>6</sub>-cholesterol pooled from the mice within a group per time point. (D) Urine samples collected from three individual NPC1 patients upon intravenous application of CD for specific treatment of NPC. Urine cholesterol concentration was determined by GC-MS-SIM and normalized to urine creatinine excretion.

### Atheroprotection by CD is LXR-dependent

We next tested whether the CD-mediated effects in isolated macrophages in vitro or in ex vivo-treated human plaque material reflect the atheroprotective effects of CD in mice. Because CD treatment lowered the systemic concentrations of LXR-modulated cytokines [IL-1 $\beta$ , IL-6, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )] (Fig. 1, H to J) and also resulted in increased *Abca1* and *Abcg1* mRNA in the aortic arches of ApoE $^{-/-}$  mice fed a cholesterol-rich diet (fig. S7), we determined whether CD-mediated atheroprotection in vivo requires LXR activation and cholesterol efflux from macrophages through ABCA1 and ABCG1. We therefore transplanted wild-type, LXR $\alpha^{-/-}\beta^{-/-}$ , or macrophage-specific ABCA1 and ABCG1 knockout (MAC-ABC<sup>DKO</sup>) bone marrow into irradiated LDLR $^{-/-}$  mice. After bone marrow engraftment, the transplanted mice were fed a cholesterol-rich diet and were concomitantly treated with CD or vehicle control for 8 weeks. CD treatment did not influence plasma cholesterol concentrations in the different transplant groups (Fig. 8, A to C). The lipoprotein profiles also remained unchanged, except that CD treatment slightly decreased the amount of HDL in LDLR $^{-/-}$  mice transplanted with MAC-ABC<sup>DKO</sup> bone marrow (fig. S8). LDLR $^{-/-}$  mice carrying wild-type bone marrow showed reduced atherosclerotic plaque size, demonstrating that CD is also effective

in the LDLR $^{-/-}$  model of atherosclerosis (Fig. 8D). Of note, CD treatment did not influence lesion development in LDLR $^{-/-}$  mice carrying LXR $\alpha^{-/-}\beta^{-/-}$  bone marrow, highlighting that LXR agonism is critical for CD-mediated atheroprotection (Fig. 8E). In contrast, deficiency of ABCA1 and ABCG1 in macrophages did not influence the effectiveness of CD treatment (Fig. 8F), suggesting that CD can bypass these cholesterol efflux pathways.

To better understand how CD-dependent LXR agonism can ameliorate atherosclerosis, we performed a genome-wide gene expression analysis on aortic tissue from LDLR $^{-/-}$  mice transplanted with wild-type or LXR $\alpha^{-/-}\beta^{-/-}$  bone marrow. GOEA of DE genes demonstrated that important pathways involved in atherogenesis, including lipid metabolism and inflammation, were regulated by CD treatment in an LXR-dependent manner (Fig. 8G). Similar to our studies on human plaques, LXR target genes were found among the top DE genes upon CD treatment (Fig. 8H). Moreover, we confirmed our observation from human plaques that CD promoted up-regulation of the NLRP3 inhibitor *Hsp90aa1* and down-regulation of NLRP3 inflammasome genes in an LXR-dependent manner (Fig. 8I). Together, these data suggest that the CD-mediated atheroprotection observed in murine atherosclerosis is dependent on LXR activation and that CD exerts multiple anti-inflammatory effects in atherosclerotic plaques.

### DISCUSSION

Here, we tested the hypothesis that increasing the solubility of cholesterol by pharmacological means can have beneficial effects on diet-induced atherosclerosis. The large effect observed and the unexpected ability of CD to promote regression of established atherosclerosis even under the extreme hypercholesterolemic conditions observed during a cholesterol-rich diet cannot be explained by simple mass action of CD alone. The results from the lipid and genomic discovery approaches combined with in vivo studies in gene-deficient mice suggest that CD exerts its potent effect mainly by reprogramming cells in atherosclerotic plaques. By increasing the amount of endogenous LXR ligands, CD acts akin to a pro-drug except that it is not metabolized itself but rather promotes the metabolism of its cargo cholesterol into pharmacologically active metabolites.

It appears that transitory changes in cholesterol metabolism and inflammatory pathways are linked and that the activity of LXR is a key rheostat in this system. For example, innate immune activation by microbial components or the acute-phase response can suppress the expression of LXR target genes, such as *ABCA1* and *ABCG1*, causing cholesterol retention, which can augment an inflammatory reaction in various ways (35, 36). It is conceivable that this type of innate immune amplification could be part of an evolutionarily conserved antimicrobial defense mechanism (23). The resulting cholesterol accumulation increases LXR agonists, which in turn can counterbalance the inflammatory response and increase cholesterol efflux, restoring cholesterol and immune homeostasis. However, because of the overabundance of proinflammatory dietary factors and an excess of cholesterol, this balance may be shifted toward chronic inflammation and cholesterol retention, which drive atherogenesis. By promoting cholesterol solubility, enhancing LXR activity, and mobilizing cholesterol efflux, CD could therefore normalize both cholesterol and immune homeostasis in the vasculature.

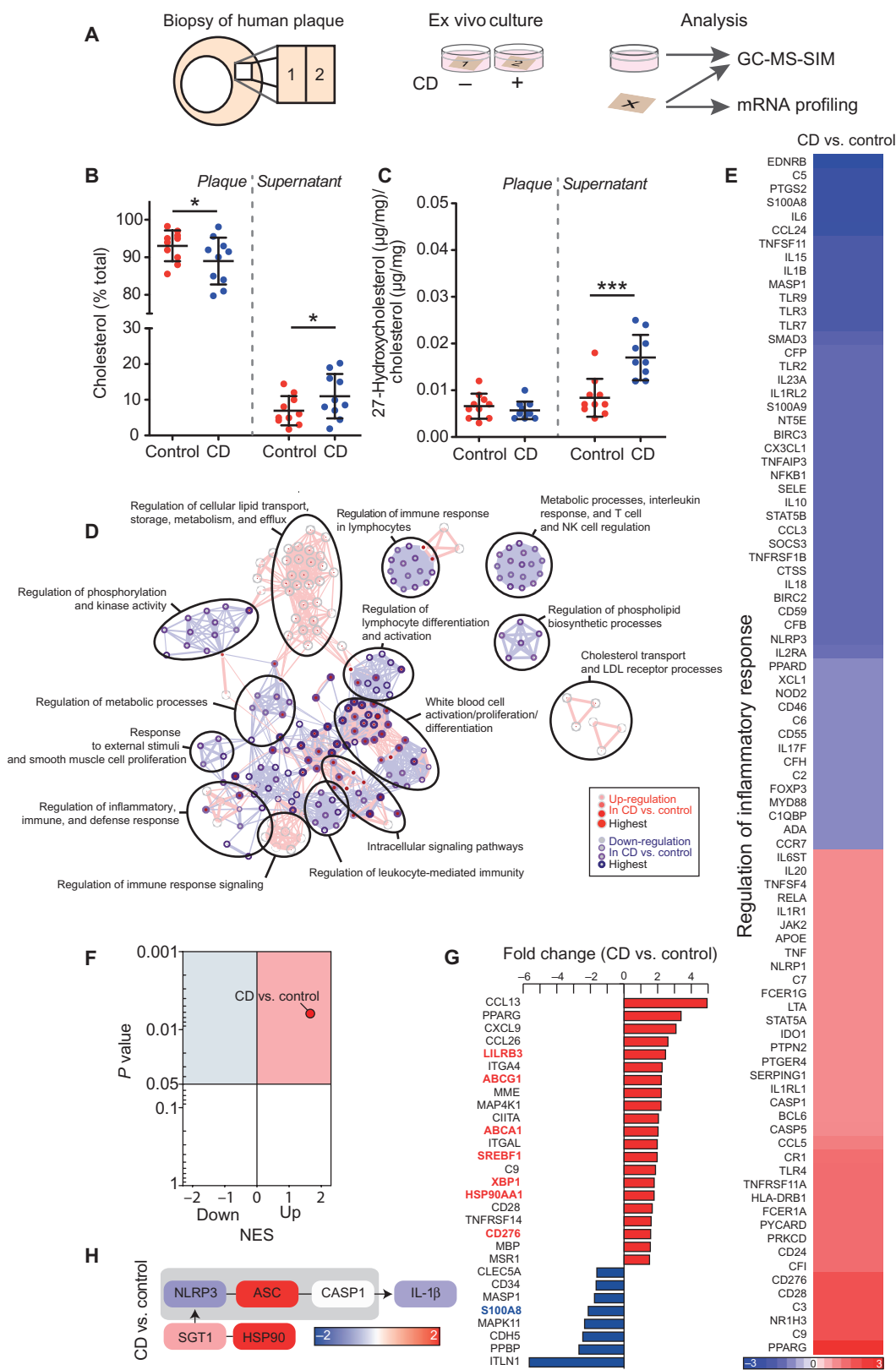
The effect of CD on macrophages resembles that of the antiatherogenic factor HDL. HDL relieves cells of excess cholesterol through ABC transporters; in addition, HDL can have marked anti-inflammatory

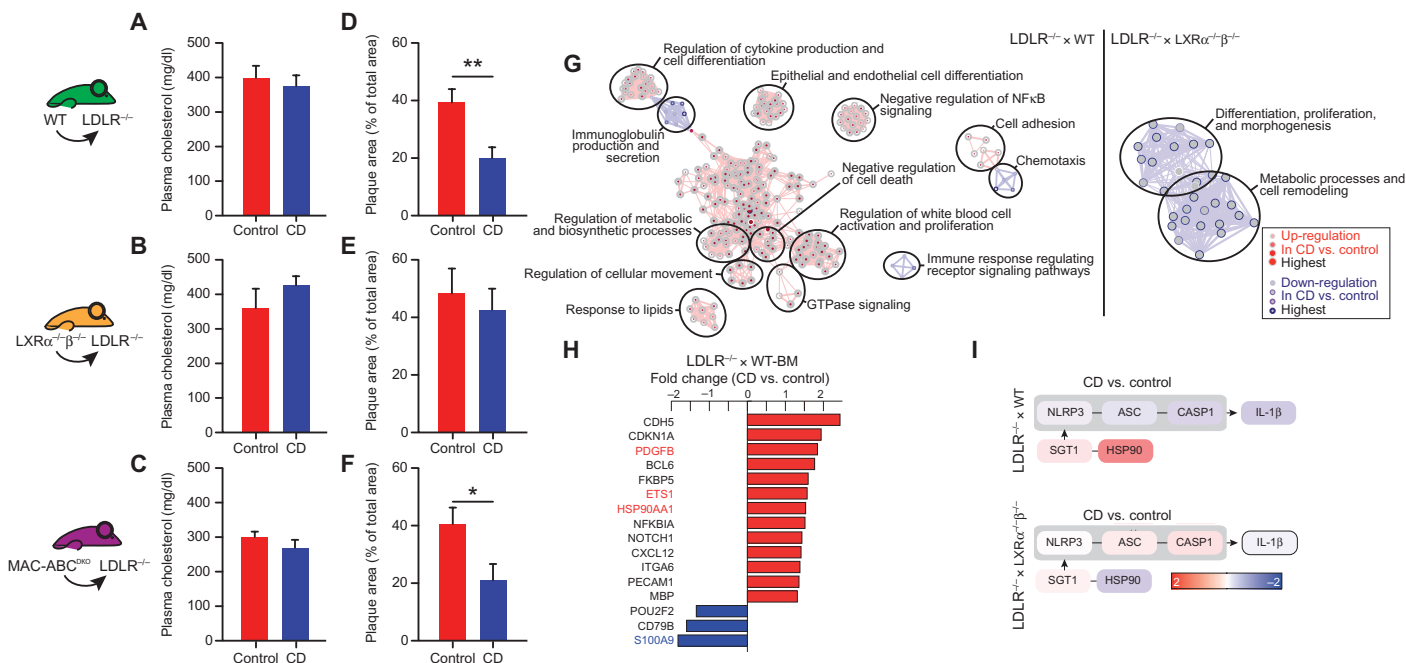
**Fig. 7. CD induces cholesterol metabolism and an anti-inflammatory LXR profile in human atherosclerotic carotid plaques.** (A) Human atherosclerotic carotid plaques obtained by carotid endarterectomy ( $n = 10$ ) were split into two macroscopically equal pieces and cultured for 24 hours with 10 mM CD or control.

Half of the plaque tissue was used for mRNA profiling with nCounter Analysis System (NanoString Technologies), and the other half and the culture supernatant were analyzed by GC-MS-SIM. (B) Cholesterol efflux from plaque tissue into supernatants displayed as percent of total cholesterol per sample. (C) Distribution of 27-hydroxycholesterol relative to cholesterol in plaque and supernatant. (D) GOEA of DE genes (fold change > 1.3,  $P < 0.05$ ) visualized as GO network, where red nodes indicate GO term enrichment by up-regulated DE genes and blue borders indicate GO term enrichment by down-regulated DE genes. Node size and border width represent the corresponding false discovery rate (FDR)-adjusted enrichment  $P$  value ( $q$  value). Edges represent the associations between two enriched GO terms based on shared genes, and edge thickness indicates the overlap of genes between neighbor nodes. Highly connected terms were grouped together and were annotated manually by a shared general term. (E) Heat map of genes involved in the GO term “regulation of inflammatory response” (GO:0050727).

Color bar indicates fold change. (F) Volcano plot of NES and enrichment  $P$  values based on GSEA for the LXR target gene set (table S2). Red circle indicates positive and significant enrichment of the LXR target gene set (NES > 1,  $P < 0.05$ ).

(G) Top DE genes determined by three-way analysis of variance (ANOVA) (fold change > 1.5,  $P < 0.05$ ). LXR target genes are colored in red or blue. (H) The expression of genes relevant to the NLRP3 inflammasome pathway. Color bar indicates fold change. (B and C) Data are shown as means  $\pm$  SEM. \*\*\* $P < 0.001$  and \* $P < 0.05$ , CD versus control (paired two-tailed Student's  $t$  test).





**Fig. 8. CD impairs atherogenesis and regulates metabolic and anti-inflammatory processes in an LXR-dependent manner.** LDLR<sup>-/-</sup> mice were transplanted with WT, LXRe<sup>-/-</sup>β<sup>-/-</sup>, or MAC-ABC<sup>DKO</sup> bone marrow. They were then fed a cholesterol-rich diet for 8 weeks and concomitantly treated with CD (2 g/kg) or vehicle control twice a week ( $n = 6$  to 8 per group). (A to C) Plasma cholesterol concentrations of CD- and vehicle-treated animals. (D to F) Atherosclerotic plaque area relative to total arterial wall area. (G to I) Descending aortas of LDLR<sup>-/-</sup> mice transplanted with WT and LXRe<sup>-/-</sup>β<sup>-/-</sup> bone marrow were used for gene expression analysis by microarray, with subsequent filtration for the genes included in the human plaque mRNA profiling. (G) GOEA of DE genes (fold change > 1.3,  $P < 0.05$ ) visualized as GO network, where red nodes indicate GO term enrichment by up-regulated DE genes and blue

borders indicate GO term enrichment by down-regulated DE genes. Node size and border width represent the corresponding FDR-adjusted enrichment  $P$  value ( $q$  value). Edges represent the associations between two enriched GO terms based on shared genes, and edge thickness indicates the overlap of genes between neighbor nodes. Highly connected terms were grouped together and were annotated manually by a shared general term. NFκB, nuclear factor κB; GTPase, guanosine triphosphatase. (H) DE genes determined by three-way ANOVA (fold change > 1.3,  $P < 0.05$ ) in aortas of LDLR<sup>-/-</sup> mice transplanted with WT bone marrow. LXR target genes are colored in red or blue. (I) The expression of genes relevant for the NLRP3 inflammasome pathway. Color bar indicates fold change. (A and F) Data are shown as means ± SEM; \*\* $P < 0.01$  and \* $P < 0.05$ , CD versus control (unpaired two-tailed Student's  $t$  test).

effects on macrophages (37). However, HDL does not activate LXR but increases the expression of activating transcription factor 3, a key repressor of innate immune pathways (37). In contrast, although CD has the ability to increase HDL-mediated RCT, it can also mobilize cholesterol for direct excretion into the urine and feces. These data suggest that CD can bypass ABCA1- and ABCG1-mediated active cholesterol efflux. Consistent with these observations, our bone marrow transplantation studies indicate that the atheroprotective effects of CD are independent of the active cholesterol efflux process mediated by ABCA1 and ABCG1. This is in line with recent findings demonstrating that synthetic LXR agonists mediate atheroprotection independent of macrophage ABCA1 and ABCG1 (38).

There are several limitations to our study. First, although our data demonstrate that CD promotes LXR activation in plaque macrophages and that LXR is required in myeloid cells for CD-mediated atheroprotection, we cannot exclude other unidentified pathways. It is likely that CD-mediated atheroprotection is multifactorial and that the differential effects of CD such as physically increasing cholesterol solubility and promoting cholesterol metabolism and efflux in macrophages, as well as its anti-inflammatory properties, cannot easily be isolated. Second, although our data identified 27-hydroxycholesterol as the primary LXR agonist upon CD treatment, other endogenous oxysterols

may also contribute to LXR activation. In this context, the functional activity of regulatory enzymes such as Cyp27A1 and the differential effects in specific cell types would be interesting. Third, although our ex vivo experiments with carotid artery plaques suggest that CD can induce atheroprotective pathways in human disease, specific clinical trials are necessary to validate these findings.

Preclinical models showing the effectiveness of LXR agonism in preventing murine atherosclerosis (39) and promoting atherosclerosis regression (40) provided promising prospects for clinical use of LXR agonists in atherosclerosis treatment, but the progression of therapeutic molecules into the clinic was hampered by liver toxicity and lipogenic effects (41). CD, on the other hand, is already in clinical use in humans for the delivery of lipophilic drugs and has not shown relevant toxicity. Hence, repurposing CD for the treatment or prevention of atherosclerosis would be feasible. Our studies provide a proof of principle that therapies aimed at increasing the solubility and removal of macrophage cholesterol could be an effective strategy for the treatment of atherosclerosis.

## MATERIALS AND METHODS

See the Supplementary Materials.

## SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. CD treatment does not influence general cardiovascular parameters.

Fig. S2. CD treatment does not alter plasma sterol concentrations in atherosclerosis regression trials.

Fig. S3. CD (10 mM) does not affect the viability of murine macrophages.

Fig. S4. CD mediates intracellular CC dissolution.

Fig. S5. CC loading of macrophages induces lipid droplet accumulation.

Fig. S6. CD does not affect *Cyp27a1* expression.

Fig. S7. CD treatment induces the expression of cholesterol efflux transporters in aortic arches of atherosclerotic mice.

Fig. S8. CD treatment does not alter murine lipoprotein profiles.

Table S1. LXR target gene list for GSEA analysis of BMDMs from wild-type and *LXRα<sup>-/-</sup>β<sup>-/-</sup>* mice.

Table S2. LXR target gene list for GSEA analysis of human atherosclerotic plaques.

Table S3. List of additional metabolic and regulatory genes (nCounter Panel-Plus).

Table S4. Original data for all figures (provided as an Excel file).

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## Cyclodextrin promotes atherosclerosis regression via macrophage reprogramming

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Editor's Summary

### Dissolving away cholesterol

Cardiovascular disease resulting from atherosclerosis is one of the most common causes of death worldwide, and additional therapies for this disease are greatly needed because not all patients can be effectively treated with existing approaches. Cyclodextrin is a common FDA-approved substance that is already used as a solubilizing agent to improve delivery of various drugs. Now, Zimmer *et al.* have discovered that cyclodextrin can also solubilize cholesterol, removing it from plaques, dissolving cholesterol crystals, and successfully treating atherosclerosis in a mouse model. Because cyclodextrin is already known to be safe in humans, this drug is now a potential candidate for testing in human patients for the treatment of atherosclerosis.

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