Correction

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HDL promotes rapid atherosclerosis regression in mice and alters inflammatory properties of plaque monocyte-derived cells

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HDL cholesterol (HDL-C) plasma levels are inversely related to cardiovascular disease risk. Previous studies have shown in animals and humans that HDL promotes regression of atherosclerosis. We hypothesized that this was related to an ability to promote the loss of monocyte-derived cells (CD68\(^+\), primarily macrophages and macrophage foam cells) from plaques. To test this hypothesis, we used an established model of atherosclerosis regression in which plaque-bearing aortic arches from apolipoprotein E-deficient (apoE\(^{−/−}\)) mice (low HDL-C, high non–HDL-C) were transplanted into recipient mice with differing levels of HDL-C and non–HDL-C: C57BL6 mice (normal HDL-C, low non–HDL-C), apoAI\(^+\)/apoAI\(^−\) mice (low HDL-C, low non–HDL-C), or apoAI\(^+\)/apoAI\(^−\) mice transgenic for human apoAI (hAI/ apoE\(^{−/−}\); normal HDL-C, high non–HDL-C). Remarkably, despite persistent elevated non–HDL-C in hAI/apoE\(^{−/−}\) recipients, plaque CD68\(^+\) cell content decreased by >50% by 1 wk after transplantation, whereas there was little change in apoAI\(^−/−\) recipient mice despite hypolipidemia. The decreased content of plaque CD68\(^+\) cells after HDL-C normalization was associated with their emigration and induction of their chemokine receptor CCR7. Furthermore, in CD68\(^+\) cells laser-captured from the plaques, normalization of HDL-C led to decreased expression of inflammatory factors and enrichment of markers of the M2 (tissue repair) macrophage stage. Again, none of these beneficial changes were observed in the apoAI\(^−/−\) recipients, suggesting a major requirement for reverse cholesterol transport for the beneficial effects of HDL. Overall, these results establish HDL as a regulator in vivo of the migratory and inflammatory properties of monocyte-derived cells in mouse atherosclerotic plaques, and highlight the phenotypic plasticity of these cells.

Arginase I | Alternative Activation

A strong inverse correlation has been established between plasma HDL cholesterol (HDL-C) and the risk of cardiovascular disease (1–5). HDL has been shown to be atheroprotective in mouse models of progression (e.g., refs. 6–9), and its role in regression, although less well characterized, is supported by studies in which HDL was infused into apolipoprotein E-deficient (apoE\(^{−/−}\)) mice (10) or was increased by adenoviral-mediated overexpression of human apoAI or apoE in LDL receptor-deficient or apoE\(^{−/−}\) mice, respectively (11, 12). In addition, infusions of HDL in the cholesterol-fed rabbit (13) or in human subjects (e.g., ref. 14) reduced plaque size.

Taken together, these studies suggest that HDL may be an effective therapy for regression of established atherosclerosis; however, the molecular effects it has on plaque cells in vivo are largely unknown. In the present article, we have adapted a mouse transplantation model of atherosclerosis regression (e.g., refs. 15 and 16) to address this important gap in knowledge. Previous studies in this model have established that transplantation of atherosclerotic aortic arches of hyperlipidemic apoE\(^{−/−}\) mice into normolipidemic WT recipients leads to a rapid decrease in plaque area (e.g., refs. 15 and 17).

By adapting this model, we have now tested the effects on plaques of either normalizing the naturally low plasma level of HDL-C in apoE\(^{−/−}\) mice or lowering plasma non–HDL-C levels in the setting of low HDL-C. The fundamental questions addressed are whether normalization of HDL-C is sufficient and required for atherosclerosis regression, and whether HDL, in addition to decreasing the content of plaque monocyte-derived cells (CD68\(^+\) cells; primarily macrophages and macrophage foam cells) alters the inflammatory phenotypes of these cells.

As will be presented, there were a number of striking findings. Overall, the data establish that in its promotion of mouse atherosclerosis regression, HDL exerts major molecular effects on plaque CD68\(^+\) cells, resulting in significant alterations of their functional properties independent of plasma non–HDL-C levels.

Results

ApoAI Deficiency Impairs Plaque Regression. Our previous results showed that when atherosclerotic aortic segments were shifted from the plasma environment of an apoE\(^{−/−}\) mouse (low HDL-C, high non–HDL-C) to that of a C57BL6 (WT) mouse (normal HDL-C, low non–HDL-C), the CD68\(^+\) cell content of the plaques decreased as rapidly as in 3 d (18, 19). To directly evaluate the importance of normalizing plasma HDL-C when non-HDL is low, apoE\(^{−/−}\) mice were fed a high fat-cholesterol diet for 16 wk to develop advanced plaques (“Baseline” mice), after which their aortic arches were transplanted into either WT or apoAI\(^−/−\) recipient mice.

As expected, the apoAI\(^−/−\) mice had HDL-C and non–HDL-C levels that were extremely low (HDL-C: 11 ± 6 mg/dL vs. 67 ± 8 WT; total C: 56 ± 13 mg/dL vs. 88 ± 19 WT). Remarkably, after transfer of atherosclerotic apoE\(^{−/−}\) aortic segments to the apoAI\(^−/−\) mice, plaque area and CD68\(^+\) cell content did not significantly decrease compared with the results in Baseline mice (Fig. 1A; representative pictures are in Fig. S1). In contrast, in the WT recipients, both parameters decreased significantly over the same 1-wk time period. These results strongly suggested that even with substantial non–HDL-C lowering, plaque regression was significantly impaired by low plasma levels of apoAI and HDL-C.

Selective Normalization of Plasma HDL-C Levels Promotes Rapid Plaque Regression. We also examined the consequences on advanced plaques of selectively normalizing plasma HDL-C. Ath-
ApoAI deficiency impairs plaque regression despite hypolipidemia, but selective normalization of plasma HDL-C levels promotes plaque regression despite hyperlipidemia. (A) After 16 wk on the Western diet, apoE−/− mice were killed and aortic arches were used for baseline determinations (Baseline), or were transplanted into WT or apoAI−/− recipients. Plaque size and the area immunostained by an anti-CD68 antibody were determined. *P < 0.05, compared with Baseline or the apoAI recipients. (B) Similar to A, but transplantation was into apoE−/−, or apoE−/− mice expressing the human apoAI transgene (humanApoAI/apoE−/−). *P < 0.05, compared with Baseline or apoE−/− recipients. For all figures, the number of mice were: Baseline, n = 10; recipients: WT, n = 11; apoAI−/−, n = 14; apoE−/−, n = 11; hAI/apoE−/−, n = 13.

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** ApoAI deficiency impairs plaque regression despite hypolipidemia, but selective normalization of plasma HDL-C levels promotes plaque regression despite hyperlipidemia. (A) After 16 wk on the Western diet, apoE−/− mice were transplanted into WT or apoAI−/− recipients. Plaque size and the area immunostained by an anti-CD68 antibody were determined. *P < 0.05, compared with Baseline or the apoAI recipients. (B) Similar to A, but transplantation was into apoE−/−, or apoE−/− mice expressing the human apoAI transgene (humanApoAI/apoE−/−). *P < 0.05, compared with Baseline or apoE−/− recipients. For all figures, the number of mice were: Baseline, n = 10; recipients: WT, n = 11; apoAI−/−, n = 14; apoE−/−, n = 11; hAI/apoE−/−, n = 13.

eosclerotic aortic segments from apoE−/− mice were transplanted into mice that were either apoE−/− (HDL-C: 26 ± 7 mg/dL; total-C: 497 ± 51 mg/dL), or apoE−/− mice expressing human apoAI (hAI/apoE−/−); HDL-C: 77 ± 16 mg/dL; total-C: 517 ± 60 mg/dL). As summarized in Fig. 1B (representative images are in Fig. S1), at 1 wk posttransplantation, in spite of persistent non-HDL hyperlipidemia, plaque area and CD68+ cell content decreased by ~50% in the hAI/apoE−/− recipients compared with the Baseline mice. In agreement with our previous studies (15, 17), neither parameter significantly decreased in apoE−/− recipient mice. Note that in the hAI/apoE−/− recipients, changes in plaque CD68+ content and size, though clearly improved, were not as large as in the WT recipient, consistent with clinical data showing that maximal regression in intravascular ultrasound studies was achieved when both plasma LDL-C was lowered and HDL-C was raised (20).

**ApoAI Deficiency Impairs, but Normalization of HDL Restores, the Ability of Ly-6Chi−Mononuclear Cells to Emigrate from Plaques.** We had previously demonstrated that depletion of CD68+ cells from plaque macrophages mediated by ly-6Chimice in vivo with fluoroscent beads to track their movements into and out of mouse plaques (22, 23). As shown in Fig. 2A, in plaques transplanted into WT recipients there was a 50% decrease in the number of Ly-6 Чи-derived cells after 1 wk. In contrast, apoAI deficiency impaired the ability of Ly-6Chi−-derived mononuclear cells to emigrate, as judged by the retention of beads. Notably, when plasma HDL-C was normalized in apoE−/− mice, there was a 40% decrease in the number of beads in the plaques compared with the initial content in the Baseline mice. These data suggest that normalizing plasma HDL-C promotes the migration of Ly-6Chi−-derived mononuclear cells from plaques in an apoAI-dependent and a non–HDL-C independent manner.

**Induction of Chemokine Receptor CCR7 Is Blunted in CD68+ Cells by apoAI Deficiency, but Is Restored by Normalization of HDL-C.** We have shown that the chemokine receptor CCR7 was induced in plaque CD68+ cells of apoE−/− aortic arches transplanted into WT mice and that CCR7 was functionally required for the emigration of these cells (19). The deficiency of plaque CD68+ cell emigration in the apoAI−/− recipients raised the possibility that CCR7 was not induced in these mice. To test this theory, plaque CD68+ cells were selected by laser-capture microdissection (LCM) and mRNA isolated. As shown in Fig. 2B, there was an approximately six times induction of CCR7 mRNA in plaque CD68+ cells from WT recipients compared with Baseline mice, but there was obvious blunting of this in the cells from apoAI−/− recipients. In contrast, in cells from hAI/apoE−/− recipients, CCR7 mRNA abundance was significantly increased compared with that in Baseline or apoE−/− recipient mice. These changes at the mRNA level for CCR7 were consistent with those at the protein level (Fig. S2).

To understand the mechanistic basis for the effects on CCR7 gene expression, we performed experiments in DC2.4 cells, which share features of monocyte-derived cells in regressing plaques (18, 25). As shown in Fig. 3, cholesterol loading with acetylated LDL suppressed CCR7 gene expression. Incubation of cells with HDL increased CCR7 gene expression, and after cholesterol loading (resembling the events in hAI/apoE−/− recipients), reversed the suppression of CCR7 gene expression. A similar pattern of results was observed with bone marrow-derived macrophages (BMDM) (Fig. S3). That a variation in CCR7 expression modulated by HDL or apoAI is functionally significant is suggested not only by the emigration in the apoAI−/− mice but by the decrease in the cholesteryl content (as judged by Oil red O staining) (Fig. S4). The increase in the expression of the sterol-regulated gene, HMGCoA reductase, in CD68+ cells from plaques in hAI/apoE−/− recipients relative to those from Baseline mice (Fig. S6).

**ApoAI Deficiency and Normalization of HDL-C Levels Differentially Affect the Expression of Inflammatory Markers in Plaque CD68+ Cells.** HDL has a number of anti-inflammatory properties in vitro (for recent reviews, see refs. 26 and 27). We previously reported that vascular cell-adhesion molecule 1 (VCAM-1) and monocyte chemotactic protein 1 (MCP-1) mRNAs, targets of NF-κB, were decreased in plaque CD68+ cells under regression conditions (19). To investigate whether similar changes were induced by normalization of plasma HDL-C, we measured at the mRNA level these and other known inflammatory factors in laser-captured CD68+ cells. As shown in Fig. 4A, in the apoAI−/− recipients, there were no significant decreases (vs. Baseline mice) in the expression of VCAM-1, intercellular adhesion molecule 1, MCP-1, and TNF-α, whereas expression decreased in the WT recipients. Notably, the normalization of plasma HDL-C levels in hAI/apoE−/− mice also reduced the expression of these inflammatory factors compared with either the Baseline or apoE−/− recipient mice. In the case of MCP-1, the result was consistent with decreased immunostaining in hAI/apoE−/− recipient mice in a previous study in which the other factors were not examined (28).

**Normalization of HDL-C Enhances the M2 Phenotype of Plaque CD68+ Cells.** In response to microenvironmental stimuli, macrophages can become polarized to inflammatory (M1) and reparative (M2)
phenotypes, (e.g., see ref. 29 for a review). Because we observed a decrease in the mRNAs for inflammatory markers in the hAI/apoE−/− recipients, we hypothesized that HDL has broad effects on the inflammatory state of plaque CD68+ cells by affecting their polarization.

Markers for M2 macrophages include arginase 1, mannose receptor, CD163, C-lectin, and Fizz 1. As shown in Fig. 4B, in the WT recipients the mRNAs for all of these markers were significantly up-regulated in plaque CD68+ cells. In the cells from apoAI−/− recipients, except for mannose receptor, the expression of these markers remained at Baseline levels. In marked contrast, normalization of plasma HDL-C levels increased the expression of M2 markers similar to what was observed in WT recipients (Fig. 4B).

To extend these results, we tested whether HDL or apoAI could induce the expression of M2 markers arginase I and Fizz 1 in BMDMs. As shown in Fig. 5, incubation with either HDL or apoAI strongly induced the expression of both markers. Because arginase I has been functionally linked to macrophage function and protection from atherosclerosis (30–32), we also confirmed in plaques (Fig. 6) and in HDL or apoAI-treated BMDMs (Fig. S7) that changes at the protein level were consistent with the arginase I mRNA data. Overall, the data suggest that a component of HDL’s anti-inflammatory properties is its serving as a M2 polarization factor of monocyte-derived cells, which would facilitate tissue repair during plaque regression.

Discussion

Given the widespread presence of atherosclerosis (33), its regression is a clinically appealing approach to reduce cardiovascular disease risk (34). We have previously reported that transplantation of an atherosclerotic aortic segment from the apoE−/− mouse into a normocholesterolemic WT recipient leads to the major loss (>75%) of plaque CD68+ cells by 1 wk (19). Compared with the plasma environment in the apoE−/− donor, three lipoprotein-related factors are changed in the WT recipient: namely, plasma levels of non–HDL-C decrease and those of HDL-C and apoE increase. In the present studies, we focused on the change in HDL-C. There were four major findings: (i) Normalization of plasma HDL-C, despite persistent non–HDL-C hyperlipidemia, resulted in a decrease in plaque size and in the depletion of plaque CD68+ cells within a week; (ii) The reduction in CD68+ cells was associated with their emigration, coincident with the induction of CCR7, a factor we previously demonstrated to be required for plaque regression (19); (iii) Normalization of HDL-C led to decreased inflammation in plaque CD68+ cells and their enrichment in markers of the M2, or tissue repair, phenotype; and (iv) All of the foregoing changes were dependent on apoAI and not on non–HDL-C hyperlipidemia.

The requirement for apoAI suggests that the benefits of HDL-C normalization on the plaques are related, at least in part, to increased reverse cholesterol transport (RCT), the process by which HDL particles brings excess cholesterol from peripheral cells to the liver. Although there are examples to the contrary (35), in the present study plasma levels of HDL-C appeared to serve as a strong functional marker of RCT. Along with the greater level of HDL-C in hAI/apoE−/− plasma relative to apoE−/− mice, there was also increased plasma apoAI [162 mg/dL vs. 39 mg/dL (9, 28)]. In fact, the plasma level of apoAI was also higher than that in the WT mice (∼110 mg/dL) (e.g., see ref. 36). The human-like subspecies of HDL that form when human apoAI is expressed in mice (36, 37) may also have contributed to enhanced RCT in the hAI/apoE−/− mouse. Overall, that many of the plaque CD68+ cell features in WT and hAI/apoE−/− recipients were similar implies that there was sufficient apoAI in the latter mice to form enough cholesterol acceptors to overcome the proatherogenic effects of non–HDL hyperlipidemia and the deficiency of apoE, as was also observed in studies of plaque progression in hAI/apoE−/− mice (e.g., refs. 7–9). Increased RCT in the hAI/apoE−/− recipients would also be consistent with the lower Oil Red O plaque staining (Fig. S5).

The above considerations also suggest a molecular mechanism by which the normalization of HDL-C may have contributed directly to the emigration of plaque CD68+ cells. Both the human and mouse CCR7 promoters have sterol response elements. If these were functional, sterol depletion would activate CCR7 transcription. That in the hAI/apoE−/− plasma environment there was sufficient loss of cholesterol to activate a sterol response element-
regulated gene is supported by finding (vs. Baseline mice) increased HMGCoA reductase mRNA in plaque CD68+ cells (Fig. S6). This proposed regulation of CCR7 by HDL is also consistent with (i) the data on CCR7 gene expression in DC2.4 (Fig. 3), BMDM (Fig. S3), and THP-1 cells (38); and (ii) increased DC2.4 chemotaxis to CCR7 ligands of cells incubated with HDL or apoAI (Fig. S4), and with increased dendritic cell migration in vivo, which depends on CCR7 function (39), in hAI/apoE−/− (vs. apoE−/−) mice (40).

Besides effects on the plaque content of CD68+ cells, the normalization of plasma HDL-C had dramatic effects on the inflammatory state of these cells, with a general decrease and increase, respectively, in markers of M1 (classically activated) or M2 (alternatively activated or tissue repair) macrophages. Although this classification scheme has been considered to be overly simplistic (e.g., ref. 41), it has been a useful start with which to broadly characterize the macrophage heterogeneity known to occur in many tissues, including atherosclerotic plaques (42). Of the M2 markers, arginase I is particularly interesting because of its implication as an antiatherosclerosis factor in animals and humans (30–32). One proposed mechanism for this is that by shunting substrate away from macrophage inducible nitric-oxide synthase, there is reduced production of damaging reactive nitrogen species (e.g., see ref. 43).

Although several factors influencing macrophage polarization in vitro have been identified, including LPS and IFN-γ (for M1) and IL4 and IL13 (for M2), comparatively little is known about the regulation in vivo. Recently, it has been shown that peroxisome proliferator-activated receptor-γ (PPAR-γ) can also drive monocytes toward the M2 state in vitro, but a PPAR-γ agonist was unable to influence in vivo the expression of M2 markers in established atherosclerotic plaque macrophages (42, 44). There is also evidence from diet-induced obese mice that adipose tissue macrophages in the lean state express M2 markers, with obesity down-regulating them and increasing the expression of M1 markers (45). Perhaps a related phenomenon in atherosclerosis is that in plaques in apoE−/− mice, macrophages appeared to have started off in the M2 state, but became M1-like as the phenotype, or on cellular cholesterol content, particularly that of the plasma membrane. The former possibility is suggested by the work of Oram and colleagues, who showed that apoAI was an activator of STAT3 (47). STAT signaling is invoked as a factor in IL4-mediated macrophage polarization to the M2 state, although only STAT6 has been examined in detail (recently reviewed in ref. 48). The latter possibility is supported by the studies from the laboratories of Tall (reviewed in ref. 49) and Parks (50), which have shown heightened responses to inflammatory stimuli when the plasma membranes of cultured macrophages were enriched in unesterified cholesterol, with attenuation after cholesterol efflux.

Another example supporting a role of apoAI or HDL in macrophage polarization was recently provided by Datta and colleagues (51), who showed that human monocyte-derived macrophages incubated with either apoAI or an apoAI peptide mimetic exhibited characteristics more of the M2 state, consistent with the results we observed in mouse BMDMs (Fig. 5). Note that all of the afore-

Fig. 4. Normalization of HDL-C levels change the gene expression of markers of inflammation and of the M2 macrophage phenotype in plaque CD68+ cells in an apoAI-dependent manner. CD68+ cells were laser captured from the plaques of donor (Baseline) mice or from grafts 1 wk after transplantation into recipients. The mRNA levels of the indicated genes associated with inflammation (A) or the M2 macrophage state (B) were measured by qRT-PCR. Data are based on two pools of RNA, each consisting of three independent mice, and are expressed as mean (± SEM) fold-change over Baseline. ICAM-1, intercellular adhesion molecule 1; MR, mannose receptor. *P < 0.05 vs. Baseline.

Fig. 5. HDL and apoAI induce in vitro the gene expression of markers of M2 macrophages. BMDMs were isolated from WT mice, incubated for 6 d in the presence of M-CSF. HDL3 or apoAI was added for 5 h and gene expression of arginase 1, Fizz 1, and cyclophilin A were assessed by qRT-PCR. Shown are the results (mean ± SEM, normalized to cyclophilin A) of three experiments, each done in duplicate or triplicate. *P < 0.01, **P < 0.001 vs. corresponding control value.
mentioned studies were conducted in vitro. To our knowledge, the present study is unique in demonstrating in any significant molecular detail the effects of HDL on the inflammatory state of monocyte-derived cells in atherosclerotic plaques.

We previously performed aortic transplantations from apoE\(^{-/-}\) mice to hAI/apoE\(^{-/-}\) recipients, but ended the experiment much later (5 mo) than in the present study (28). Decreases in plaque size and CD68\(^{-}\) cell content were found, but the other parameters we now report were not examined. Although we were initially surprised to observe changes in the plaque CD68\(^{-}\) content that in 1 wk approached those measured after 5 mo, this rapidity is consistent with both an HDL infusion study in apoE\(^{-/-}\) mice (10) and our aortic transplantation studies in WT recipients (18, 19). As mentioned in Results, however, the magnitude of the decrease in plaque CD68\(^{-}\) cell content in the hAI/apoE\(^{-/-}\) recipient mice after 1 wk was not as great as in the WT recipients, suggesting that for optimal regulation of atherosclerosis in apoE\(^{-/-}\) mice, both an increase in HDL and a lowering of non-HDL are necessary. This finding would agree with a meta-analysis of clinical studies showing that plaque regression was most pronounced with substantial reductions in plasma LDL-C levels and the greatest increases in HDL-C (20). Furthermore, the relative lack of efficacy of low non-HDL-C to promote atherosclerosis regression in the absence of apoAI may relate to the clinical observation that low HDL-C, despite statin-lowered LDL-C levels, raises the risk of cardiovascular events (52). Both the preclinical and clinical data foster the speculation that there is some minimum threshold of RCT required to realize the full benefits of aggressive non–HDL-C reduction.

In summary, the normalization of HDL-C promotes plaque regression with profound changes in the content and characteristics of the monocyte-derived cell population in plaques of apoE\(^{-/-}\) mice. These effects were observed in the face of continuing non-HDL hyperlipidemia and were dependent on apoAI. The extension of these studies is likely to further inform our knowledge of the atheroprotective mechanisms of HDL, especially those related to the regulation of macrophage inflammatory states.

Materials and Methods

Animals and Aortic Transplantation. All procedures were approved by the Animal Care and Use Committee of the New York University School of Medicine. The aortic arch transplantation model has been described previously (15, 16). Basically, a donor arch from an atherosclerotic mouse is interpositioned with the abdominal aorta in the recipient mouse and blood flow is directed through the graft. To develop advanced plaques, male apoE\(^{-/-}\) (C57BL/6) mice were weaned at 1 mo onto a 21% (wt/wt) fat, 0.15% cholesterol Western diet (Research Diets). The diet was continued for 16 wk. Mice were then divided into one group (pretransplant, n = 10) for baseline analyses and another group to be donors of aortic arch segments. The recipients were male mice and were either apoAI\(^{-/-}\) (n = 14), WT (n = 11), apoE\(^{-/-}\) (n = 11), or hAI/apoE\(^{-/-}\) (n = 13) C57BL/6 mice, all maintained on standard chow diet. Recipient mice were killed 1 wk after transplantation.

Lipid and Lipoprotein Analyses. Plasma total cholesterol levels were determined by enzymatic assays (Infinity Total Cholesterol Reagent, Sigma). Plasma HDL-C was determined by precipitating non–HDL-C (Wako Diagnostic) and then assaying the remaining cholesterol with the Infinity Total Cholesterol Reagent.

Plaque Assessment. The pretransplant and grafted arches were removed after perfusion of cold PBS at 100 mm Hg, embedded in OCT, and frozen. Serial sections (6–μm thick) were cut and stained for CD68 (rat anti-mouse; Serotec) as previously described (19). In some cases, staining was done for CCR7 or arginase I. Primary antibodies were a rabbit monoclonal to CCR7 (Abcam) or a rabbit polyclonal to arginase I (Santa Cruz Biotechnology). The secondary antibody for both was FITC-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories). Negative controls were performed with an irrelevant primary antibody.

Morphometric measurements were performed on digitized images of CD68-stained serial sections of each arch by using Imagepro Plus software. At least five sections per vessel were analyzed and the mean value used as the summary parameter.

Laser Capture Microdissection. To isolate CD68\(^{+}\) cells from plaques, LCM was performed with the PixCell II instrument (Arcturus Bioscience), as we have previously reported (53, 54). Briefly, at 100–μm intervals, 6–μm frozen sections were immunostained for CD68 and used as templates for the next five serial sections. RNA was isolated by the Qiagen RNAeasy MicroIsolation kit and treated with DNase. The RNA quality verified with the Agilent 2100 Bioanalyzer. Each RNA sample from laser-captured cells represents a pool of three mice.

Quantitative Real Time RT-PCR. To measure the mRNA abundances of specific genes of interest, the input for each assay was 100 pg of total RNA. The primer and probe sequences for all genes tested was previously described (19, 29, 42, 55). Data were normalized with a housekeeping gene, GAPDH, and changes were expressed relative to changes in the nontransplanted control vessel.

Labeling of Blood Monocytes. For selective labeling of Ly-6Chigh (CCR2\(^{+}\)) monocytes, 250 μL of liposomes containing clodronate were intravenously injected into the donor mice, followed by 250 μL of fluorescent microspheres intravenously 24 h later (22, 23). One week after transplantation, beads were counted in plaques using a Leica fluorescent microscope (light range, 450–490 nm).

Effects of HDL and apoAI on BMDM. BMDM were isolated from tibia and femur of 6- to 8-wk-old C57/BL6 WT mice. Bone marrow cells were incubated for 6 d in DMEM supplemented with 10% FBS and 10 nM macrophage colony-stimulating factor (M-CSF). Twelve hours before treatment with HDL\(_{3}\), or apoAI, cells were incubated in DMEM supplemented with 5% delipidated serum and 10 ng/mL M-CSF. HDL\(_{3}\) or apoAI was added (100 μg/mL) for 5 h. Gene expression of arginase I, Fizz 1, and cycophilin A were assessed by qRT-PCR.

HDL\(_{3}\) was isolated by sequential ultracentrifugation of human plasma. ApoAI was obtained after delipidation of HDL\(_{3}\). All HDL and apoAI preparations used were endotoxin free. Purity of HDL\(_{3}\) was assessed by FPLC that of apoAI by and SDS/PAGE/Red Ponceau staining.

Effects of Cholesterol Loading and HDL-Mediated Cholesterol Efflux on CCR7 Gene Expression in Viro. LDL was acetylated following a published protocol (58). An immature dendritic cell line, DC2.4 (59), was kindly provided by Tom Moran (Mount Sinai School of Medicine, New York, NY). The incubations were: 0.2% BSA-DMEM or the same medium with AcLDL (100 μg/mL for 24 h), HDL\(_{3}\) (50 μg/mL for 24 h), or AcLDL (100 μg/mL for 24 h), and subsequently with HDL\(_{3}\) (50 μg/mL for 24 h).

Statistical Analysis. Data are expressed as mean ± SEM. Data were typically analyzed by one-way ANOVA (GraphPad Software), with multiple comparison testing done post hoc. P < 0.05 was considered significant.
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Fig. S1. Representative images of plaques from the different experimental groups. Sections of aortic plaques from mice from the indicated groups were stained with anti-CD68 antibody and counterstained with hematoxylin. Magnification is 10×.

Fig. S2. Aortic sections from mice in each group were indirectly immunostained for CCR7 (Materials and Methods) using a fluorescently tagged secondary antibody. Representative laser-confocal microscopic images are displayed (40×); L, lumen. The wavy lines are autofluorescent internal elastic lamina.
Fig. S4. HDL or apoAI increases chemotaxis of DC2.4 cells to CCR7 ligands CCL19 and CCL21. Cultures of the immature dendritic cell line, DC 2.4 were treated were pretreated with HDL, 50 μg/mL, or ApoA1 (25 μg/mL) for 24 h before performing chemotaxis assays. Because LXR is up-regulated during regression (1), to simulate this, 1 μM LXR agonist (T0901317) was included in all groups. Chemotaxis buffer (DMEM) with 100 ng/mL CCL19 and 100 ng/mL CCL21 (R&D Systems) was added to the lower chambers of Transwell (Neuroprobe; 5-μm pore) plates. Then, 1 × 10^4 cells were added to the upper chambers and placed at 37 °C (5% CO_2, 95% air). Three hours later, the cells that migrated toward the lower chamber were counted. Assays were performed in triplicate and averaged. Displayed are the means and SEM; the bar with an asterisk indicates P < 0.01 for differences among the means by ANOVA.

Fig. S3. CCR7 gene expression is regulated in bone marrow-derived macrophages (BMDM) by cholesterol loading and efflux. BMDM were prepared as in Fig. 6, then treated and assessed as in Fig. 4. Shown are the mean values of CCR7 mRNA (±SEM) normalized to cyclophilin A mRNA from a representative experiment performed in triplicate. *P < 0.01 vs. basal state. The bar with an asterisk indicates P < 0.01 for differences among the means by ANOVA.


Fig. S5. Plaque cholesteryl ester content in each group as assessed by Oil Red O staining. Plaque sections from the mice described in Fig. 1 were stained by Oil Red O to detect cholesteryl esters using a standard protocol (1). After digitization of the images, the area stained in each section was determined with ImagePro Plus software. Displayed are the means ± SEM. From each mouse, at least three sections/plaque were analyzed.


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Fig. S6. HMGCoA Reductase gene expression in plaque CD68+ cells from Baseline mice and hAI/apoE−/− recipients. CD68+ cells were laser-captured from plaque sections from Baseline (apoE−/− donor; n = 10) and hAI/apoE−/− (n = 13) mice and RNA isolated as described in Fig. 2B. The mRNA abundance of the classic sterol response element-regulated gene HMGCoA reductase was then measured by qRT-PCR. Shown are the means ± SEM. *P < 0.01.

Fig. S7. HDL and apoAI induce in vitro the protein expression of arginase I. BMDM were isolated and treated as in Fig. 5, but instead of measuring the level of arginase I mRNA, the cell lysates were used for Western blot analysis of arginase I protein and hsp90 protein (control) abundance, using primary antibodies from Santa Cruz (1:500) and Cell Signaling (1:1,000), respectively. Detection of signal was by the ECL system (Pierce). Quantification of the resulting images was by densitometry. Two separate experiments were conducted with similar results.