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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−/− mice (low HDL-C, low non–HDL-C), or apoE−/− mice transgenic for human apoAI (hAIApoE−/−; normal HDL-C, high non–HDL-C). Remarkably, despite persistent elevated non–HDL-C for hAIApoE−/− 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 HDLC normalization was associated with their emigration and induction of their chemokine receptor CCR7. Furthermore, in C57BL6 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) macrophase 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.

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 adenosine-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-C 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 plasma 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. 


The authors declare no conflict of interest.

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References

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<sup>−/−</sup> mice were killed and aortic arches were used for baseline determinations (Baseline), or were transplanted into WT or apoAI<sup>−/−</sup> recipients. Plaque size and the area immunostained by an anti-CD68 antibody were determined. ∗p < 0.05, compared with Baseline or the apoAI<sup>−/−</sup> recipients. (B) Similar to A, but transplantation was into apoE<sup>−/−</sup>, or apoE<sup>−/−</sup> apoAI transgenic (human apoAI/apoE<sup>−/−</sup>−) mice expressing the human apoAI transgene (human apoAI/apoE<sup>−/−</sup>−). ∗p < 0.05, compared with Baseline or apoE<sup>−/−</sup>− recipients. For all figures, the number of mice were: Baseline, n = 10; recipients: WT, n = 11; apoAI<sup>−/−</sup>, n = 14; apoE<sup>−/−</sup>−, n = 11; hAI/apoE<sup>−/−</sup>−, n = 13.

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<sup>−/−</sup> mice were transplanted into WT or apoAI<sup>−/−</sup>− recipients. Plaque size and the area immunostained by an anti-CD68 antibody were determined. ∗p < 0.05, compared with Baseline or the apoAI<sup>−/−</sup>− recipients. (B) Similar to A, but transplantation was into apoE<sup>−/−</sup>−, or apoE<sup>−/−</sup>− apoAI transgenic (human apoAI/apoE<sup>−/−</sup>−) mice expressing the human apoAI transgene (human apoAI/apoE<sup>−/−</sup>−). ∗p < 0.05, compared with Baseline or apoE<sup>−/−</sup>− recipients. For all figures, the number of mice were: Baseline, n = 10; recipients: WT, n = 11; apoAI<sup>−/−</sup>, n = 14; apoE<sup>−/−</sup>−, n = 11; hAI/apoE<sup>−/−</sup>−, n = 13.

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

**A**  
**B**

The Ly-6C<sup>hi</sup> (CCR2<sup>hi</sup>) subset of circulating monocytes is linked to the progression of atherosclerosis (21–24), and these cells can be selectively labeled in vivo with fluorescent beads to track their movements into and out of mouse plaques (22, 23). As shown in Fig. 2 A, in plaques transplanted into WT recipients there was a 50% decrease in the content of labeled Ly-6C<sup>hi</sup>-derived cells after 1 wk. In contrast, apoAI deficiency impaired the ability of Ly-6C<sup>hi</sup>-derived mononuclear cells to emigrate, as judged by the retention of beads. Notably, when plasma HDL-C was normalized in apoE<sup>−/−</sup> 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-6C<sup>hi</sup>-derived mononuclear cells from plaques in an apoAI-dependent and a non–HDL-C independent manner.

**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<sup>−/−</sup> mice were transplanted into WT or apoAI<sup>−/−</sup>− recipients. Plaque size and the area immunostained by an anti-CD68 antibody were determined. ∗p < 0.05, compared with Baseline or the apoAI<sup>−/−</sup>− recipients. (B) Similar to A, but transplantation was into apoE<sup>−/−</sup>−, or apoE<sup>−/−</sup>− apoAI transgenic (human apoAI/apoE<sup>−/−</sup>−) mice expressing the human apoAI transgene (human apoAI/apoE<sup>−/−</sup>−). ∗p < 0.05, compared with Baseline or apoE<sup>−/−</sup>− recipients. For all figures, the number of mice were: Baseline, n = 10; recipients: WT, n = 11; apoAI<sup>−/−</sup>, n = 14; apoE<sup>−/−</sup>−, n = 11; hAI/apoE<sup>−/−</sup>−, n = 13.
recipients, we hypothesized that HDL has broad effects. In mice, there was also increased plasma apoAI [162 mg/dl].

Cell features in WT and hAI/apoE mouse. Overall, that many of mice (e.g., refs. 7) could induce the expression of M2 markers arginase I and Fizz 1. As shown in Fig. 4, the expression of these markers remained at Baseline levels. In marked contrast, non-HDL-C hyperlipidemia. Results: (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 HDL-C+ 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 bring 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−/− mouse, 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-

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 I, 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 HDL-C+ 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.
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 the regulation of the M2 macrophage state (48).

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.
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 regression 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 (pretreatment, n = 10) for basal 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 (Wake Diagnostics) 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.

Labeling of Blood Monocytes. For selective labeling of Ly-6C+ (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 C57BL/6 WT mice. Bone marrow cells were incubated for 6 d in DMEM supplemented with 10% FBS and 10 ng/mL macrophage colony-stimulating factor (M-CSF). Twelve hours before treatment with HDL or apoAI, cells were incubated in DMEM supplemented with 5% delipidated serum and 10 ng/mL M-CSF. HDL or apoAI was added (100 μg/mL) for 5 h. Gene expression of arginase 1, Fizz 1, and cyclophilin A were assessed by qRTPCR.

Effects of Cholesterol Loading and HDL-Mediated Cholesterol Efflux on CCR7 Gene Expression in Vitro. 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), HDL3 or apoAI was added (100 μg/mL) for 5 h. Gene expression of arginase 1, Fizz 1, and cyclophilin A were assessed by qRTPCR.

HDL3 was isolated by sequential ultracentrifugation of human plasma. ApoAI was obtained after delipidation of HDL3. All HDL and apoAI preparations used were endotoxin free. Purity of HDL3 was assessed by FPLC that was isolated by sequential ultracentrifugation of human plasma. 

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