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CalDAG-GEFI deficiency reduces atherosclerotic lesion development in mice

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Abstract

Objective—Platelets are important to the development and progression of atherosclerotic lesions. However, relatively little is known about the contribution of platelet signaling to this pathological process. Our recent work identified two independent, yet synergistic signaling pathways that lead to the activation of the small GTPase Rap1; one mediated by the guanine nucleotide exchange factor, CalDAG-GEFI (CDGI), the other by P2Y12, a platelet receptor for ADP and the target of anti-platelet drugs. In this study, we evaluated lesion formation in atherosclerosis-prone low-density lipoprotein receptor deficient ($Ldlr^{-/-}$) mice lacking CDGI and/or P2Y12 in hematopoietic cells.

Approach and Results—Lethally irradiated $Ldlr^{-/-}$ mice were reconstituted with bone marrow from $Caldaggef1^{-/-}$ ($cdgI^{-/-}$), $p2y12^{-/-}$, or $cdgI^{-/-}p2y12^{-/-}$ (DKO) mice and fed a high fat diet for 12 weeks. $Ldlr^{-/-}$ chimeras deficient for CDGI and/or P2Y12 developed significantly smaller atherosclerotic lesions in the aortic sinus and in aortas when compared to the $Ldlr^{-/-}$/WT controls. We also observed a significant reduction in platelet-leukocyte aggregates in blood from hypercholesterolemic $Ldlr^{-/-}/cdgI^{-/-}$ and $Ldlr^{-/-}/p2y12^{-/-}$ chimeras. Consistently, fewer macrophages and neutrophils were detected in the aortic sinus of $Ldlr^{-/-}/cdgI^{-/-}$ and $Ldlr^{-/-}/p2y12^{-/-}$ chimeras. Compared to controls, the plaque collagen content was significantly higher in $Ldlr^{-/-}$ chimeras lacking CDGI. Interestingly, no statistically significant additive effects were seen in $Ldlr^{-/-}$/DKO chimeras when compared to chimeras lacking only CDGI.

Conclusion—Our findings suggest that CDGI is critical for atherosclerotic plaque development in hypercholesterolemic Ldlr−/− mice due to its contribution to platelet-leukocyte aggregate formation and leukocyte recruitment to the lesion area.

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Disclosures

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Keywords

platelets; signaling; Rap1; CalDAG-GEFI; atherosclerosis

Introduction

Atherosclerosis is a chronic inflammatory vascular disease involving various cells, such as vascular cells, leukocytes and platelets. The presence of circulating activated platelets has been reported in the blood of patients with unstable atherosclerosis [1, 2], stable coronary disease [3] and hypercholesterolemia [4]. Platelet depletion or blockade of the main platelet adhesion receptors, GPIbα or αIIbβ3, in apolipoprotein E-null (apoE$^{-/-}$) mice profoundly reduced leukocyte accumulation in the arterial intima and attenuated atherosclerotic lesion formation [5]. Thus, platelets seem to be a proximal sensor of the vascular injury before the development of atherosclerotic lesions.

Platelet activation has long been postulated to contribute to the development of atherosclerotic plaques, although the mechanism by which this might occur is not clear. Activated platelets in blood bind leukocytes mainly via a platelet P-selectin – leukocyte PSGL-1 interaction [6], and platelet–leukocyte aggregates are well-documented in patients and mice with atherosclerosis [7, 8]. Once formed, platelet-leukocyte aggregates promote endothelial cell (EC) activation and EC P-selectin-dependent leukocyte rolling [9]. Indeed, deletion of P-selectin in platelets and/or ECs leads to significantly impaired early atherosclerotic lesion development in mice [10,11]. Platelets are also a reservoir for cytokines (eg. CXCL4, IL1β, CD40L), which can increase vascular permeability and leukocyte arrest/extravasation[12,13]. Interaction between activated platelets and endothelial cells triggers cytokine secretion (eg. CCL2), increased surface expression of inflammatory adhesion molecules (eg. VCAM1) and the release of microvesicles by endothelial cells [6]. The initial platelet tethering depends on the interaction of glycoprotein (GP) VI with subendothelial collagen and GPIb-V-IX with von Willebrand factor (vWF) bound to the surface of activated endothelial cells [14]. Following cellular activation, integrin αIIbβ3 is critical for firm adhesion of platelets to the exposed subendothelium and to activated endothelial cells [15].

Studies from our group established a central role for Ca^{2+} and diacylglycerol regulated guanine nucleotide exchange factor I (CalDAG-GEFI, CDGI, RasGRP2) in the inside-out activation of integrins on platelets and neutrophils [16,17,18]. CDGI catalyzes the activation of the small GTPase Rap1. In platelets, the Rap1B isoform accounts for ~90% of the total Rap protein [19], and its importance in αIIbβ3 activation was demonstrated in studies with Rap1b-deficient mice [20]. Our recent studies identified a 2-pathway model for Rap1-dependent integrin activation downstream of PLC activation in platelets [21]. CDGI is a high-affinity sensor for Ca^{2+}, which mediates the rapid but reversible activation of αIIbβ3 [22]. In the absence of CDGI, Rap1/integrin activation is delayed but sustained and depends on signaling by protein kinase C and the platelet receptor for ADP, P2Y12 [23]. Importantly, apoE$^{-/-}$ mice deficient in P2Y12 exhibit reduced plaque formation when on high fat diet (HFD) [24]. Furthermore, treatment of atherosclerotic patients with the P2Y12 inhibitor, clopidogrel, reduced the number of P-selectin-positive platelets and platelet-leukocyte

aggregates in blood [25, 26]. In mice, an anti-atherogenic effect of clopidogrel was reported by some investigators [27, 28], while others could not reproduce these results [29, 30].

Based on these observations, we hypothesized that platelet Rap1 signaling, mediated by CDGI and P2Y12, contributes to plaque development in atherosclerosis-prone Ldlr$^{-/-}$ mice, and that deletion of CDGI in hematopoietic cells leads to impaired lesion development in hypercholesterolemic Ldlr$^{-/-}$ mice.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Generation and characterization of Ldlr$^{-/-}$ mice lacking CalDAG-GEFI and/or P2Y12 in blood cells only

At 4 weeks of age, Ldlr$^{-/-}$ male mice were lethally irradiated and reconstituted with bone marrow from either WT, cdgI$^{-/-}$, p2y12$^{-/-}$ or cdgI$^{-/-}$ p2y12$^{-/-}$ double knockout (DKO) mice. After 6 weeks of normal chow diet, leukocyte counts and platelet counts were not significantly different between transplanted animals (Supplemental Fig. I). The mice were then put on high fat diet (HFD) for 12 weeks, and the blood lipid levels and platelet activation response were assessed. Total cholesterol, triglyceride, VLDL, HDL and BWs were similar among all groups after 12 weeks of HFD (Table1). Platelet counts were similar between the different groups of chimeric mice (Fig. 1A). In response to stimulation of the thrombin receptor, protease-activated receptor 4 (PAR4), or the collagen receptor, GPVI, platelets from Ldlr$^{-/-}$/cdgI$^{-/-}$ and Ldlr$^{-/-}$/p2y12$^{-/-}$ mice showed reduced P-selectin exposure and integrin activation compared with Ldlr$^{-/-}$/WT platelets (Fig. 1B). As expected, agonist-induced platelet activation was abolished in Ldlr$^{-/-}$/DKO mice (Fig. 1B).

Platelet adherence to fibrous-cap-derived collagen or the damaged endothelium is a nidus for inflammatory and progenitor cell accumulation at atherosclerotic sites leading to atheroprogression. Therefore, we examined platelet accumulation on a collagen surface under flow. Anticoagulated whole blood from hypercholesterolemic mice was perfused over immobilized collagen at arterial (1200 s^{-1}) shear rates. Blood from Ldlr$^{-/-}$/WT chimeric mice formed three-dimensional thrombi that stained strongly for surface P-selectin, a marker of platelet activation (Fig. 1C–E). In contrast, thrombus formation at arterial shear rates was almost completely abolished in blood isolated from both Ldlr$^{-/-}$/cdgI$^{-/-}$ and Ldlr$^{-/-}$/DKO mice. Small thrombi consistent with activated (P-selectin-positive) platelets were observed when blood from Ldlr$^{-/-}$/p2y12$^{-/-}$ chimeric mice was perfused over collagen. These findings are consistent with our previous work showing significantly impaired adhesion under flow conditions for platelets lacking CDGI or P2Y12 [21].

While integrin activation, and to some extent granule secretion, are dependent on Rap1 signaling, we wanted to directly assess Rap1 activation and determine the effect of cholesterol on platelet sensitivity to activation. When stimulated with the weaker agonist, ADP, platelet aggregation and Rap1 activation were abolished in CdgI$^{-/-}$ or p2y12$^{-/-}$

platelets, both in the presence and absence of exogenous cholesterol (Fig. 2). Interestingly, cholesterol loading of wild-type platelets enhanced ADP-induced Rap1 activation and aggregation and delayed reversibility, with no apparent effect in platelets deficient for CalDAG-GEFI or P2Y12.

Reduced atherosclerotic lesion formation in hypercholesterolemic Ldlr$^{-/-}$/cdgI$^{-/-}$ and Ldlr$^{-/-}$/p2y12$^{-/-}$ chimeric mice

We next determined the effect of hematopoietic CDGI and/or P2Y12 deficiency on atherosclerosis in the aorta or aortic sinus of hypercholesterolemic mice. By morphometric analysis, we determined that lesions in the aortic sinus of Ldlr$^{-/-}$/cdgI$^{-/-}$ chimeras were ~42% smaller than those of Ldlr$^{-/-}$/WT control chimeras (0.18 ± 0.02 mm^2 vs 0.31 ± 0.05 mm^2, p< 0.001)(Fig. 3A,B). Similarly, lesions in the aortic sinus of Ldlr$^{-/-}$/p2y12$^{-/-}$ chimeras were significantly smaller than those in controls (0.22 ± 0.10 mm^2, p<0.01). Finally, Ldlr$^{-/-}$/DKO chimeras had 48% smaller lesions compared to Ldlr$^{-/-}$/WT controls (0.16 ± 0.02 mm^2 vs 0.31 ± 0.05 mm^2, p< 0.001). Aortic root lesion size was similar between hypercholesterolemic Ldlr$^{-/-}$/cdgI−/−, Ldlr$^{-/-}$/p2y12$^{-/-}$ and Ldlr$^{-/-}$/DKO chimeric mice, with no additive effect in mice lacking both CDGI and P2Y12.

Atherosclerotic lesion development was also evaluated by *en face* analysis of the aortic arch and abdominal aorta. Analyses of the aortic arches showed a ~two-fold reduction in lesion size for Ldlr$^{-/-}$/cdgI$^{-/-}$, Ldlr$^{-/-}$/p2y12$^{-/-}$ and Ldlr$^{-/-}$/DKO chimeras when compared to Ldlr$^{-/-}$/WT chimeras (5.93 ± 0.66%, 4.68 ± 0.65%, 4.42 ± 0.56% versus 9.26 ± 1.21%) (Fig. 4A). Similarly, atherosclerotic lesions in the abdominal aortas of Ldlr$^{-/-}$/cdgI$^{-/-}$, Ldlr$^{-/-}$/p2y12$^{-/-}$ and Ldlr$^{-/-}$/DKO chimeras were ~two-fold smaller compared to Ldr$^{-/-}$/WT chimeras (1.35 ± 0.28%, 1.29 ± 0.30%, 1.60 ± 0.35% versus 3.48 ± 0.80%) (Fig. 4B). No additive effects were observed in double vs. single knockout mice. Together, these results suggest a significant role for both CDGI and P2Y12 signaling in bone marrow-derived cells in promoting atherosclerosis throughout the arterial tree.

Reduced platelet-neutrophil interactions in blood from hypercholesterolemic Ldlr $^{-/-}$/ cdgI$^{-/-}$ mice

Activated platelets are known to form pro-inflammatory platelet-leukocyte aggregates in circulation [8,7], and these cellular aggregates are associated with increased atherosclerosis and acute coronary syndrome[3]. To test whether deficiency in CDGI and/or P2Y12 affects the formation of such aggregates, we measured platelet-neutrophil aggregates (PNAs), identified as GPIb$^+$Ly6G$^+$ events by flow cytometric analysis, in blood from the above described Ldlr$^{-/-}$ chimeric mice. PNAs were significantly increased in Ldlr$^{-/-}$/WT mice fed a HFD for 12 weeks when compared to mice on a normal diet (Fig. 5A). In contrast, hypercholesterolemia did not induce any increase in circulating PNAs in Ldlr$^{-/-}$/cdgI$^{-/-}$, Ldlr$^{-/-}$/p2y12$^{-/-}$ or Ldlr$^{-/-}$/DKO mice. Consistent with this finding, plasma levels of platelet factor 4, a marker of systemic platelet activation, were significantly reduced in Ldlr$^{-/-}$/cdgI$^{-/-}$ and Ldlr$^{-/-}$/p2y12$^{-/-}$ chimeras when compared to controls (Table 1). Platelet-neutrophil adhesion was also investigated in blood from hypercholesterolemic mice perfused over collagen at a shear rate of 400s^{-1}. In these experiments, αIIbβ3-mediated platelet aggregate formation was inhibited to better visualize PNA formation. Firm

neutrophil adhesion to adherent platelets under flow was significantly reduced in Ldlr $^{-/-}$/cdgI$^{-/-}$ and Ldlr$^{-/-}$/DKO blood (Fig. 5B). A trend to lower PNA formation was also observed in Ldlr$^{-/-}$/p2y12$^{-/-}$ blood. Together, these findings suggest that CDGI and P2Y12 may contribute to leukocyte recruitment into the developing plaque.

Decreased inflammation and plaque remodeling in hypercholesterolemic Ldlr$^{-/-}$cdgI$^{-/-}$ mice

We next investigated whether macrophage (anti-CD68) and/or neutrophil (anti-Ly6G) accumulation in areas of plaque development was affected in Ldlr $^{-/-}$/cdgI$^{-/-}$ and Ldlr$^{-/-}$/p2y12$^{-/-}$ mice. In line with the decreased lesion area, we observed markedly reduced intimal macrophage abundance in the aortic roots of Ldlr$^{-/-}$/cdgI$^{-/-}$ and Ldlr$^{-/-}$/DKO chimeras when compared with Ldlr$^{-/-}$/WT controls (0.10 ± 0.007%, 0.10 ± 0.006% versus 0.20 ± 0.006%) (Fig. 6A–B, supplemental Fig II). A less pronounced but significant reduction in macrophage content was observed in Ldlr$^{-/-}$/p2y12$^{-/-}$chimeric mice. Similarly, we observed that the neutrophil content in the aortic sinus was significantly reduced in Ldlr$^{-/-}$/cdgI$^{-/-}$ and Ldlr$^{-/-}$/DKO chimeras. A small but insignificant reduction in neutrophil accumulation was also observed in the aortic sinus of Ldlr$^{-/-}$/p2y12$^{-/-}$ chimeras (Fig. 7A–B). However, no marked differences in leukocyte numbers per lesion area were observed for any of the genotypes (Supplemental Fig III), suggesting that loss of CDGI, and to a lesser extent P2Y12, may limit atherosclerotic lesion formation by impairing the recruitment of monocytes and neutrophils into the vessel wall at athero-susceptible sites (Figs. 6–7).

In mice and humans, atherosclerotic plaque progression is associated with an increase in smooth muscle cell migration and proliferation within the subintima, where these cells produce and deposit extracellular matrix components, primarily collagen. Macrophages and neutrophils contain and release proteases such as elastase, cathepsin G and myeloperoxidase that can degrade extracellular matrix proteins, resulting in plaque remodeling. Interestingly, collagen abundance was significantly greater in aortic sinus lesions of hypercholesterolemic Ldlr$^{-/-}$/cdgI$^{-/-}$ and Ldlr$^{-/-}$/DKO chimeric mice when compared to Ldlr$^{-/-}$/WT controls (0.48 ± 0.07% and 0.47 ± 0.05% vs 0.27 ± 0.01%, Fig. 8), while no significant difference in collagen levels was observed between hypercholesterolemic Ldlr$^{-/-}$/WT and Ldlr$^{-/-}$/p2y12$^{-/-}$ chimeric mice (Fig. 8). As leukocyte densities were not significantly different between any groups of mice (Supplemental Fig. III), these findings suggest that, in addition to its role in leukocyte recruitment and atherogenesis, CDGI signaling may contribute to leukocyte activation and plaque destabilization.

Discussion

It is now widely accepted that platelets contribute to a pro-adhesive and pro-inflammatory environment during atherosclerosis. As shown by intravital microscopy, platelets adhere to the inflamed endothelium before lesions can be detected [5]. Once adherent and activated, platelets can deposit chemokines on the luminal surface of the vessel wall. Activated platelets also express adhesion receptors important for the recruitment of leukocytes. Importantly, both leukocyte recruitment and atherosclerotic plaque formation were markedly reduced in thrombocytopenic mice or mice with select platelet adhesion defects [5]. However,

the contribution of Rap1 signaling, a critical node in platelet activation, to this process has not been established. To address this question, we studied atherosclerotic plaque development in Ldlr−/− chimeric mice lacking key regulators of platelet Rap1 signaling. We provide evidence that signaling by the Rap-GEF, CDGI, is critical in promoting both atherosclerotic plaque formation and remodeling in $Ldlr^{-/-}$ mice. We further demonstrate that plaque formation, but not composition, depends on signaling via the ADP receptor, P2Y12, an important pathway leading to Rap1 activation in platelets.

CDGI is an important regulator of Rap1 activity in murine platelets and neutrophils. While our studies clearly show that CDGI signaling in hematopoietic cells is critical during atherosclerotic plaque development in mice, we cannot provide a conclusive answer for whether reduced plaque formation in $Ldlr^{-/-}/cdgI^{-/-}$ chimeras is due to altered function of platelets, neutrophils, or both. However, for several reasons we think that CDGI signaling in platelets plays a critical role during plaque development. First, CDGI signaling is critical to αIIbβ3 integrin activation and platelet adhesion, important processes in atherosclerosis. Second, the CDGI-independent pathway to Rap1 activation and platelet adhesion requires signaling by P2Y12, a surface receptor that is not expressed on hematopoietic cell types other than platelets; plaque formation was also markedly impaired in $Ldlr^{-/-}/p2y12^{-/-}$ chimeras. Finally, both platelet activation and platelet-leukocyte aggregate formation were significantly reduced in both $Ldlr^{-/-}/cdgI^{-/-}$ and $Ldlr^{-/-}/p2y12^{-/-}$ chimeras, providing a plausible explanation for the reduced accumulation of leukocytes in athero-prone areas of hypercholesterolemic mice. Future studies in mice lacking CDGI or the two Rap1 isoforms specifically in platelets or neutrophils will be required to clarify, on a cellular level, how this signaling pathway contributes to atherosclerotic lesion development, and whether the effects observed in $Ldlr^{-/-}/cdgI^{-/-}$mice are indeed caused by a defect in Rap1 signaling.

Interestingly, our studies also uncovered some interesting differences in plaque composition between $Ldlr^{-/-}/cdgI^{-/-}$ and $Ldlr^{-/-}/p2y12^{-/-}$ mice, as plaques from $Ldlr^{-/-}/cdgI^{-/-}$ chimeras were high in collagen content. It is well-known that neutrophils secrete cytokines and proteases that result in: (1) endothelial cell dysfunction and vascular permeability, (2) monocyte recruitment and foam cell formation, and (3) endothelial erosion and weakening of the fibrous cap. In humans, neutrophils are often found in rupture-prone plaques, i.e. lesions with a large lipid core and low collagen content[31]. Thus, the increased collagen content in lesions of $Ldlr^{-/-}/cdgI^{-/-}$ chimeras may, at least in part, be the result of impaired neutrophil activation, a conclusion that was not experimentally tested in this study. Future studies in mice with cell type-specific deletion of CDGI will be necessary to clarify these points.

A critical role for P2Y12 in atherosclerotic plaque development has been reported by several groups, using both genetic and pharmacological disruption of receptor function; however, the results using the P2Y12 inhibitor clopidogrel have been variable depending on the experimental conditions. Our results on the role of P2Y12 in atherosclerosis are in accordance with a recent publication by Li et al., showing that deficiency of P2Y12 in $apoE^{-/-}$ mice fed a HFD for 20 weeks results in decreased atherosclerotic lesion formation[24]. Both our study and that of Li et al. also document a significant reduction of plasma PF4 levels in P2Y12-deficient hypercholesterolemic mice, indicative of reduced

platelet activation in these animals. In contrast, treatment of apoE−/− mice with the P2Y12 inhibitor clopidogrel bisulfate was associated with inconsistent results. While two groups found significant protection from atherosclerosis in clopidogrel-treated mice[27,28], other groups could not reproduce these findings[29, 30]. Pharmacological inhibition is more likely to give variable results than a genetic approach. We can only speculate that differences in the experimental conditions and the inhibitor dose may be responsible for the discrepant results. The genetic approach eliminates some of these potential inconsistencies and thus should be considered more definitive.

An interesting observation in our studies is that the atherosclerotic lesion area in Ldlr$^{-/-}$/DKO chimeras was not statistically different from that in Ldlr$^{-/-}$/cdgI$^{-/-}$ or Ldlr$^{-/-}$/p2y12$^{-/-}$ chimeric mice, although a trend to smaller plaques was observed in the aortic sinus of DKO mice. Given that deficiency in CDGI or P2Y12 only partially inhibits Rap1-mediated platelet responses (Fig. 1), one could have expected additive effects of deleting both molecules at the same time. It is important to remember, however, that synergistic activity by both pathways is required for Rap1 activation to occur in platelets that are activated with weak agonists, such as ADP (Fig. 2), or threshold concentrations of strong agonists[23, 17]. Thus, our results suggest that only weak stimulation of platelets occurs at sites of plaque development, a conclusion that is consistent with the fact that the endothelial lining is intact at early stages of atherosclerosis[32].

Significant progress has been made in our understanding of the molecular mechanisms regulating plaque development and destabilization in experimental atherosclerosis. However, most of these new concepts have not been validated in humans, and translation of these findings into novel strategies to prevent atherosclerosis is lacking. Direct targeting of receptors critical for leukocyte or platelet adhesion, such as β2 and β3 integrins, is not a viable strategy, as these receptors are too important for the physiologic function of these cells. Targeting of agonist receptors, such as GPCRs, would seem a better approach, but redundancy among these receptors may limit the effectiveness of such interventions. Based on our studies, CDGI represents a novel therapeutic target to limit atherosclerotic lesion development. Rap1 signaling is critical for integrin activation both in platelets and neutrophils. CDGI is an important activator of Rap1, which operates downstream of most agonist receptors expressed on these cells. CDGI is important for the rapid inside-out activation of integrins, required for platelet adhesion under conditions of high shear stress; at lower shear stress, however, platelet adhesion is supported by CDGI-independent Rap1 signaling, mediated by PKC and P2Y12. Thus, CDGI signaling is particularly important for platelet adhesion in areas of high shear stress, such as found in the coronary arteries. Expression of CDGI is limited to few cell types, including platelets, neutrophils and neurons in certain areas in the brain. However, dogs[33] and humans[34] with loss-of-function mutations in CDGI are characterized by impaired function of platelets but not other cells. Based on these findings and our current work, we propose that inhibition of CDGI signaling may be a powerful approach to safely prevent atherosclerosis and atherothrombosis.

In summary, our findings reveal a critical role for the Rap1-GEF, CDGI, in promoting atherosclerotic plaque development in hypercholesterolemic Ldlr$^{-/-}$ mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard Abbreviations and Acronyms

ADP	adenosine diphosphate
apoE	apolipoprotein E
CDGI	CalDAG-GEFI
DKO	double knockout
EC	endothelial cell
GP	glycoprotein
GPCR	G-protein-coupled receptor
HFD	high fat diet
Ldlr	low-density lipoprotein receptor
PF4	platelet factor 4

References

1. Aukrust P, Muller F, Ueland T, Berget T, Aaser E, Brunsvig A, Solum NO, Forfang K, Froland SS, Gullestad L. Enhanced levels of soluble and membrane-bound cd40 ligand in patients with unstable angina. Possible reflection of t lymphocyte and platelet involvement in the pathogenesis of acute coronary syndromes. Circulation. 1999; 100:614–620. [PubMed: 10441098]
2. Fitzgerald DJ, Roy L, Catella F, FitzGerald GA. Platelet activation in unstable coronary disease. N Engl J Med. 1986; 315:983–989. [PubMed: 3531859]
3. Furman MI, Benoit SE, Barnard MR, Valeri CR, Borbone ML, Becker RC, Hechtman HB, Michelson AD. Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. J Am Coll Cardiol. 1998; 31:352–358. [PubMed: 9462579]
4. Broijersen A, Hamsten A, Eriksson M, Angelin B, Hjemdahl P. Platelet activity in vivo in hyperlipoproteinemia--importance of combined hyperlipidemia. Thromb Haemost. 1998; 79:268–275. [PubMed: 9493574]
5. Massberg S, Brand K, Gruner S, Page S, Muller E, Muller I, Bergmeier W, Richter T, Lorenz M, Konrad I, Nieswandt B, Gawaz M. A critical role of platelet adhesion in the initiation of atherosclerotic lesion formation. J Exp Med. 2002; 196:887–896. [PubMed: 12370251]
6. Projahn D, Koenen RR. Platelets: Key players in vascular inflammation. J Leukoc Biol. 2012; 92:1167–1175. [PubMed: 22923486]

7. Zarbock A, Polanowska-Grabowska RK, Ley K. Platelet-neutrophil-interactions: Linking hemostasis and inflammation. Blood Rev. 2007; 21:99–111. [PubMed: 16987572]
8. Huo Y, Schober A, Forlow SB, Smith DF, Hyman MC, Jung S, Littman DR, Weber C, Ley K. Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein e. Nat Med. 2003; 9:61–67. [PubMed: 12483207]
9. Dole VS, Bergmeier W, Mitchell HA, Eichenberger SC, Wagner DD. Activated platelets induce weibel-palade-body secretion and leukocyte rolling in vivo: Role of p-selectin. Blood. 2005; 106:2334–2339. [PubMed: 15956287]
10. Burger PC, Wagner DD. Platelet p-selectin facilitates atherosclerotic lesion development. Blood. 2003; 101:2661–2666. [PubMed: 12480714]
11. Johnson RC, Chapman SM, Dong ZM, Ordovas JM, Mayadas TN, Herz J, Hynes RO, Schaefer EJ, Wagner DD. Absence of p-selectin delays fatty streak formation in mice. J Clin Invest. 1997; 99:1037–1043. [PubMed: 9062362]
12. Shi G, Field DJ, Long X, Mickelsen D, Ko KA, Ture S, Korshunov VA, Miano JM, Morrell CN. Platelet factor 4 mediates vascular smooth muscle cell injury responses. Blood. 2013; 121:4417–4427. [PubMed: 23568488]
13. Koenen RR, Weber C. Platelet-derived chemokines in vascular remodeling and atherosclerosis. Semin Thromb Hemost. 2010; 36:163–169. [PubMed: 20414831]
14. Massberg S, Gawaz M, Gruner S, Schulte V, Konrad I, Zohlnhofer D, Heinzmann U, Nieswandt B. A crucial role of glycoprotein vi for platelet recruitment to the injured arterial wall in vivo. J Exp Med. 2003; 197:41–49. [PubMed: 12515812]
15. Massberg S, Schurzinger K, Lorenz M, Konrad I, Schulz C, Plesnila N, Kennerknecht E, Rudelius M, Sauer S, Braun S, Kremmer E, Emambokus NR, Frampton J, Gawaz M. Platelet adhesion via glycoprotein iib integrin is critical for atheroprogression and focal cerebral ischemia: An in vivo study in mice lacking glycoprotein iib. Circulation. 2005; 112:1180–1188. [PubMed: 16103235]
16. Crittenden JR, Bergmeier W, Zhang Y, Piffath CL, Liang Y, Wagner DD, Housman DE, Graybiel AM. Caldag-gefi integrates signaling for platelet aggregation and thrombus formation. Nat Med. 2004; 10:982–986. [PubMed: 15334074]
17. Stefanini L, Roden RC, Bergmeier W. Caldag-gefi is at the nexus of calcium-dependent platelet activation. Blood. 2009; 114:2506–2514. [PubMed: 19628710]
18. Bergmeier W, Goerge T, Wang HW, Crittenden JR, Baldwin AC, Cifuni SM, Housman DE, Graybiel AM, Wagner DD. Mice lacking the signaling molecule caldag-gefi represent a model for leukocyte adhesion deficiency type iii. J Clin Invest. 2007; 117:1699–1707. [PubMed: 17492052]
19. Torti M, Lapetina EG. Structure and function of rap proteins in human platelets. Thromb Haemost. 1994; 71:533–543. [PubMed: 8091376]
20. Chrzanowska-Wodnicka M, Smyth SS, Schoenwaelder SM, Fischer TH, White GC 2nd. Rap1b is required for normal platelet function and hemostasis in mice. J Clin Invest. 2005; 115:680–687. [PubMed: 15696195]
21. Stolla M, Stefanini L, Roden RC, Chavez M, Hirsch J, Greene T, Ouellette TD, Maloney SF, Diamond SL, Poncz M, Woulfe DS, Bergmeier W. The kinetics of alphaiibbeta3 activation determines the size and stability of thrombi in mice: Implications for antiplatelet therapy. Blood. 2011; 117:1005–1013. [PubMed: 20971951]
22. Stefanini L, Bergmeier W. Caldag-gefi and platelet activation. Platelets. 2010; 21:239–243. [PubMed: 20218908]
23. Cifuni SM, Wagner DD, Bergmeier W. Caldag-gefi and protein kinase c represent alternative pathways leading to activation of integrin alphaiibbeta3 in platelets. Blood. 2008; 112:1696–1703. [PubMed: 18544684]
24. Li D, Wang Y, Zhang L, Luo X, Li J, Chen X, Niu H, Wang K, Sun Y, Wang X, Yan Y, Chai W, Gartner TK, Liu J. Roles of purinergic receptor p2y, g protein-coupled 12 in the development of atherosclerosis in apolipoprotein e-deficient mice. Arterioscler Thromb Vasc Biol. 2012; 32:e81–89. [PubMed: 22628428]
25. Klinkhardt U, Bauersachs R, Adams J, Graff J, Lindhoff-Last E, Harder S. Clopidogrel but not aspirin reduces p-selectin expression and formation of platelet-leukocyte aggregates in patients

with atherosclerotic vascular disease. Clin Pharmacol Ther. 2003; 73:232–241. [PubMed: 12621388]

26. Cha JK, Jeong MH, Lee KM, Bae HR, Lim YJ, Park KW, Cheon SM. Changes in platelet p-selectin and in plasma c-reactive protein in acute atherosclerotic ischemic stroke treated with a loading dose of clopidogrel. J Thromb Thrombolysis. 2002; 14:145–150. [PubMed: 12714834]
27. Afek A, Kogan E, Maysel-Auslender S, Mor A, Regev E, Rubinstein A, Keren G, George J. Clopidogrel attenuates atheroma formation and induces a stable plaque phenotype in apolipoprotein e knockout mice. Microvascular research. 2009; 77:364–369. [PubMed: 19323972]
28. Heim C, Gebhardt J, Ramsperger-Gleixner M, Jacobi J, Weyand M, Ensminger SM. Clopidogrel significantly lowers the development of atherosclerosis in apoe-deficient mice in vivo. Heart and vessels. 2015
29. West LE, Steiner T, Judge HM, Francis SE, Storey RF. Vessel wall, not platelet, p2y12 potentiates early atherogenesis. Cardiovascular research. 2014; 102:429–435. [PubMed: 24510394]
30. Schulz C, Konrad I, Sauer S, Orschiedt L, Koellnberger M, Lorenz R, Walter U, Massberg S. Effect of chronic treatment with acetylsalicylic acid and clopidogrel on atheroprogression and atherothrombosis in apoe-deficient mice in vivo. Thromb Haemost. 2008; 99:190–195. [PubMed: 18217153]
31. Ionita MG, van den Borne P, Catanzariti LM, Moll FL, de Vries JP, Pasterkamp G, Vink A, de Kleijn DP. High neutrophil numbers in human carotid atherosclerotic plaques are associated with characteristics of rupture-prone lesions. Arterioscler Thromb Vasc Biol. 2010; 30:1842–1848. [PubMed: 20595650]
32. Stary HC, Blankenhorn DH, Chandler AB, Glagov S, Insull W Jr, Richardson M, Rosenfeld ME, Schaffer SA, Schwartz CJ, Wagner WD, et al. A definition of the intima of human arteries and of its atherosclerosis-prone regions. A report from the committee on vascular lesions of the council on arteriosclerosis, american heart association. Arteriosclerosis and thrombosis : a journal of vascular biology / American Heart Association. 1992; 12:120–134. [PubMed: 1731855]
33. Boudreaux MK, Catalfamo JL, Klok M. Calcium-diacylglycerol guanine nucleotide exchange factor i gene mutations associated with loss of function in canine platelets. Translational research : the journal of laboratory and clinical medicine. 2007; 150:81–92. [PubMed: 17656327]
34. Canault M, Ghalloussi D, Grosdidier C, Guinier M, Perret C, Chelghoum N, Germain M, Raslova H, Peiretti F, Morange PE, Saut N, Pillois X, Nurden AT, Cambien F, Pierres A, van den Berg TK, Kuijpers TW, Alessi MC, Tregouet DA. Human caldag-gefi gene (rasgrp2) mutation affects platelet function and causes severe bleeding. J Exp Med. 2014; 211:1349–1362. [PubMed: 24958846]

Significance

Atherosclerosis is characterized by a chronic inflammatory process, which leads to a remodeling of the inflamed vasculature and the formation of a plaque. The rupture of this plaque leads to platelet activation and thrombosis (atherothrombosis). In addition, platelets were identified as key players during plaque development, as they support leukocyte recruitment to the lesion area. However, relatively little is known about the platelet signaling machinery required in this process. In this study, we demonstrate a critical role for the Rap-GEF, CalDAG-GEFI, in promoting vascular inflammation and atherosclerotic lesion development in hypercholesterolemic mice. Our studies suggest that targeting CalDAG-GEFI would impair atherosclerotic lesion development and be beneficial in the prevention of atherothrombosis.

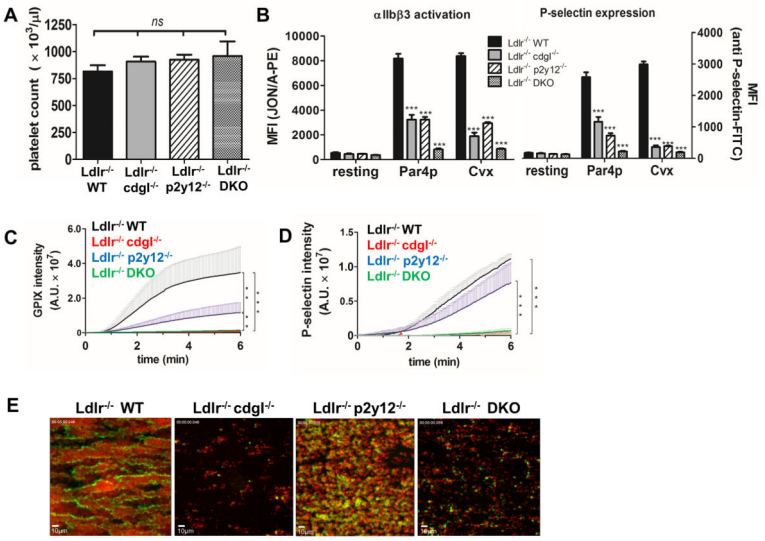

Figure 1. Impaired platelet activation in hypercholesterolemic Ldlr$^{-/-}$cdgI$^{-/-}$ and Ldlr$^{-/-}$p2y12$^{-/-}$ chimeric mice

All studies were done with chimeric mice on high fat diet (12 weeks). **(A)** Peripheral platelet count. **(B)** αIIbβ3 integrin activation (JON/A-PE) and P-selectin exposure (anti P-selectin-FITC) in platelets from the indicated chimeric mice after activation with Par4p (μM) or convulxin ng/ml (Cvx). **(C–E)** Platelet adhesion to collagen under flow. Whole blood from the indicated chimeric mice was perfused for 5 minutes over a collagen-coated surface at a shear rate of 1200s^{-1}. Real-time quantification of platelet accumulation (C) and surface expression of P-selectin (D) was performed by fluorescence videomicroscopy. Representative images (E) are shown for each genotype at t = 5 min (red, platelets and green, P-selectin). Data are the mean ± SEM of at least 5–10 mice per group. **P< 0.01, ***P<0.001.

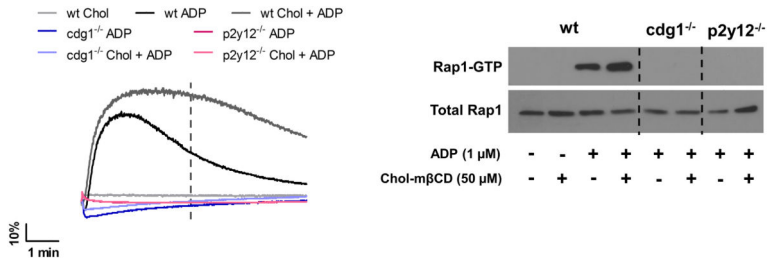

Figure 2. Effect of cholesterol on Rap1 activation in platelets

(A) Aggregation of washed platelets in response to ADP (1μM) and/or cholesterol-mβCD (50μM). Dotted line indicates the time point when samples were extracted for determination of Rap1-GTP levels. **(B)** Detection of Rap1-GTP and total Rap1 in the indicated platelet preparations. Data are representative of 3 independent experiments.

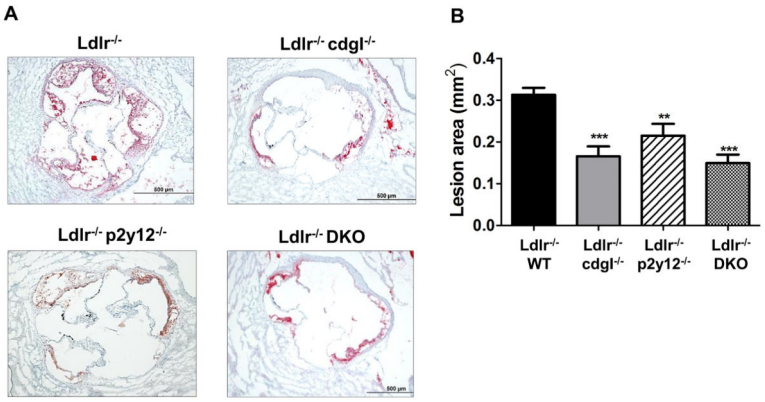

Figure 3. Reduced atherosclerotic lesion formation in the aortic sinus of hypercholesterolemic $Ldlr^{-/-}cdgl^{-/-}$ and $Ldlr^{-/-}p2y12^{-/-}$ chimeric mice

(A) Representative images of oil red O staining of the aortic sinus of the indicated hypercholesterolemic mice. **(B)** Quantification of lesion area. Data are the mean ± SEM of at least 10 mice per group. **P< 0.01, ***P<0.001 versus $Ldlr^{-/-}$ WT chimeras.

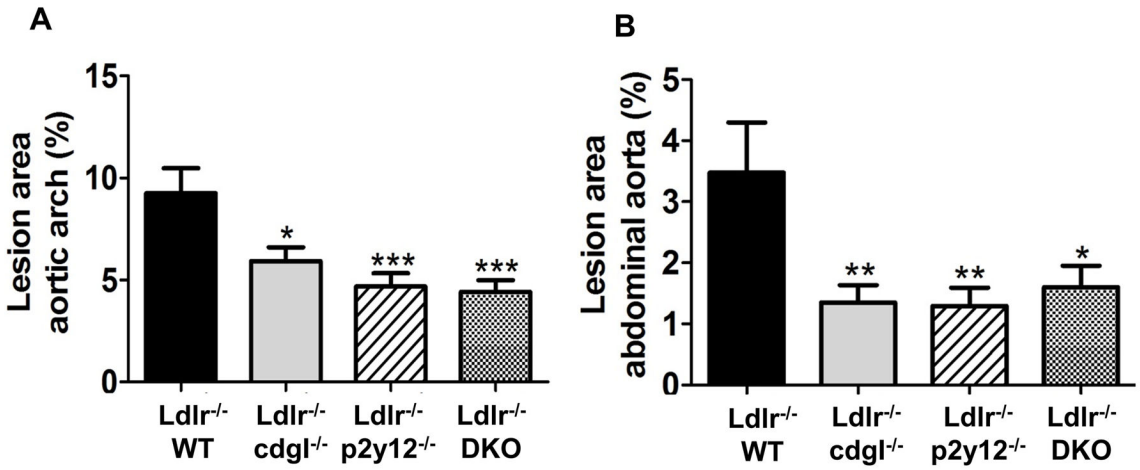

Figure 4. Reduced atherosclerotic lesion formation in the aorta of hypercholesterolemic $Ldlr^{-/-}cdgI^{-/-}$ and $Ldlr^{-/-}p2y12^{-/-}$ chimeric mice

Quantification of atherosclerosic lesion size in aortic arches **(A)** and abdominal aortas **(B)** of the indicated mice. *P<0.05, **P< 0.01, ***P<0.001 versus $Ldlr^{-/-}$/WT chimeras. Data are the mean ± SEM of at least 10 mice per group.

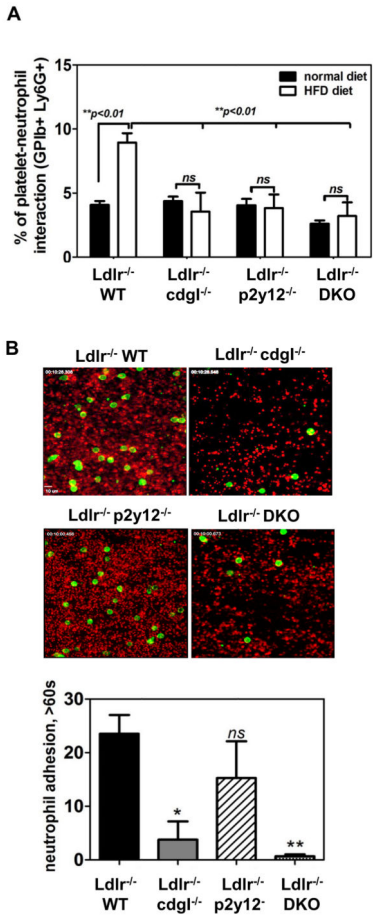

Figure 5. Reduced platelet-neutrophil interactions in hypercholesterolemic Ldlr$^{-/-}$cdgI$^{-/-}$ and Ldlr$^{-/-}$p2y12$^{-/-}$ chimeric mice

(A) Quantification of platelet-neutrophil aggregates in whole blood from the indicated hypercholesterolemic mice. **(B)** Whole blood from the indicated hypercholesterolemic mice (12 weeks of high fat diet) was perfused over a collagen-coated surface at a shear rate of 400s^{-1}. Firm neutrophil adhesion (green) to collagen-bound platelets (red) was quantified. *P<0.05, **P< 0.01, ns indicates not significant. Data are the mean ± SEM of at 5–10 mice per group.

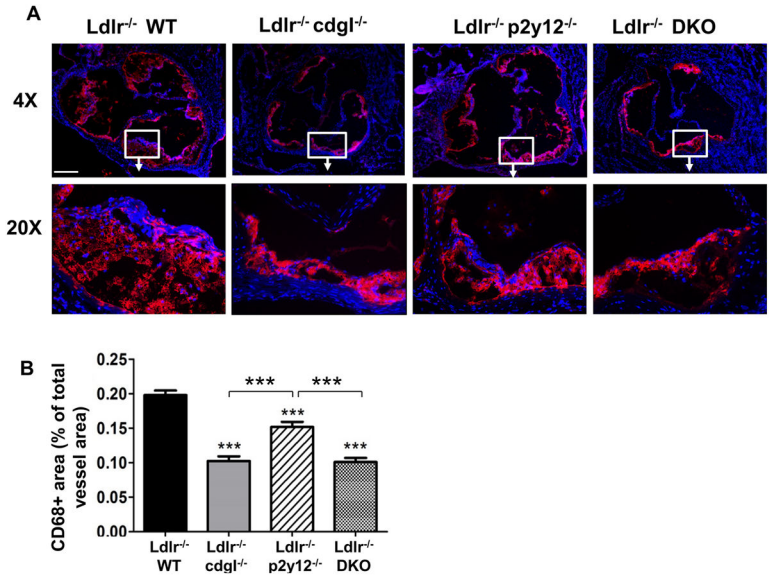

Figure 6. Decreased macrophage content per vessel area in hypercholesterolemic Ldlr$^{-/-}$cdgl$^{-/-}$ mice

Tissue sections were stained for macrophages (CD68 positive cells, red) and nuclei (DAPI, blue). **(A)** Representative images. **(B)** Quantification of CD68-postive area in the cross section of aortic sinuses from each genotype. ***P<0.001. Data are the mean ± SEM of 5–10 mice per group. Scale bar = 250μm.

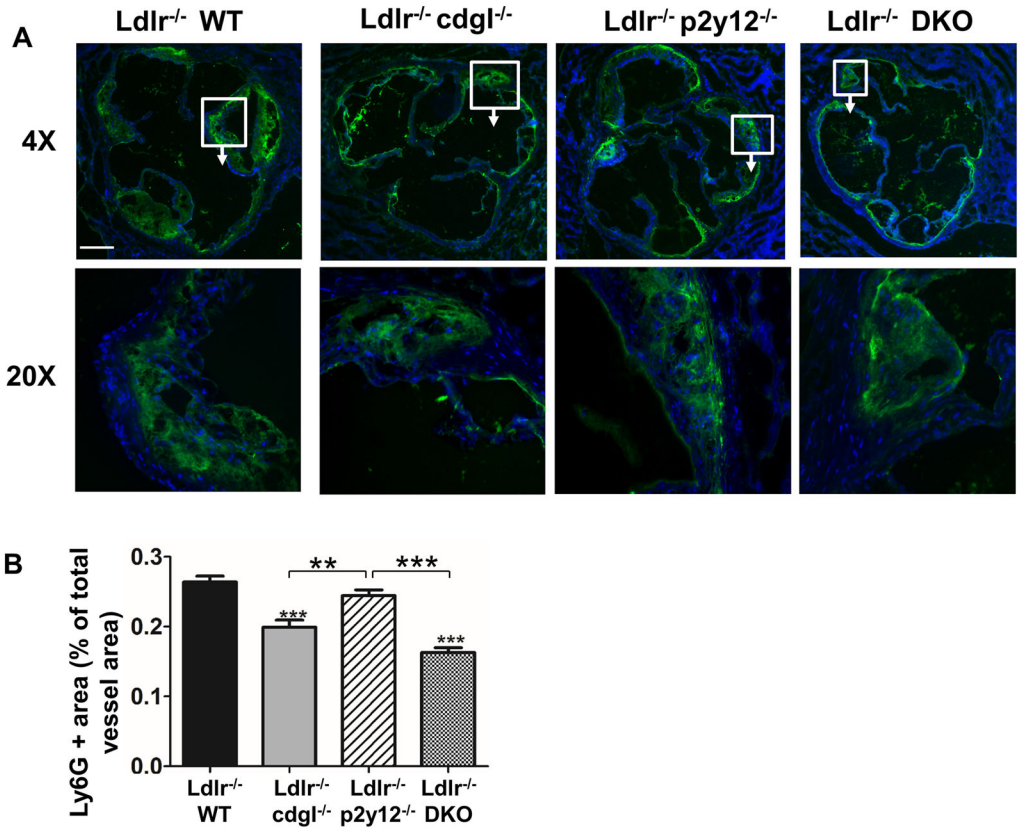

Figure 7. Decreased neutrophil content per vessel area in hypercholesterolemic Ldlr$^{-/-}$cdgI$^{-/-}$ mice

Tissue sections were stained for neutrophils (Ly6G positive cells, green) and nuclei (DAPI, blue). **(A)** Representative images. **(B)** Quantification of Ly6G-postive area in the cross section of aortic sinuses from each genotype. **P <0.01, ***P<0.001. Data are the mean ± SEM of 5–10 mice per group. Scale bar = 250μm.

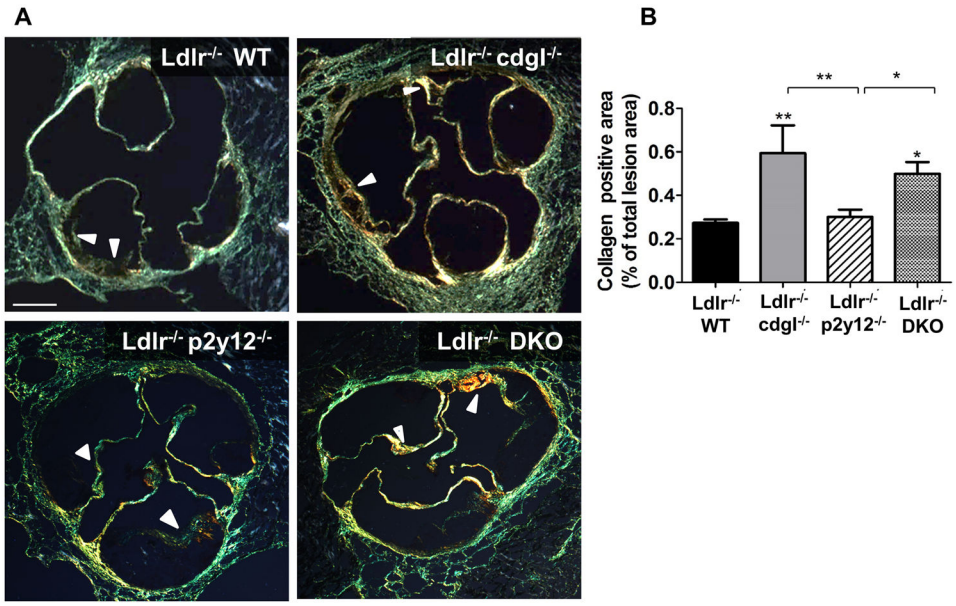

Figure 8. Increased collagen content in hypercholesterolemic Ldlr$^{-/-}$cdgI$^{-/-}$ mice

Tissue sections were stained for collagen (picrosirius red). **(A)** Representative images **(B)** Quantification of collagen-positive area in the cross sections of aortic sinus from each genotype. *P<0.05, **P <0.01. Data are the mean ± SEM of 5–10 mice per group. Scale bar = 250μm.

Table 1

Body weight, lipid profiles and platelet factor 4 levels.

	LDLr$^{-/-}$ WT	**LDLr$^{-/-}$ cdgI$^{-/-}$**	**LDLr$^{-/-}$ p2y12$^{-/-}$**	**LDLr$^{-/-}$ DKO**
body weight (g)	27 ± 0.9	28 ± 1.1	29 ± 0.9	28 ± 1.3
total cholesterol (mg/dl)	1122 ± 78	1397 ± 151	1182 ± 69	1144 ± 115
total triglyceride (mg/dl)	614 ± 53	718 ± 164	540 ± 55	468 ± 47
total VLDL (mg/dl)	98 ± 8.5	117 ± 25	89 ± 6.5	80 ± 4.9
total LDL (mg/ml)	944 ± 74	1201 ± 136	1012 ± 69	982 ± 114
total HDL (mg/dl)	79 ± 1.2	78 ± 2.3	80 ± 1.2	80 ± 1.3
platelet factor 4 (ng/ml)	78 ± 6.9	48 ± 2.4***	58 ± 3.3**	51 ± 1.5***