Cholesterol efflux in megakaryocyte progenitors suppresses platelet production and thrombocytosis

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Platelets have a key role in atherogenesis and its complications. Both hypercholesterolemia and increased platelet production promote atherothrombosis; however, a potential link between altered cholesterol homeostasis and platelet production has not been explored. Here we show that transplantation of bone marrow deficient in ABCG4, a transporter of unknown function, into Ldlr−/− mice resulted in thrombocytosis, accelerated thrombosis and atherosclerosis. Although not detected in atherosclerotic lesions, Abcg4 was highly expressed in bone marrow megakaryocyte progenitors (MkPs). Abcg4−/− MkPs had defective cholesterol efflux to high-density lipoprotein (HDL), increased cell surface expression of the thrombopoietin (TPO) receptor (c-MPL) and enhanced proliferation. These consequences of ABCG4 deficiency seemed to reflect disruption of negative feedback regulation of c-MPL signaling by the E3 ligase c-CBL and the cholesterol-sensing LYN kinase. HDL infusion reduced platelet counts in Ldlr−/− mice and in a mouse model of myeloproliferative neoplasm in an ABCG4-dependent fashion. HDL infusions may offer a new approach to reducing atherothrombotic events associated with increased platelet production.

Atherothrombotic events resulting in heart attack and stroke are the leading cause of morbidity and mortality globally. Platelets are involved in multiple steps leading to atherothrombosis, both in the promotion of atherosclerotic plaque growth and also in the formation of thrombi on ruptured or eroded plaques. Increased numbers and activation of platelets both contribute to atherothrombotic risk, and increased platelet production may underlie these processes. A striking example of increased platelet production occurs in myeloproliferative neoplasms such as myelofibrosis and essential thrombocytosis, in which mutations in the gene encoding c-MPL or in the genes encoding its downstream signaling elements lead to excessive production of megakaryocytes and thrombocytosis. More generally, increased platelet production, denoted by increased platelet volume and increased numbers of circulating reticulated platelets, is a major risk factor for atherosclerotic cardiovascular disease and may precipitate acute coronary syndromes.

Increased amounts of low-density lipoprotein and decreased amounts of HDL are also well known major risk factors for atherothrombosis. The atheroprotective functions of HDL are thought to be mediated by its ability to promote cholesterol efflux from cells in the arterial wall in a process that is facilitated by the ATP-binding cassette transporters ABCA1 and ABCG1 (ref. 11). Although hypercholesterolemia has been associated with increased platelet production, the underlying mechanisms are unclear. Moreover, potential mechanisms linking defective cholesterol efflux pathways to platelet production have not been explored.

The ATP-binding cassette transporter ABCG4, which is highly homologous to ABCG1, promotes cholesterol efflux to HDL when overexpressed in cultured cells. However, ABCG4 is not expressed in macrophage foam cells, and its in vivo function and potential effects on atherogenesis remain unknown. Abcg4 expression has been detected in the brain and hematopoietic tissues such as fetal liver and bone marrow. To uncover how ABCG4 might act in the hematopoietic system, we assessed the effects of ABCG4 deficiency on hematopoietic function and atherogenesis in a hypercholesterolemic mouse model of atherosclerosis.

RESULTS

ABCG4 deficiency accelerates atherosclerosis and thrombosis

We assessed hematopoietic parameters and atherogenesis in a hypercholesterolemic mouse model of atherosclerosis by reconstituting irradiated Ldlr−/− mice with bone marrow from wild-type (WT) or Abcg4−/− mice. We also performed atherosclerosis studies in Ldlr−/− mice transplanted with Abcg1−/− bone marrow. After the mice had been fed a high-fat, high-cholesterol diet (WTD) for 12 weeks, atherosclerotic lesion size was significantly increased in the aorta of Ldlr−/− mice receiving bone marrow transplantation (BMT) with ABCG4-deficient bone marrow (Fig. 1a). In contrast, mice receiving ABCG1-deficient bone marrow did not show increased advanced atherosclerosis (Fig. 1a), consistent with previous studies. Histological analysis of the lesions showed typical, macrophage foam cell–rich atherosclerotic lesions with no differences in morphology.
Figure 1 ABCG4 deficiency in bone marrow increases platelet count and accelerates atherosclerosis and thrombosis. Shown are results from Ldrl−/− mice transplanted with donor bone marrow cells from WT, Abcg4−/−, Abcg1−/− or Abca1−/− mice and fed a WTD diet for 12 weeks. (a) Quantification of proximal aortic root lesion area (with each symbol representing an individual mouse and the means of each group shown as horizontal lines) by morphometric analysis of H&E-stained sections. (b) Representative lacZ-stained proximal aortas from mice receiving Abca1−/− Abcg1−/− or Abcg4−/− bone marrow. Original magnification, ×40. Scale bars, 50 μm. (c) Platelet counts from Ldrl−/− mice receiving WT or Abcg4−/− bone marrow (BM). Data are shown as the means ± s.e.m. (n = 12 mice per group). (d) Cell surface expression of CD11b in platelet-associated Ly6-C hi monocytes or neutrophils in WTD-fed Ldrl−/− recipient mice. Anti-CD41, CD41-specific antibody; ISO, isotype-matching control antibody. (e,f) Concentrations of plasma platelet-derived microparticles (e) and percentages of reticulated platelets (f) in WTD-fed Ldrl−/− recipient mice. (g) Microthrombosis formation on collagen in a flow chamber under shear flow using blood from WT-fed Ldrl−/− recipient mice. Chamber surface coverage by the thrombi (fluorescence positive) was quantified. (h) FeC13-induced carotid artery thrombosis in vivo in WTD-fed Ldrl−/− recipient mice. n = 11 mice per group. Data (d-h) are shown as the means ± s.e.m. *P < 0.05 for the comparisons between genotypes, †P < 0.05 for basal compared to treatment. Statistical significance was determined by one-way analysis of variance (ANOVA) (a) or t-test (c-h).

between the BMT groups (Supplementary Fig. 1a). The Abcg4 knockout mice used were generated using a lacZ knock-in allele at the Abcg4 locus14. However, we found no lacZ-positive cells in lesions of mice receiving Abcg4−/− bone marrow (Fig. 1b). As a positive control, we stained aortic lesions from Ldrl−/− mice receiving Abca1−/− Abcg1−/− bone marrow (also generated with a lacZ knock-in allele in the Abcg1 locus)19 and found lacZ-positive cells indicating Abcg1 expression in the lesions. Plasma lipid and lipoprotein concentrations were similar in recipients of WT or Abcg4−/− bone marrow (Supplementary Fig. 1b–d), as were leukocyte, monocyte (Supplementary Fig. 1c,f), total lymphocyte, B cell and T cell counts (data not shown). Platelet counts were 52% greater in Abcg4−/− bone marrow recipients compared with recipients of WT bone marrow (Fig. 1c). We observed mild anemia and reticulocytosis in the Ldrl−/− mice receiving Abcg4−/− bone marrow (Supplementary Fig. 1g,h).

Activated platelets contribute directly to atherogenesis4, in part by promoting activation and adhesion of monocytes to the arterial endothelium3,4. The numbers of platelet-neutrophil and platelet–Ly6-C hi monocyte aggregates were increased in hypercholesterolemic mice receiving Abcg4−/− bone marrow compared to those receiving WT bone marrow (Supplementary Fig. 1i). These aggregated leukocytes from the recipients of Abcg4−/− bone marrow expressed higher levels of CD11b (as determined by mean fluorescence intensity (MF1)), a key cell adhesion molecule that facilitates adhesion to the endothelium20 (Fig. 1d), indicating they were more activated than in the WT bone marrow recipients. Depletion of platelets by injection of CD41-specific antibodies, which markedly reduced platelet count in Ldrl−/− mice receiving WT or Abcg4−/− bone marrow (data not shown), reduced aggregate numbers and leukocyte CD11b expression (Fig. 1d and Supplementary Fig. 1i). Platelet microparticles promote atherogenesis by facilitating chemokine deposition onto the arterial endothelium and recruiting monocytes to lesions21. The numbers of platelet-derived microparticles were threefold higher in hypercholesterolemic mice receiving Abcg4−/− bone marrow than in those receiving WT bone marrow (Fig. 1e). Circulating amounts of reticulated platelets correlate directly with platelet reactivity22 and are strongly associated with increased risk of myocardial infarction in humans23,24. There was also a significant increase in the percentage of reticulated platelets in mice receiving Abcg4−/− bone marrow (Fig. 1f), consistent with increased platelet production and turnover25. These findings are consistent with previous studies in which infusions of activated platelets increased atherosclerotic lesion formation4 and suggest that increased endogenous platelet production in recipients of Abcg4−/− bone marrow leads to accelerated atherogenesis.

Thrombocytosis and increased amounts of reticulated platelets would also be expected to promote thrombosis. Mice are resistant to spontaneous thrombosis on atherosclerotic plaques. Thus, to assess thrombogenicity, we evaluated thrombus formation in whole blood using an ex vivo perfusion chamber model. Compared to WT controls, we found a marked increase in Abcg4−/− platelet adhesion and aggregation to a collagen-coated surface under shear-flow conditions (Fig. 1g). We also examined arterial thrombosis in vivo using a carotid artery thrombosis model. Carotid artery occlusion by a thrombus after injury with FeCl3 was significantly accelerated in mice receiving Abcg4−/− bone marrow compared to those receiving WT bone marrow (Fig. 1h). Together these findings indicate an increased propensity to thrombus formation in hypercholesterolemic mice with bone marrow ABCG4 deficiency.

ABCG4 is expressed in platelet progenitors

We first considered that ABCG4 might be acting in platelets to influence cholesterol efflux and platelet numbers. However, we did not detect Abcg4 mRNA in WT platelets or lacZ staining in platelets of Abcg4−/− mice (data not shown). In Abcg4−/− mice, there was no
alteration in cholesterol efflux by platelets to HDL or in platelet cholesterol concentrations (Supplementary Fig. 2a,b), indicating that ABCG4 does not act in platelets to regulate circulating platelet numbers. Thus, the mechanisms by which ABCG4 acts to regulate platelet numbers seem to be distinct from those reported for the scavenger receptor SR-BI25.

The phenotype of ABCG4-deficient mice, including prominent thrombocytosis, mild anemia and increased numbers of reticulated platelets, platelet and leukocyte aggregates and platelet microparticles, resembles that of essential thrombocytosis26, a myeloproliferative neoplasm in which mutations in the genes encoding c-MPL or JAK2 in bone marrow progenitors lead to excessive proliferation of platelet progenitors and increased platelet production27. Platelets are produced by megakaryocytes in the bone marrow and spleen, and megakaryocytes are derived from megakaryocyte-erythroid progenitors (MEPs). We hypothesized that ABCG4 might be expressed in bone marrow platelet progenitors and could be involved in the regulation of their proliferation and in megakaryocytopoiesis. After separation of bone marrow hematopoietic cell populations by FACS (Supplementary Fig. 3), we detected Abcg4 mRNA primarily in MEPs (Fig. 2a), with a lower level of expression in the common myeloid progenitor (CMP) population. We found very low or no Abcg4 expression in other cell types (Fig. 2a). The restricted expression of Abcg4 to MEPs contrasts with the expression of Abca1 and Abcg1, which are highly expressed in hematopoietic stem and progenitor cells (HSPCs) but not MEPs. To test whether Abcg4 is the main cholesterol efflux transporter expressed in MEPs, we treated mice with a liver X receptor (LXR) activator in the attempt to induce Abcg4 expression; however, there was little expression of these two genes in MEPs of the treated mice, suggesting that Abcg4 is the dominant transporter in these cells (Supplementary Fig. 4a,b).

Recent studies have shown that the MEP population contains CD41+ cells with megakaryocyte progenitor potential, as well as CD71+ cells with erythroid progenitor potential28. We further sorted the MEP population into CD41+CD71lo, CD41+CD71hi (MkP) and CD41loCD71lo (MEP) cells from the parent MEP cell populations from WT and Abcg4−/− bone marrow shown in Supplementary Figure 4. Abcg4−/− expression in MEPs and MkPs as assessed by immunofluorescence confocal microscopy. Cells were stained with isotype control antibody or antibody to ABCG4 (anti-ABCG4; green) and DAPI (nuclei; blue). Scale bar, 5 µm. (e) Confocal microscopy of WT MkPs immunostained with antibodies to ABCG4 (green), 58K Golgi or TGN38 (red) and DAPI (blue). Scale bars, 5 µm. The arrows point to structures highly positive for ABCG4 or marker staining. (f,g) Quantification of the indicated bone marrow (BM) cell populations (f) and cell surface c-MPL expression (g) in Ldlr−/− recipient mice transplanted with WT or Abcg4−/− bone marrow and fed WTD for 12 weeks (n = 5 mice per group). Also shown in g is a representative histogram for c-MPL expression. (h) Megakaryocyte-colony-forming unit (MK-CFU) assay using hematopoietic progenitor cells (HPCs) harvested from WT and Abcg4−/− mice (n = 5 mice per group). The micrographs show megakaryocyte colonies positively stained for acetylcholinesterase activity. Scale bars, 50 µm. (i) Platelet counts in WT and Abcg4−/− mice (n = 5 mice per group) receiving a single dose of TPO (50 µg per kg body weight) or vehicle control. Data (a,c,f-i) are shown as the means ± s.e.m. *P < 0.05 (t test) for WT compared to Abcg4−/−. alteration in cholesterol efflux by platelets to HDL or in platelet cholesterol concentrations (Supplementary Fig. 2a,b), indicating that ABCG4 does not act in platelets to regulate circulating platelet numbers. Thus, the mechanisms by which ABCG4 acts to regulate platelet numbers seem to be distinct from those reported for the scavenger receptor SR-BI25.

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isotype-matched control antibody (Fig. 2d). Notably, ABCG4 staining partially colocalized with Golgi and, particularly, trans-Golgi markers (Fig. 2e), whereas we detected no colocalization with c-MPL (plasma membrane), Lamp2 (lysosome) or calnexin (endoplasmic reticulum) (Supplementary Fig. 5 and data not shown). Thus, ABCG4 is selectively expressed in the MEP and MkP populations and seems to localize partly to the trans Golgi.

ABCG4 deficiency increases MkP proliferation

The percentages of MkPs and CD41<sup>lo</sup>CD71<sup>lo</sup> MEPs, but not of HSPCs or CMPs, were significantly increased in the bone marrow of hypercholesterolemic recipients of Abcg4<sup>−/−</sup> bone marrow when compared to recipients of WT bone marrow (Fig. 2f). The numbers of CD41<sup>lo</sup>CD71<sup>lo</sup> erythrocyte progenitors were also increased in these mice (data not shown). mRNA levels of Gata1, Pu.1 (also known as Sfi1), Eklf (also known as Klf1) and Flt1, transcription factors known to have crucial roles in the regulation of MEP, MkP and erythrocyte progenitor cell proliferation and differentiation, were similar in Abcg4<sup>−/−</sup> CD41<sup>lo</sup>CD71<sup>lo</sup> MkPs and CD41<sup>lo</sup>CD71<sup>lo</sup> erythrocyte progenitors (data not shown), suggesting that the lineage choice of Abcg4<sup>−/−</sup> hematopoietic cells is not markedly altered.

TPO is the most important growth factor regulating megakaryocyte and platelet lineage development in vivo. We did not observe any change in plasma TPO concentrations in mice receiving Abcg4<sup>−/−</sup>-bone marrow compared to those receiving WT bone marrow (Supplementary Fig. 6a). However, we found increased expression of c-MPL on the surface of Abcg4<sup>−/−</sup> MkPs and CD41<sup>lo</sup>CD71<sup>lo</sup> MEPs (Fig. 2g) but not on megakaryocytes or platelets (Supplementary Fig. 6b,c). This is consistent with the expression profile of Abcg4 and the hypothesis that increased MkP proliferation is the underlying mechanism of thrombocytosis in ABCG4-deficient mice. Indeed, increased EdU incorporation into DNA was increased in MEPs from Abcg4<sup>−/−</sup>-mice compared to those from WT mice (Supplementary Fig. 6d). Colony-formation assays showed a 2.5-fold increase in the number of megakaryocyte colonies arising from ABCG4-deficient compared to WT bone marrow in response to TPO (Fig. 2h). Moreover, the number of megakaryocytes was increased in the bone marrow and spleen of Ldlr<sup>−/−</sup> mice receiving Abcg4<sup>−/−</sup>-bone marrow compared to those receiving WT bone marrow (Supplementary Fig. 7a,b).

Increased TPO-induced platelet production in Abcg4<sup>−/−</sup> mice

Platelet counts are tightly regulated by a negative feedback mechanism in which c-MPL at the surface of megakaryocytes and platelets serves as a clearance sink for TPO and thus limits the increase in platelet count that results from increased TPO–c-MPL signaling in bone marrow cells<sup>29,30</sup>. TPO administration to mice may overwhelm the negative feedback regulatory mechanism, uncovering the effects of increased c-MPL activity<sup>31</sup>. To test the hypothesis that ABCG4 deficiency in MEPs and MkPs results in increased cell surface expression of c-MPL, increased sensitivity of cells to TPO and enhanced platelet production, we administered TPO to WT and Abcg4<sup>−/−</sup> mice. The increase in the number of platelets was much more pronounced in Abcg4<sup>−/−</sup>-mice (2.1-fold) compared to WT mice (1.4-fold) (Fig. 2i). These results indicate that ABCG4 deficiency renders mice more responsive to TPO in vivo, consistent with the idea that increased c-MPL expression on MkPs is the mechanism underlying increased platelet production in Abcg4<sup>−/−</sup>-mice.

ABCG4 promotes cholesterol efflux from MkPs to HDL

We next investigated potential mechanisms linking ABCG4 deficiency to increased expression of c-MPL and increased proliferation and expansion of MkPs. We first examined cellular cholesterol efflux from WT and Abcg4<sup>−/−</sup>-MkPs using a fluorescent cholesterol analog (BODIPY-cholesterol)-based flow cytometry assay. ABCG4 deficiency was associated with reduced cholesterol efflux to reconstituted HDL (rHDL) in Abcg4<sup>−/−</sup>-MkPs (Fig. 3a). BODIPY-cholesterol concentrations in Abcg4<sup>−/−</sup>-MkPs were also significantly increased (Fig. 3b). These findings could indicate that ABCG4-deficiency resulted in defective cholesterol efflux or that membranes of Abcg4<sup>−/−</sup>-cells have a higher affinity, capacity or both for cholesterol. A substantial portion of the BODIPY-cholesterol that accumulated in Abcg4<sup>−/−</sup>-MkPs was in the plasma membrane (Fig. 3c). Free cholesterol content as assessed by filipin staining was also significantly increased in the plasma membrane of Abcg4<sup>−/−</sup>-MkPs (Fig. 3d). Cholesterol accumulation is
known to suppress the expression of cholesterol-responsive genes\(^{33}\). Accordingly, expression of the cholesterol-responsive genes \(Ldrl\) and \(Hmgcs1\) was significantly \((P < 0.05)\) decreased in \(Abcg4^{-/-}\) relative to the WT MEPs; however, this effect was not observed in granulocyte-macrophage progenitors, which do not express \(Abcg4\) (Supplementary Fig. 8a,b). Thus, despite our findings showing that \(Abcg4\) localizes to the Golgi (Fig. 2e), \(Abcg4\) deficiency results in defective cholesterol efflux to HDL and an increase in cell cholesterol content, including in the plasma membrane. These results are consistent with studies suggesting that sterol-rich plasma membrane lipid raft domains can be segregated from non-raft domains in the \(trans\) Golgi\(^{33}\).

To determine whether an increase in cellular cholesterol content can recapitulate the effects of \(Abcg4\) deficiency, we loaded cells with cholesterol-cyclodextrin complexes. This led to increased proliferation of WT and \(Abcg4^{-/-}\) MkPs, paralleling increased cell surface c-MPL expression (Fig. 3c,f). After treatment of the cells with cyclodextrin to remove cellular cholesterol, proliferation and the cell surface expression of c-MPL in \(Abcg4^{-/-}\) MkPs were significantly reduced to levels similar to those in cyclodextrin-treated WT MkPs (Fig. 3f). Although rHDL significantly reduced WT MkP proliferation and cell surface expression of c-MPL, it had no effect in \(Abcg4^{-/-}\) MkPs, consistent with the cholesterol efflux data (Fig. 3a).

In addition, removal of cellular cholesterol by cyclodextrin reversed the increase in the number of megakaryocyte colonies associated with ABCG4 deficiency (Supplementary Fig. 8e). These findings suggest that ABCG4 acts to modulate MkP cell surface c-MPL expression and cell proliferation by regulation of membrane cholesterol content.

**Decreased downregulation of TPO receptor in \(Abcg4^{-/-}\) MkPs.**

We next studied mechanisms linking changes in cellular cholesterol concentrations to altered c-MPL expression in MkPs. Previous studies have shown that TPO binding to its receptor, c-MPL, results in activation of a negative feedback loop in which c-CBL–mediated ubiquitinylation leads to receptor internalization, degradation or both\(^{34}\). c-CBL phosphorylation in response to the activation of growth factor receptors is required to mediate negative feedback regulation\(^{35}\). We assessed whether such negative feedback regulation is defective in \(Abcg4^{-/-}\) MkPs. In response to TPO treatment, the increase in the amount of c-CBL tyrosine phosphorylation was markedly blunted in \(Abcg4^{-/-}\) compared to WT MkPs (Fig. 4a), whereas the amount of total c-CBL was unchanged (data not shown). Treatment of WT MkPs with a proteasome inhibitor, MG132, increased c-MPL expression.
Figure 5 rHDL suppresses platelet production in an ABCG4-dependent fashion in vivo. (a–c) Platelet counts determined by hematology analyzer (a) and the abundance of bone marrow MkPs (b) and bone marrow MkP c-MPL expression (c) determined by flow cytometry in WTD-fed Ldlr−/− recipient mice transplanted with WT or Abcg4−/− bone marrow 5 d after receiving a single infusion of vehicle or rHDL (100 mg apoA-I per kg body weight; n = 5 mice per group). *P < 0.05 (t test) for effect of rHDL infusion. (d) Platelet counts monitored weekly in WT mice transplanted with donor c-MplW55L−/−-transduced bone marrow cells from WT (n = 10) or Abcg4−/− (n = 10) mice (n = 5 mice per subgroup). The mice were given two infusions of rHDL (100 mg apoA-I per kg body weight) or vehicle, one at week 9 and one at week 10, as indicated. *P < 0.05 for WT compared to Abcg4−/−. †P < 0.05 for treatment effects. Statistical significance was determined by t test. (e,f) Platelet counts before (pre) and 5 d after (post) infusion (e) and total platelets after infusion (f) in patients with peripheral vascular disease who received a single infusion of rHDL (80 mg per kg body weight) or placebo. Data are presented as the mean decrease in total platelet numbers after infusion (n = 7 per group) (f). *P < 0.05 for placebo compared to HDL; NS, not significant. Data (a–f) are shown as the mean ± s.e.m. (g) Schematic model depicting the involvement of ABCG4 in the regulation of c-MPL expression in MkPs and MkP proliferation, leading to effects on platelet production, thrombosis and atherosclerosis.

to a level similar to that in Abcg4−/− cells (Fig. 4b), consistent with the idea that in Abcg4−/− there is decreased proteosomal degradation of c-MPL compared to WT cells. Cholesterol loading by cholesterol-cyclodextrin complexes reduced c-CBL phosphorylation, whereas removal of cellular cholesterol by cyclodextrin increased c-CBL phosphorylation in both WT and Abcg4−/− MkPs (Fig. 4c). Although rHDL treatment increased the amount of phosphorylated c-CBL in WT MkPs, it did not alter c-CBL phosphorylation in Abcg4−/− MkPs (Fig. 4c), consistent with the inability of rHDL to modulate c-MPL expression and cell proliferation of Abcg4−/− MkPs. These findings suggest that impaired cholesterol efflux in Abcg4−/− MkPs results in defective c-CBL-mediated feedback downregulation of c-MPL by TPO.

LYN kinase modulates proliferative responses of MkPs

The kinase(s) catalyzing c-CBL tyrosine phosphorylation in response to TPO are not known. SRC-family kinases (SKs) such as LYN, FYN and c-SRC are known to phosphorylate tyrosine residues of c-CBL36, leading to its activation, and SFK inhibitors have been shown to increase cell surface c-MPL expression through undefined mechanisms29. We hypothesized that the activity of SFKs is decreased in Abcg4−/− MkPs, leading to decreased c-CBL phosphorylation. Consistent with this suggestion, treatment of WT and Abcg4−/− MkPs with SU6656, an inhibitor of LYN, FYN and c-SRC37, markedly decreased c-CBL phosphorylation, increased cell surface expression of c-MPL and abolished the difference in response to TPO between WT and Abcg4−/− MkPs (Fig. 4d,e). TPO activation of c-MPL increases the kinase activity of LYN and FYN but not other SFKs38. LYN kinase is palmitoylated and membrane associated, and its activity is increased by decreased membrane cholesterol content39. Notably, Lyn−/− mice show increased megakaryocytopenosis with mild thrombocytosis40 and mild anemia with reticulocytosis41, defects that bear a striking resemblance to those of Abcg4−/− mice. Thus we hypothesized that LYN might be the dominant tyrosine kinase catalyzing c-CBL tyrosine phosphorylation in response to TPO. TPO-treated Lyn−/− MkPs showed decreased c-CBL phosphorylation and increased cell surface c-MPL expression (Fig. 4f,g) and cell proliferation compared to WT MkPs (Fig. 4h), demonstrating that LYN has a key role in regulating the tyrosine phosphorylation of c-CBL and in MkP proliferation in response to TPO. Cholesterol loading by cholesterol-cyclodextrin complexes decreased c-CBL phosphorylation, increased c-MPL expression and enhanced cell proliferation in WT MkPs but had no effect in Lyn−/− MkPs (Fig. 4f–h). Treatments with either cyclodextrin or rHDL to induce cholesterol efflux decreased the proliferation of WT MkPs. In contrast, Lyn−/− MkPs showed increased proliferation that was unresponsive to either cholesterol loading or deletion treatments (Fig. 4j). These findings indicate an essential role of LYN kinase in mediating the effects of cholesterol loading and unloading on c-CBL phosphorylation as well as the effects of TPO on c-MPL expression and MkP proliferation.

A LYN kinase activator reduces c-MPL expression

To further assess the possible involvement of LYN in the negative regulation of surface c-MPL expression on MkPs, we tested the effects of pharmacological LYN activation. Treatment of bone marrow cells from hypercholesterolemic Ldlr−/− recipient mice with tolmidone, a compound that selectively increases LYN kinase activity in vivo42, reduced cell surface c-MPL expression in both WT and Abcg4−/− MkPs (Fig. 4i) and completely reversed the increased cell surface expression of c-MPL in Abcg4−/− MkPs from normocholesterolemic mice (Supplementary Fig. 9). Together these observations suggest that in the presence of excessive membrane cholesterol accumulation,
decreased LYN kinase activity leads to diminished c-CBL–mediated downregulation of c-MPL by TPO.

We assessed known TPO-mediated signaling pathways that could potentially be activated in Abcg4−/− MkPs. Both basal and TPO-stimulated amounts of phosphorylated ERK1/2 and phosphorylated Akt were significantly higher in Abcg4−/− compared to WT MkPs; the amounts of phosphorylated STAT5 were also increased, albeit nonsignificantly (Fig. 4j). This pattern is similar to that seen with LYN deficiency40.

HDL infusion decreases MkP proliferation and platelet count

To test whether HDL administration can reduce MkP proliferation and platelet counts in vivo, we infused a preparation of rHDL that has been shown previously to reduce coronary atheroma volume in humans43 into WTD-fed Ldlr−/− mice with or without ABCG4 deficiency. rHDL, but not saline, infusion significantly decreased platelet counts by ~30% in Ldlr−/− but not Abcg4−/− Ldlr−/− mice (Fig. 5a). HDL infusion also decreased decreased c-MPL expression on MkPs, decreased numbers and proliferation of MkPs and decreased megakaryocyte counts in spleen and bone marrow in Abcg4−/− mice; however, rHDL had no effect in Abcg4−/− mice (Fig. 5b,c) and Supplementary Fig. 10a–c). These findings demonstrate an essential role of ABCG4 in mediating the ability of rHDL to reduce MkP proliferation and platelet counts.

We further explored the therapeutic potential for rHDL to reduce platelet counts by testing the effects of HDL infusion in a mouse model of myelofibrosis and essential thrombocytosis. In this model, mice are transplanted with bone marrow cells transduced with a retrovirus expressing an activating mutant form of c-MPL (c-MPLW515L), found in human myeloproliferative neoplasms27,44. Such c-MPL mutations are found in a subset of patients with myelofibrosis (~10%) and essential thrombocytosis (~4–5%) and cause proliferation of MEPs, megakaryocyte expansion and thrombocytosis9,27. The activity of this mutant form of c-MPL requires cell surface localization45. Because cell surface c-MPL expression was increased in Abcg4−/− mice (Fig. 2g), we hypothesized that c-MPL activity might be enhanced by ABCG4 deficiency. Indeed, thrombocytosis developed more rapidly and was more pronounced in mice transplanted with Abcg4−/− bone marrow cells with retroviral-mediated expression of c-MPLW515L compared to mice that received WT c-MPLW515L bone marrow cells (Fig. 5d). Although rHDL infusions effectively reversed thrombocytosis in WT c-MPLW515L bone marrow–transplanted mice, the same treatment had no effect on platelet counts in mice transplanted with Abcg4−/− c-MPLW515L bone marrow cells.

To test whether rHDL infusion in humans could reduce platelet numbers, we analyzed data obtained from a previously reported study involving patients with peripheral vascular disease46. This revealed that infusion of rHDL, but not placebo, was associated with a significant reduction of platelet counts (Fig. 5e), when normalized to baseline platelet values, the change was still significantly different between the groups (Fig. 5f). The reduction of platelets in this study was moderate, and platelet counts were maintained in the normal range (Fig. 5e).

Together these findings suggest that HDL and ABCG4 promote cholesterol efflux from MkPs and thus facilitate the negative feedback regulation of c-MPL by TPO in MkPs (Fig. 5g).

DISCUSSION

Our findings show that defective cholesterol homeostasis in megakaryocyte progenitor cells promotes megakaryocyte formation, platelet overproduction, arterial thrombosis and atherogenesis. Increased membrane cholesterol concentrations in megakaryocyte progenitors lead to increased amounts and signaling of the TPO receptor. ABCG4 is highly expressed in MkPs, and its deficiency leads to cholesterol accumulation, MkP proliferation and increased platelet production. The ability of rHDL to suppress MEP and MkP proliferation and platelet counts in vivo was dependent on ABCG4, probably reflecting the cell type–restricted pattern of expression of cholesterol efflux–promoting ABC transporters. Therapeutic interventions such as rHDL infusions have the potential to reverse excessive megakaryo-cytopoiesis in states of platelet overproduction, such as those that occur in myeloproliferative neoplasms.

The idea that cellular sterol metabolism is intimately connected to proliferative responses is longstanding47. The requirement for new membrane synthesis during cell proliferation leads to activation of cholesterol biosynthesis involving cleavage of sterol regulatory element binding transcription factor 2 (SREBP-2) and transcriptional induction of cholesterol biosynthetic genes48. Recent studies have linked control of cell proliferation to cholesterol efflux pathways mediated by ABCA1, ABCG1 or both29,49–51. However, specific molecular mechanisms linking cellular cholesterol accumulation to altered growth factor receptor signaling have not been defined. Our studies suggest that LYN kinase may act as a membrane cholesterol sensor, acting upstream of c-CBL to modulate its downregulation of c-MPL. This hypothesis is supported by previous studies showing that LYN kinase activity is modulated by altered membrane cholesterol concentrations39. LYN is palmitoylated, and palmitoylation-defective LYN shows decreased association with cholesterol-rich membranes but an increased ability to mediate tyrosine phosphorylation of immunoglobulin receptors52. We showed that infusions of cholesterol-poor rHDL were associated with a reduction in platelet counts in a previous small study involving patients undergoing treatment for peripheral vascular disease, suggesting the potential human relevance of our findings. Moreover, in a recent human genome-wide association study, SNPs in or near the c-CBL (also called CBL) gene were associated with platelet counts53. Interestingly, ABCG4 is in tight linkage disequilibrium with c-CBL, and SNPs associated with platelet counts could be influencing expression of c-CBL and/or ABCG4 (ref. 53). Our findings suggest a potential mechanism linking expression of ABCG4 to the regulation of platelet counts involving defective c-CBL–mediated feedback regulation of c-MPL and thus support the concept that these genes act in megakaryocytes or their progenitors to regulate platelet production53.

There is tremendous interest in the development of new therapies that increase plasma HDL concentrations as potential treatments for atherosclerotic cardiovascular disease. The achievement of this goal has been challenging, as highlighted by the recent failure of treatments that increase HDL concentrations in clinical trials, such as the CETP inhibitors torcetrapib and dalcetrapib or extended release niacin54,55. However, approaches that actively increase the flux of cholesterol from macrophages and other cells remain promising treatments to reduce coronary atherosclerosis13,56. Our study suggests that such treatments may have the beneficial effects of suppressing MEP and MkP proliferation.

Thrombocytosis in essential thrombocytosis and myelofibrosis is currently treated with low-dose aspirin, and high-risk patients with essential thrombocytosis (>60 years old or having experienced a previous thrombotic event) are treated with genotoxic agents such as hydroxyurea57. There remains a need for new therapies for patients with myelofibrosis given their poor overall outcome and limited
therapeutic options58. Our findings suggest that rHDL infusion may specifically reverse c-MPL-dependent MEP proliferation and aberrant megakaryopoiesis underlying thrombocytosis in essential thrombocytopathy and myelofibrosis. Moreover, increased platelet production is a cardiovascular risk factor and has been implicated more generally in the precipitation of atherothrombotic events. Thus, rHDL infusions could complement existing treatments that directly target platelets or clotting factors. rHDL infusions may have multiple beneficial effects in the setting of acute coronary syndromes, including the removal of cholesterol, the suppression of inflammation in plaques and the suppression of excessive myeloid cell production and extramedullary hematopoiesis, as well as limiting the overproduction of platelets59,60.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

ACKNOWLEDGMENTS

A.J.M. was supported by a postdoctoral fellowship from the American Heart Association (12POST11890019). This study was supported by US National Institutes of Health grant HL107653 (A.R.T.). We thank A.L. DeFranco and C.A. Lowell of the University of California, San Francisco for providing Lyn+ bone marrow cells and W. Tong of the University of Pennsylvania for providing the antibody to c-MPL for flow cytometry analysis.

AUTHOR CONTRIBUTIONS

A.J.M., N. Bijl and N.W. conceived the study, designed, performed and analyzed the experiments and wrote the manuscript. L.Y.-C., C.B.W., N. Bhagwat, A.R., Y.W. and J.A.S. designed, performed and analyzed experiments. R.L.L. and H.N. provided intellectual input and assisted with the preparation of the manuscript. A.R.T. conceived the study and contributed to writing the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice and treatments. The Institutional Animal Care and Use Committee of Columbia University approved all the mouse studies. Abcg4−/−, Abeg1−/−, Abca1−/− and Abcg1−/− mice in a C57Bl/6 background were created as described14,19 and used in this study. Abcg4−/− mice were backcrossed onto C57Bl/6 mice for more than ten generations. WT (C57Bl/6) and Ldr−− (B6.129S7-Ldrtm1Her) mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). For bone marrow transplantation studies, bone marrow from WT, Abcg4−/−, Abeg1−/−, Abca1−/− or Abcg1−/− mice was transplanted into WT or Ldr−− recipient mice as described19. For atherosclerosis studies, bone marrow–transplanted recipient mice were fed a Western diet (TD88137, Harlan Teklad) for the indicated period of time. Bone marrow–specific retroviral expression of murine c-MPL W515L was established as described44 using WT C57Bl/6 mice as the recipient and WT C57Bl/6 or Abcg4−/− mice as the bone marrow donor. Where indicated, vehicle (saline), rHDL or TPO (R&D Systems) was injected at the indicated dose into the mice through the tail vein. rHDL (CSL-111) was provided by CSL Behring AG, Bern, Switzerland; CSL-111 is composed of human apoA-I and phosphatidylcholine from soy bean in a ratio of 1:150. All patients gave their informed consent to the study, which was approved by the Human Ethics Committee of the Alfred Hospital and conducted in accordance with the principles of the Declaration of Helsinki 2000.

Femurs and tibia of Ldr−/− mice used to prepare Ldr−/− bone marrow cells were kindly provided by A.L. DeFranco of the University of California, San Francisco. The mice were created as described61 and backcrossed at least 15 generations onto the C57Bl/6 background. MG132 (474790) and SU6656 (572635) were from EMD Millipore (Darmstadt, Germany). These compounds were dissolved in DMSO as 10 mM (MG132) or 10 mg/ml (SU6656) stocks and diluted to the indicated concentrations in cell culture medium.

Histochemistry. Tissues and proximal aortas were serially paraffin sectioned and stained with H&E for morphological analysis as described19. The aortic lesion size of each mouse was calculated as the mean of the lesion areas in five aortic sections. Bone samples were decalcified with EDTA solution before cryosectioning. Antibody to von Willebrand factor (Dako, A0082, 1:500 dilution) was used to stain megakaryocytes in bone and spleen sections. In situ staining of frozen sections of mouse bone, spleen or proximal aorta was carried out using a β-Galactosidase Staining Kit (Cell Signaling Technology, Danvers, MA).

Complete blood count. Complete blood counts were quantified using whole blood collected from tail bleeding. A FORCYTE Veterinary Hematology Analyzer (Oxford Science, Inc.) was used for the analysis.

Plasma and cellular lipids. Plasma lipoprotein cholesterol and triglyceride concentrations were determined by colorimetric enzymatic assays using assay kits from Wako Diagnostics (Japan). Platelets were isolated from platelet-rich plasma by centrifugation at ~3,500 r.p.m. for 10 min in platelet-poor plasma, and platelet cholesterol content was measured by gas chromatography after lipid extraction.

Cholesterol efflux. For platelet cholesterol efflux studies, platelets were isolated from platelet-rich plasma by centrifugation at ~3,500 r.p.m. for 10 min in an Eppendorf centrifuge. Platelet-rich plasma was prepared from a low-speed spin of EDTA-treated mouse plasma, and platelet cholesterol content was measured by gas chromatography after lipid extraction.

Flow cytometry–based proliferation studies. Blood leukocytes and bone marrow HSPCs were stained and analyzed or sorted as described38. Briefly, bone marrow cells from mouse femurs and tibias were stained with a cocktail of antibodies to lineage-committed cells (CD45R, CD19, CD11b, CD3e, TER-119, CD2, CD8, CD4 and Ly-6C/G, all FITC conjugated; eBioscience), with antibodies to Sca1 (Biologend) and c-Kit (eBioscience) to identify HSPC populations and LSK (Lin−Sca1−c-Kit+) cells and with antibodies to CD16/32 (FcγRII/III) and CD11b (BD Biosciences) to separate CMP (Lin−Sca1−c-Kit−CD16/32−FcyRII/III−) and MEP (Lin−Sca1−c-Kit−CD16/32−FcyRII/III+) cell populations. All antibodies were used at 1:200 dilution. For DNA content analysis (G2M phase), bone marrow cells were fixed and stained with DAPI (Invitrogen) before flow cytometry analysis. To determine in vivo cell proliferation, Edu (Invitrogen; 1 mg per mouse) was injected into mice through the tail vein 24 h before the mice were euthanized. Cells were immunostained as described above in preparation for flow cytometry. Cells were then fixed and permeabilized using 0.1% saponin (wt/vol; Fluka) and 1% FCS (vol/vol) in IC fixation buffer (eBiosciences) for 30 min. Cells were then washed and stained with Alexa Fluor–conjugated azides using the Click-iT system (Invitrogen). Proliferation was quantified as the percentage of Edu+ cells by flow cytometry.

Quantification of reticulated platelets. Undiluted EDTA-anticoagulated blood (5 μl) was mixed with a phycocerythrin (PE)-conjugated antibody to CD41 (eBiosciences, 14-0411-82) and the fluorescent DNA dye thiazole orange (final concentration 1 μg ml−1) and incubated at room temperature for 20 min. Samples were then fixed by adding 1 ml of 1% formaldehyde in PBS. Data acquisition using logarithmic amplification of light scatter and fluorescence signals was performed. PE-positive cells were gated in a thiazole orange versus PE dot plot.

Real-time qPCR. RNA extraction, complementary DNA synthesis and qPCR of HSPCs were performed as described42. The quality of RNA samples was determined using an Agilent 2100 Bioanalyzer and an RNA 6000 LabChip (Agilent Technologies). The primer sequences used for qPCR are shown in Supplementary Table 1.

MK-CFU assay. Primary bone marrow HSPCs obtained by FACS were plated in methylcellulose-based medium (5,000 cells per assay) containing TPO (50 ng ml−1), interleukin-6 (IL-6) (20 ng ml−1) and IL-3 (10 ng ml−1) and incubated for 8 d according to the manufacturer's protocol (Megacult-C, Stemcell Technologies). Cultures were fixed, and megakaryocyte colonies were visualized by staining for acetylcholinesterase activity. Nuclei were counterstained with Harris' hematoxylin. Colonies containing more than three megakaryocytes were scored as MK-CFUs.

ABCG4-specific antibody. The rabbit antibody to ABCG4 was custom made by Pacific Immunology (CA, USA) against a synthetic ABCG4 peptide.
(KKVNHITAEQRFSHLPRKR). Monospecific anti-peptide antibodies were purified using a peptide-affinity column. The specificity of the antibody for ABCG4 protein was assessed by immunofluorescence microscopy, which showed specific immunofluorescence signals in HEK293 cells expressing ABCG4 but not HEK293 cells transfected with mock vectors (data not shown).

Rabbit polyclonal antibody to c-MPL was kindly provided by W. Tong of the University of Pennsylvania, and the specificity of the antibody against cell surface c-MPL in flow cytometry has been reported previously62,63.

**Neutrophil and monocyte platelet aggregates.** Blood was collected through the tail vein into EDTA-lined tubes on ice to prevent leukocyte activation. Red blood cells (RBCs) were lysed, and the washed cells were then stained with CD45 (Invitrogen), CD115 (eBioscience), Gr1 (Ly6-C/G; BD Biosciences), CD11b (eBioscience) and CD41 (eBioscience) at 1:200 dilution for 30 min on ice. The cells were carefully washed, resuspended in FACS buffer and run on an LSRII flow cytometer to detect leukocyte platelet interactions and leukocyte activation. Viable cells were selected on the basis of forward and side scatter characteristics, and then CD45+ leukocytes were selected. Ly6-C+ monocyte platelet aggregates were identified as CD115+Gr1hi (Ly6-Chi) and CD41+. Neutrophil-platelet aggregates were identified as CD11b+Ly6-G+ and CD41+. Platelet-dependent activation of Ly6-C+ monocytes and neutrophils was measured as CD11b+ MFI after subtracting the expression of CD11b on Ly6-Chi or neutrophils, which stained negative for platelets (CD41+).

**Platelet-derived microparticles.** Equal amounts of mouse plasma (20 µl) were diluted with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) binding buffer (80 µl) and then incubated with annexin V (BD Biosciences, 556420) and antibody to CD41. Equal amounts of 1-µm beads (Invitrogen) were added to the sample as a size standard, which was then run on an LSRII flow cytometer. Platelet-derived microparticles were detected as particles less than 1 µm in size that stained positive for CD41 and annexin V. A standard amount of beads was acquired to ensure accurate counting in each sample. Data were converted to the number of microparticles per 1 µl of whole blood.

**FeCl3-induced carotid artery thrombosis.** Mice were anesthetized, and a cervical incision was made to expose the common carotid artery. A miniature Doppler flow probe (TS420 transit-time perivascular flow meter, Transonic Systems Inc.) was placed on the carotid artery to monitor blood flow. The injury to the artery was induced by a piece of Whatman paper (2 mm × 2 mm) saturated with 5% FeCl3. The time until the cessation of the blood flow was recorded as the occlusion time.

**Ex vivo flow chamber assay.** Heparin (5 U ml−1)-anticoagulated whole blood was incubated with 1 µM of the fluorescent dye DiOC6 (Sigma, St. Louis, Mo, USA) for 10 min at 37 °C. The fluorescently labeled whole blood was then perfused over a collagen-coated glass cover surface (microcapillary glass tube coated with 100 µm ml−1 Horm collagen (Nycoderm) overnight) at a controlled shear rate (1,800 s−1) using a syringe pump for 3 min. Adherent platelets and aggregates in the chamber were washed and examined under an inverted fluorescence microscope, and micrographs of adhered platelets were recorded for analysis. Flow chamber surface coverage by the thrombi was calculated using ImageJ.

**c-MPL expression.** After harvesting bone marrow progenitor cells, RBCs were lysed, and the cells were resuspended in FACS buffer. Bone marrow cells were stained for Mkp8 as stated above, and c-MPL or isotype control antibodies were included. Cells were then washed and stained with a fluorescently conjugated secondary rabbit-specific antibody to detect the antibody to c-MPL for a further 30 min on ice. Following this, the cells were washed, resuspended in FACS buffer and run on an LSRII flow cytometer. MEPS were identified as Lin−c-Kit+CD16/32−CD34+CD41+ and Mkp8 were identified as Lin−c-Kit+CD16/32−CD34+CD41+.

Expression of c-MPL on late-stage megakaryocytes was detected by staining bone marrow cells with a cocktail of lineage markers (Sca1, CD127, CD45R, CD19, CD3e, TER-119, CD2, CD8 and Ly-6C/G, all FITC conjugated; eBioscience), CD41 (eBioscience) and c-MPL or isotype control as above. After staining with the antibodies, the bone marrow cells were then fixed and permeabilized using BD cytofix/perm buffer for 20 min on ice followed by washing with BD cytofix/perm wash buffer. Cells were then resuspended in FACS buffer containing propidium iodide to determine megakaryocyte ploidy. Expression of c-MPL was measured on total and late-stage megakaryocytes (defined as 32N and 64N).

Expression of c-MPL on platelets was assessed by obtaining platelet-rich plasma and staining with CD41 and c-MPL as outlined above. The surface expression of c-MPL on platelets was then quantified by MFI normalized to the isotype control.

**c-Cbl phosphorylation.** Bone marrow progenitor cells were stimulated with TPO at the indicated concentration for the specified period of time at 37 °C and then immediately diluted with ice-cold buffer and placed on ice to prevent further changes in phosphorylation. Cells were then centrifuged at 800g for 2 min, and the pellet was resuspended in BD fix buffer (BD Biosciences) for 10 min on ice. The cells were washed with BD flow cytometry staining buffer, centrifuged and then resuspended in BD cytofix/perm buffer III for 20 min. After this, the cells were washed and resuspended in BD staining buffer and incubated with lineage (Sca1, CD127, CD45R, CD19, CD11b, CD3e, TER-119, CD2, CD8, CD4 and Ly-6G, all FITC; eBioscience) and progenitor cell markers (c-Kit, CD16/CD32 (FcγRII/III), CD34, CD41 and antibody to p-c-Cbl (Tyr700 human, Tyr98 mouse; BD Biosciences)) or an isotype control for 30 min on ice. The cells were then washed, resuspended in FACS buffer and run on an LSRII. The amount of phosphorylated c-Cbl was normalized against that of isotype control staining.

**Immunofluorescence confocal microscopy.** Mkps collected by FACS from WT or Abcg4−/− bone marrow cells were attached to glass slides by a brief spin in a CytoSpin4 (Thermo Scientific) according to the manufacturer’s instructions. The cells were then fixed with 2% paraformaldehyde, permeabilized with 1% Triton X-100 in PBS for 1 min and incubated with 4% BSA in PBS plus 0.1% saponin to block nonspecific binding sites. Diluted primary antibodies against ABCG4 or cellular organelle markers (58K Golgi protein–specific antibody, Novus Biologicals; TGN38-specific antibody, BD Biosciences; c-MPL–specific antibody, Sigma–Aldrich; Lamp2-specific antibody, Novus Biologicals) were then added to the cells in 1:200 dilution and incubated at room temperature for 2 h. After washing, fluorescent secondary antibodies (1:400 dilution) were added and incubated for 1 h. Where indicated, the washed cells were counterstained with or without DAPI and examined with a fluorescence confocal microscope.

**Statistics.** For aortic morphometric atherosclerotic lesion quantification and analysis, two-way analysis of variance (ANOVA) was used. For comparison of one group with another, for instance in the c-CBL phosphorylation time course experiment (Fig. 4a), a t test was used. For comparison of various treatments on different genotypes, one-way ANOVA was used.