Cholesterol metabolism and hematopoiesis interaction in atherothrombosis

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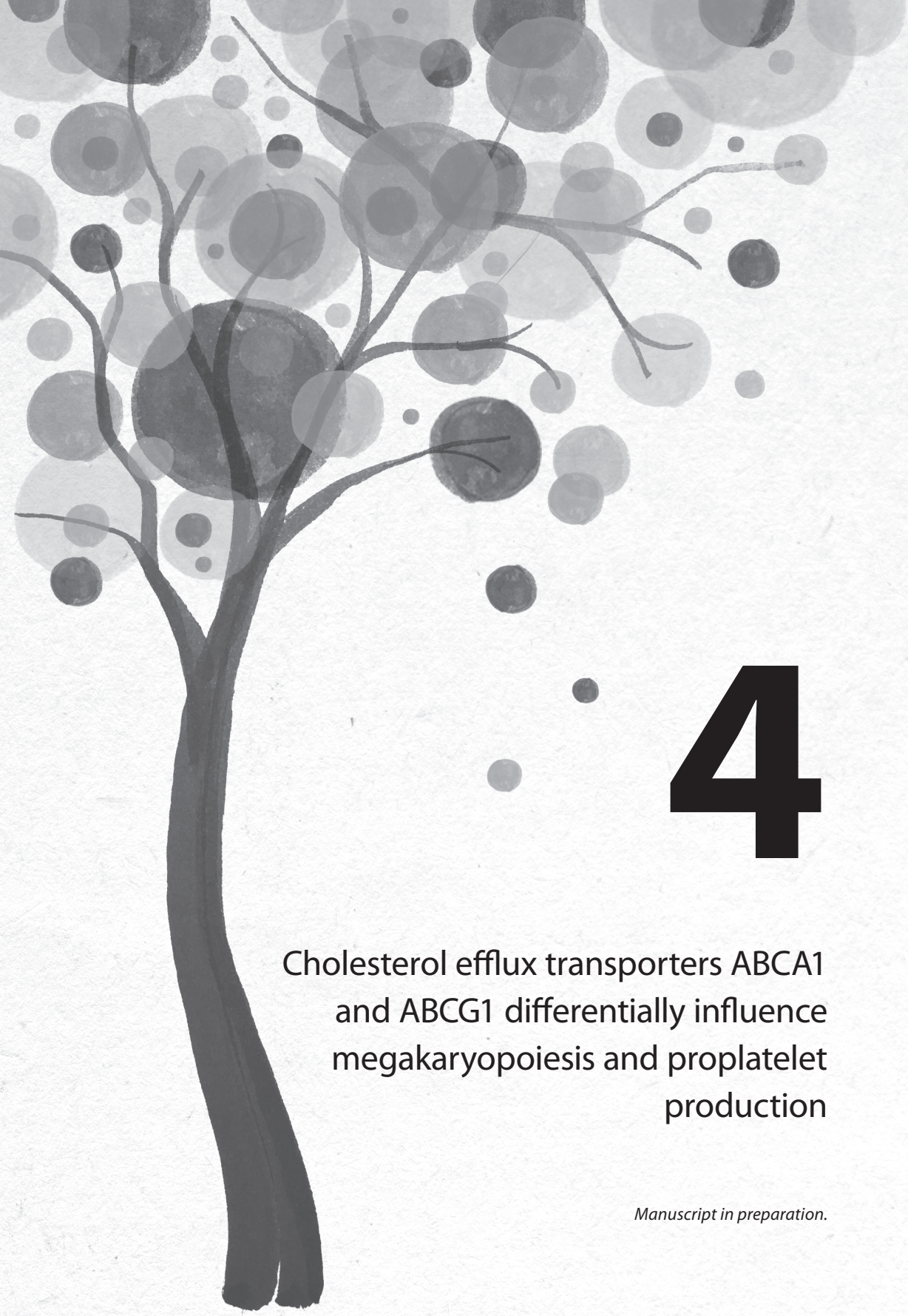

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Cholesterol efflux transporters ABCA1 and ABCG1 differentially influence megakaryopoiesis and proplatelet production

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ABSTRACT

Platelets, produced by megakaryocytes in the bone marrow, play an important role in atherothrombosis. Cholesterol efflux capacity of megakaryocytes can influence megakaryocyte formation, function and platelet production. In this study, we investigated whether disruption of the cholesterol efflux transporters ATP-Binding cassette transporter A1 (ABCA1) and ABCG1 influence megakaryopoiesis and proplatelet production. ABCA1/ABCG1 DKO mice displayed similar amounts of megakaryocytes in the bone marrow compared to control mice. Interestingly, there was a significant increase ($p<0.01$) in proplatelet production by megakaryocytes from ABCA1/ABCG1 DKO mice in bone marrow explants. Moreover, combined deletion of ABCA1 and ABCG1 had no effect on basal platelet counts or thrombopoietin stimulated platelet production. Notably, platelets of ABCA1/ABCG1 DKO mice did display an increase in platelet volume and volume distribution width. However, this increase was not associated with changes in the unesterified cholesterol content of ABCA1/ABCG1 DKO megakaryocytes. Single ABCA1 and ABCG1 knockout (KO) mice also had similar bone marrow megakaryocyte numbers compared to wild-type mice. Surprisingly, the amount of proplatelet forming megakaryocytes in single ABCG1 KO bone marrow explants was substantially higher as compared to ABCA1/ABCG1 DKO explants (AUC: 3-fold vs 1.6-fold compared to wild-type mice). Moreover, megakaryocytes from ABCA1 KO mice surprisingly showed decreased proplatelet formation, explaining the suppressed increase in proplatelet formation upon combined deletion of ABCA1 and ABCG1. In conclusion, we show that the cholesterol efflux transporters ABCA1 and ABCG1 have differential effects on proplatelet formation. Our findings suggest that ABCA1 and ABCG1 do not play a major role in megakaryocyte cholesterol efflux towards extracellular cholesterol acceptors, but may play a crucial role in the regulation of the final steps of thrombopoiesis.

INTRODUCTION

Platelets are key players in atherothrombosis, as they are the first responders to changes in the endothelial integrity and exposure of sub-endothelial structures [1]. Platelet characteristics such as density and volume are associated with increased risk of acute coronary syndromes [2–5]. Moreover, mean platelet volume, a determinant of platelet reactivity, is implied as both a causal as well as a prognostic factor in cardiovascular disease [6].

Platelets are produced by megakaryocytes in the bone marrow through the process of thrombopoiesis. Dyslipidemia alters megakaryocyte functioning, and can in this way modulate the risk of atherothrombosis via the megakaryocyte-platelet hemostatic axis [7]. High-cholesterol diet-fed rabbits and guinea pigs develop megakaryocytes with increased ploidy and size [8,9]. Moreover, hypercholesterolemia in humans is associated with higher megakaryocyte ploidy, indicative of altered megakaryocyte maturation, and higher mean platelet volume [10]. Together, these data imply regulation of megakaryocyte cholesterol homeostasis as a potential therapeutic target to modulate platelet characteristics and prevent or treat cardiovascular disease.

Cellular cholesterol homeostasis is maintained through a delicate balance involving the uptake of lipoprotein-derived cholesterol, de novo synthesis and cholesterol efflux to apolipoprotein A1 (ApoA1) and high-density lipoproteins (HDL). Previous research has indicated that cholesterol efflux capacity of megakaryocytes can influence megakaryocyte formation, function and platelet production. Murphy et al showed that the cholesterol efflux transporter ATP-Binding cassette transporter G4 (ABCG4), a half-size ABC transporter, is highly expressed in megakaryocyte progenitors [11]. ABCG4 is predominantly found in the trans-Golgi, where it may act indirectly to influence plasma membrane cholesterol content and HDL-mediated cholesterol efflux [11]. In agreement, ABCG4 knockout (KO) megakaryocyte progenitors showed defective cholesterol efflux to HDL, and increased unesterified cholesterol accumulation, with prominent accumulation in the plasma membrane [11]. This is accompanied by increased surface expression of the thrombopoietin receptor c-MPL and enhanced proliferation of megakaryocyte progenitor cells, leading to increased platelet production.

The most studied cholesterol efflux transporters are ABCA1 and ABCG1. ABCA1 is a full transporter, and is ubiquitously present on cellular plasma membranes, and mediates cholesterol efflux to lipid poor ApoA1, but not large HDL particles [12]. In contrast ABCG1 needs to dimerize with another ABC transporter to become functional [13]. ABCG1 mediates cholesterol efflux to mature HDL particles, but not lipid-free apolipoproteins [14–16]. Interestingly, whereas ABCA1 is present on the plasma membrane and directly binds to circulating ApoA1 [17], ABCG1 is involved in intracellular sterol movement. It increases the availability of cholesterol for efflux to lipoprotein acceptors and the regulation of endoplasmic reticulum-localized cholesterol biosynthesis genes [17,18]. ABCA1 and ABCG1

are highly expressed in hematopoietic stem cells and myeloid progenitors, but expression in megakaryocyte-erythrocyte progenitors is decreased [11]. Notably, stimulation of the nuclear oxysterol receptor liver X receptor (LXR) upregulates ABCA1 and ABCG1 in megakaryocyte-erythrocyte progenitors, suggesting that these transporters may play a physiological role in the control of megakaryocyte cholesterol homeostasis in a cholesterol-loading context, i.e. under hyperlipidemic conditions [11].

Because of their differential localization and possible roles in (intra)cellular cholesterol transport, we hypothesize that ABCA1 and ABCG1 may have roles in megakaryocyte functioning that are distinct from ABCG4. In this study, we have for the first time examined the roles of these transporters in megakaryopoiesis and platelet production. Hereto, we used unique ABCA1/ABCG1 double knockout (DKO) mice that as a result of a severe disruption of the cellular cholesterol efflux pathway show massive cholesterol accumulation in various tissues rich in macrophages despite low serum cholesterol levels [22].

MATERIALS AND METHODS

Mice

8-35 weeks old, male and female wild-type C57Bl6/J, ABCA1 KO, ABCG1 KO and ABCA1/ABCG1 double KO (DKO) mice were bred in house at the Gorlaeus Laboratories (Leiden, the Netherlands). All mice were fed a regular chow diet (Special Diet Services, Witham, UK) and were group housed in filter top cages. All animal experiments were performed in accordance with the national guidelines for animal experimentation. All experimental protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

TPO challenge

Blood was drawn from the tail vein of female mice prior to, and 2 and 5 days post intraperitoneal injection with 17.5 µg/kg rmTPO (Peprotech, Londen, UK) in PBS.

Platelet analysis

Blood obtained from the tail vein or orbital exsanguination of male and female mice was collected in EDTA-coated tubes. Platelet counts and characteristics were analyzed using an automated XT-2000iV veterinary hematology analyzer (Sysmex Europe GMBH, Norderstedt, Germany).

Bone marrow explants and proplatelet formation

Ex vivo megakaryocyte maturation and proplatelet production in the native bone marrow environment of female mice was measured using the bone marrow explant

method originally developed by Eckly and colleagues [19]. Bone marrow, isolated by flushing femurs and tibia with PBS, was cut in 0.5 mm thick sections. 5-6 sections were transferred into an incubation chamber and incubated at 37°C for 6 hours in Hepes Tyrodes buffer containing 3.5 g/L human serum albumin (MP biomedicals, Santa Ana, California, USA) and 1 g/L glucose (pH 7.3) supplemented with 5% serum from the donor mouse. Proplatelet-forming megakaryocytes were counted every 1.5 hours using phase contrast microscopy.

Bone marrow megakaryocyte counts

Bone marrow from female mice was obtained by flushing the femurs and tibia with PBS, fixed for 24 h in 3.7% neutral-buffered formalin (Formal-Fixx, Shandon Scientific Ltd, UK), and embedded in paraffin for sectioning. Bone marrow was sectioned using Leica RM2235 microtome at 5 µm thickness with a 25 µm interval. Serial sections were routinely stained with hematoxylin and eosin for general histology. Megakaryocyte counts were analyzed by a blinded observer using a Leica DM-RE microscope and LeicaQwin software (Leica Ltd, Cambridge, UK). Six fields of 200x200µm were analyzed in each of four sections per mouse.

Megakaryocyte cultures

Single cell suspensions of lineage negative cells ($2.0x10^6$ cells/mL) from female mice were isolated from bone marrow of 8-12 week old female wild-type mice using a Hematopoietic Progenitor Cell Enrichment Set (DM-558451; BD Biosciences, San Jose, CA, USA). The lineage negative cells were cultured in 700 µL DMEM (Lonza, Basel, Switserland) supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and unless specified otherwise, with 50 ng/mL murine thrombopoietin (mTPO, Stem Cell Technologies, Vancouver, Canada) and 70 U/mL heparin (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 12-well culture plates for up to 4 days [20].

Gene expression analysis

Total RNA was isolated from bone marrow and in vitro-cultured megakaryocytes of female mice by phenol-chloroform extraction. Equal amounts of RNA were reverse transcribed and subsequently real-time quantitative PCR analysis was executed on the cDNA using an ABI Prism 7500 apparatus (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Ribosomal Protein 37 (RPL37), Acidic Ribosomal Phosphoprotein P0 (36B4), and Peptidylprolyl Isomerase A (PPIA), were used as reference genes for normalization for bone marrow. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene for megakaryocyte cultures. Primer sequences are available upon request.

Flow cytometry

A single cell suspension of bone marrow cells from female mice was obtained by straining flushed bone marrow from the femurs and tibiae through a 70 µm nylon mesh (Greiner Bio-One, Kremsmünster, Austria) with PBS. Bone marrow from scavenger receptor BI (SR-BI) KO mice was used as a positive control for unesterified cholesterol accumulation [21]. Fc receptors were blocked with an unconjugated antibody against CD16/CD32 (Clone 93, BioLegend, San Diego, California, USA). Cell viability in all samples was determined using Fixable Viability Dye eFluor® 780 (ThermoFisher Scientific, Waltham, Massachusetts, USA). To determine megakaryocyte free cholesterol content, $5*10^6$ cells per sample were stained with Brilliant Violet 421™-conjugated anti-CD41 (clone MWReg30, BioLegend), fixed with 0.5% paraformaldehyde and stained with filipin III (Cayman Chemical, Ann Arbor, Michigan, US). Flow cytometric analysis was performed on a Cytoflex S flow cytometer (Beckman Coulter, Brea, California, USA) and the acquired data were analyzed using FlowJo software (FlowJo LLC, Ashland, Oregon, USA). Gates were set according to unstained controls and fluorescence minus one (FMO) controls.

Statistical analysis

Statistical analysis was performed using Graphpad Prism software (San Diego, USA, http://www.graphpad.com). Outlier detection was performed using Grubbs' test for outliers. The significance of differences was calculated using a two-tailed unpaired t-test or two-way analysis of variance (ANOVA) where appropriate. Probability values (p) <0.05 were considered significant.

RESULTS

The expression of the ABC transporters ABCA1 and ABCG1 was investigated during ex vivo megakaryopoeisis from lineage negative cells isolated from wildtype C57Bl6/J mice. Megakaryocyte maturation was confirmed by measuring the expression of megakaryocyte marker genes *Itga2b* (CD41), *Gp9* (CD42a) and *Gp1bb* (CD42c) (Figure 1A). Although immature megakaryocytes (1 day after the start of differentiation) tended to express ABCA1 at a higher level than ABCG1 (p=0.16), fully mature megakaryocytes (4 days after start of differentiation) expressed similar levels of ABCA1 and ABCG1 mRNA (Figure 1B).

To establish the potential relevance of ABCA1 and ABCG1 for megakaryocyte cellular cholesterol homeostasis and function we studied megakaryopoeisis and platelet characteristics in wild-type and ABCA1/ABCG1 DKO mice. ABCA1/ABCG1 DKO mice displayed similar amounts of megakaryocytes in the bone marrow (Figure 2A). Interestingly, there was a significant increase (1.7-fold, p<0.01 at 6 hours; AUC: 33±3 vs 52±7, p<0.01) in

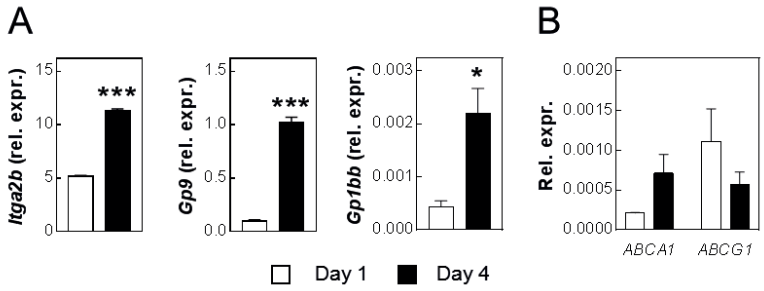

Figure 1: ABCA1 and ABCG1 are expressed in in vitro cultured megakaryocytes. (A) Relative mRNA expression of megakaryocyte markers *Itga2b* (CD41), *Gp9* (CD42a) and *Gp1bb* (CD42c) in in vitro bone marrow stem cell-derived megakaryocytes 1 and 4 days after start of differentiation (n=3-4/time point). (B) Relative mRNA expression of *ABCA1* and *ABCG1* in in vitro bone marrow stem cell-derived megakaryocytes 1 and 4 days after start of differentiation (n=2-4/time point). Data represent means±SEM. *p<0.05, ***p<0.001.

proplatelet production by megakaryocytes from ABCA1/ABCG1 DKO as compared to wildtype mice in bone marrow explants (Figure 2B).

It was previously shown that bone marrow ABCG4 deficiency alters the thrombopoietin response, through unesterified cholesterol accumulation in megakaryocyte progenitors, leading to higher platelet counts [11]. Notably, no accumulation of unesterified cholesterol was detected in megakaryocytes from ABCA1/ABCG1 DKO and wild-type mice (Figure 2C), while our positive controls (megakaryocytes from hypercholesterolemic scavenger receptor BI (SR-BI) KO mice [21] did show significant unesterified cholesterol accumulation (median fluorescence intensity: 8398±258 in SR-BI KO vs 5531±217 and 4932±308 in wild-type and ABCA1/ABCG1 DKO mice). Moreover, combined deletion of ABCA1 and ABCG1 had no effect on basal platelet counts (Figure 2D) or thrombopoietin stimulated platelet production (Figure 2E). These combined data indicate that the mechanisms by which ABCA1 and ABCG1 deletion influence megakaryopoiesis and platelet production are different from ABCG4 deletion. Notably, in support with an effect on proplatelet production, ABCA1/ABCG1 DKO mice displayed an increase in platelet volume (p<0.001; Figure 1F) and platelet distribution width (p<0.001; Figure 1G).

Single ABCG1 KO mice also exhibited virtually identical numbers of bone marrow megakaryocytes compared to wild-type mice (Figure 3A). Surprisingly, when measuring proplatelet production, we found a striking 2.2-fold increase in the amount of proplatelet forming megakaryocytes in ABCG1 KO bone marrow explants (p<0.001 at 6 hours; AUC: 33±3 vs 99±11, p<0.001; Figure 3B), which was substantially higher as compared to ABCA1/ABCG1 DKO explants (AUC: 3-fold vs 1.6-fold compared to wild-type mice). ABCG1 KO mice had equal platelet counts (Figure 3C), mean platelet volume (Figure 3D), and platelet distribution width compared to wild-type mice (Figure 3E).

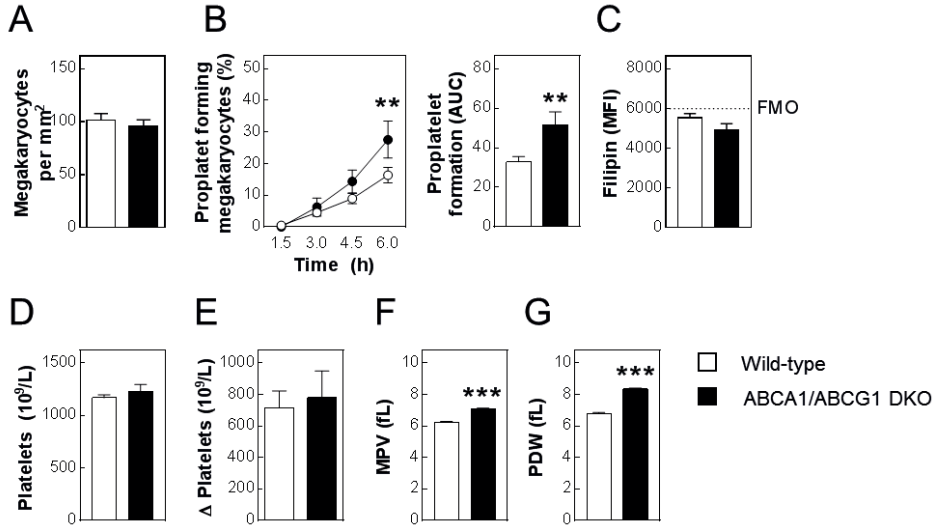

Figure 2: Double deficiency of ABCA1 and ABCG1 alters proplatelet production and platelet characteristics. (A) Quantification of bone marrow megakaryocytes in bone marrow sections from wild-type (n=3) and ABCA1/ABCG1 DKO mice (n=5). (B) Percentage of proplatelet-forming megakaryocytes in an ex vivo native bone marrow environment of wild-type (n=15) and ABCA1/ABCG1 DKO (n=4) mice, and area-under-the-curve (AUC). (C) Filipin median fluorescent intensity (MFI) of $CD41^+$ cells of wild-type (n=11) and ABCA1/ABCG1 DKO mice (n=10). FMO indicates the Fluorescence Minus One control. (D) Basal platelet counts of wild-type (n=7) and ABCA1/ABCG1 DKO mice (n=4). (E) Platelet increase 5 days after thrombopoietin challenge (wild-type: n=7, ABCA1/ABCG1 DKO: n=6). (F) Basal mean platelet volume (MPV) and (G) platelet distribution width (PDW) (wild-type: n=7, ABCA1/ABCG1 DKO: n=4). Data represent means±SEM. $^{**}p<0.01$, $^{***}p<0.001$.

Similar as in single ABCG1 KO and ABCA1/ABCG1 DKO mice, bone marrow megakaryocyte numbers in single ABCA1 KO mice were equal to the amounts in wild-types (Figure 4A). However, in contrast to the observations in single ABCG1 KO and ABCA1/ABCG1 DKO mice, megakaryocytes of single ABCA1 KO mice surprisingly showed decreased proplatelet formation (AUC: 17±2 vs 33±3for wild-type, $p<0.05$.; Figure 4B). As previously published by Lhermusier et al [22], we observed a slight, non-significant increase in platelet counts in the circulation of ABCA1 KO mice ($p=0.18$; Figure 4C). Moreover, in agreement with the earlier study [22], and as observed in the ABCA/ABCG1 DKO mice in the current study, mean platelet volume ($p<0.01$; Figure 4D) and platelet distribution width were increased in single ABCA1 KO mice ($p<0.01$; Figure 4E).

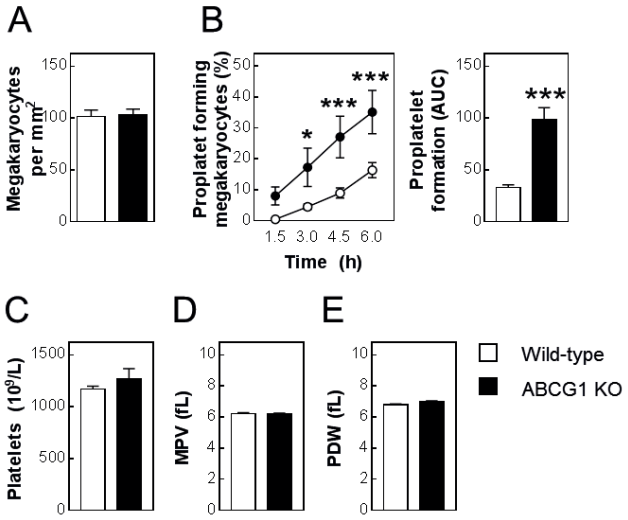

Figure 3: ABCG1 deficiency increases proplatelet production but not platelet levels and characteristics. (A) Quantification of bone marrow megakaryocytes in bone marrow sections from wild-type (n=3) and ABCG1 KO (n=3) mice. (B) Percentage of proplatelet-forming megakaryocytes in an ex vivo native bone marrow environment of wild-type (n=15) and ABCG1 KO (n=5) mice, and area-under-the-curve (AUC). (C) Basal platelet counts, (D) mean platelet volume (MPV), and (E) platelet distribution width (PDW) of wild-type (n=7) and ABCG1 KO (n=3) mice. Data represent means±SEM. *p<0.05, ***p<0.001.

DISCUSSION

In the current study we investigated the impact of ABCA1 and ABCG1 deficiency on proplatelet production. We show that absence of the cholesterol efflux transporters ABCA1 and ABCG1 alters proplatelet production by megakaryocytes. Moreover, our studies suggest that modulation of the function of individual cholesterol efflux transporters may translate into an overall differential effect on platelet production.

Previous work by Murphy et al has shown that cholesterol efflux facilitated by ABCG4 is important for megakaryocyte progenitor proliferation and platelet production [11]. ABCG4 is highly expressed in megakaryocyte progenitors, whereas the expression of ABCA1 and ABCG1 is much less in this cell type [11]. However, despite this relatively low expression, we observed strong effects of deletion of these transporters on megakaryocyte functioning and proplatelet production. Combined deletion of ABCA1 and ABCG1 has detrimental effects on the cholesterol efflux capacity of myeloid cells leading to excessive cholesterol accumulation, even at low plasma cholesterol conditions associated with ABCA1 deficiency [23]. Interestingly, no cholesterol accumulation was observed in megakaryocytes of ABCA1/ABCG1 DKO mice.

Platelets from ABCA1 KO and ABCA1/ABCG1 DKO mice are slightly larger in size. Moreover, platelets lacking ABCA1 exhibit aggregation and secretion defects in response to

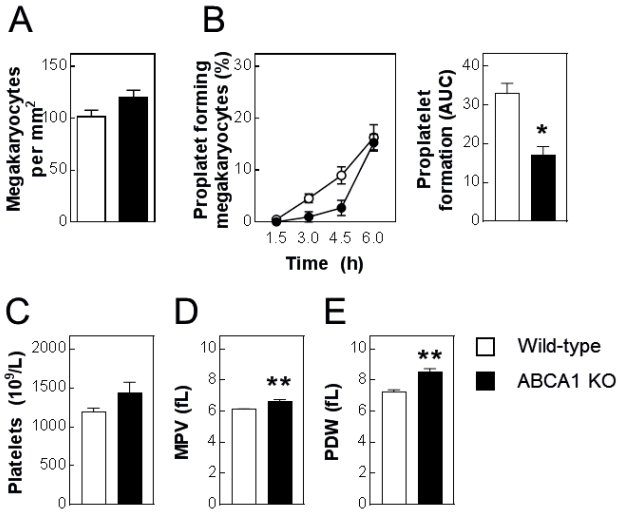

Figure 4: ABCA1 deficiency decreases proplatelet production and alters platelet characteristics. (A) Quantification of bone marrow megakaryocytes in bone marrow sections from wild-type (n=3) and ABCA1 KO (n=2) mice. (B) Percentage of proplatelet-forming megakaryocytes in an ex vivo native bone marrow environment of wild-type (n=15) and ABCA1 KO (n=3) mice, and area-under-the-curve (AUC). (C) Basal platelet counts, (D) mean platelet volume (MPV), and (E) platelet distribution width (PDW) of wild-type (n=4) and ABCA1 KO (n=5) mice. Data represent means±SEM. *p<0.05, **p<0.01.

low concentrations of thrombin and collagen [22]. Bone marrow transplantation of wild-type bone marrow into ABCA1 KO mice suggested that, at least in part, this platelet phenotype is driven by the virtual absence of HDL cholesterol in the ABCA1 KO mice [22]. In line with the notion that plasma cholesterol can alter platelet function and characteristics, accumulation of HDL cholesterol in SR-BI KO mice leads to severe thrombocytopenia, as well as an increased platelet size and reactivity. Studies by us and others have shown that this phenotype is associated with increased platelet clearance rates due to intracellular unesterified cholesterol accumulation, as well as decreased proplatelet formation [21,24,25]. ABCG1 KO mice, which exhibit normal plasma cholesterol levels, do not have altered platelet characteristics. Importantly, despite the observed alterations in proplatelet formation in absence of ABCA1 and ABCG1 in the current study, this does not translate into differences in platelet counts. This indicates that platelet counts are not solely dependent on megakaryocyte platelet production rates.

The most important and novel finding of the current study is that deletion of ABCA1 or ABCG1 has independent effects on megakaryocyte functioning, as demonstrated by the differential effects on proplatelet formation. ABCA1 deficiency inhibits the early stages of proplatelet formation, whereas in stark contrast, absence of ABCG1 strongly increases proplatelet formation. In accordance with this notion, combined deletion

of both transporters leads to an effect that is intermediate between the effects of the individual single KOs.

Proplatelet formation is a terminal process, and after the entirety of the megakaryocyte cell body is converted into proplatelets, the nucleus is extruded and degraded [26]. Currently, there is still an insufficient understanding of signals that trigger proplatelet formation. However, it does require an apoptotic phase and caspase activation [26]. Although for long it remained unsure how the apoptotic process required for proplatelet formation was initiated, studies by Lopez et al have shown that transient ER stress activation triggers this process. In support, ER stress induction peaks at the proplatelet formation stage [27]. Moreover, treatment with salubrinal, which protects cells from ER stress-induced apoptosis, dose dependently inhibits the production of proplatelets from megakaryocytes [27]. Conversely, induction of ER stress by the ER stressor thapsigargin enhances the formation of functional platelet-like particles from MEG-01 megakaryocyte cell line cells [28]. Therefore, it is likely that ER stress induced apoptosis is the driving force behind proplatelet formation. Although the precise cellular localization of ABCG1 in megakaryocytes remains elusive, in myeloid cells it is involved in intracellular trafficking of cholesterol over the ER membrane and endosomes, in addition to a role in cholesterol sensing [17,18]. ABCG1 deficiency induces ER stress in endothelial cells [30]. Moreover, blockade of cholesterol trafficking to the ER reversed ER stress-induced apoptosis of ABCG1 deficient endothelial cells, suggesting that activation of ER stress in ABCG1 KO endothelial cells depends on trafficking of unesterified to the ER membrane [30]. It is therefore interesting to speculate that deletion of ABCG1 in megakaryocytes enhances the ER stress response necessary for terminal megakaryocyte maturation, and in that way accelerates proplatelet formation.

Conversely, can the observed decrease in proplatelet formation in the single ABCA1 KO megakaryocytes be explained by a decrease in ER stress response? ABCA1 facilitates retrograde sterol transport from the plasma membrane to the ER for sterol sensing [31]. In vitro, lack of ABCA1 in mouse embryonic fibroblasts is associated with hyperactivation of the SREBP-2 pathway, even in the presence of an increase in cellular cholesterol [31]. These data suggest that ABCA1 deficiency decreases ER cholesterol levels and disrupts sterol sensing in the ER. Possibly, a decrease in ER cholesterol could delay the ER stress response in ABCA1 KO megakaryocytes, explaining the decrease in proplatelet formation in these cells. Further studies on the effect of intracellular cholesterol trafficking and sterol sensing on terminal megakaryocyte maturation and proplatelet formation are warranted to provide further mechanistic support for this hypothesis.

In conclusion, we show that the cholesterol efflux transporters ABCA1 and ABCG1 have differential effects on megakaryocyte functioning, proplatelet formation and platelet characteristics. Our findings suggest that ABCA1 and ABCG1 do not play a major role in

megakaryocyte cholesterol efflux towards extracellular cholesterol acceptors, but may play a crucial role in the regulation of the final steps of thrombopoiesis.

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