Cholesterol metabolism and hematopoiesis interaction in atherothrombosis

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3

Hypercholesterolemia impairs megakaryopoiesis and platelet production in scavenger receptor BI knockout mice

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ABSTRACT

Thrombocytopenia in scavenger receptor BI (SR-BI) knockout mice is suggested to result from augmented platelet clearance induced by elevated intracellular unesterified cholesterol (UC) levels. We hypothesize that SR-BI deficiency may also influence platelet production at the level of its precursor cell in the bone marrow, the megakaryocyte. In this study, we compared megakaryopoiesis and platelet production in SR-BI knockout and wild-type mice. In line with our hypothesis, megakaryocytes from SR-BI knockout mice exhibited UC accumulation while no accumulation of UC was detectable in wild-type megakaryocytes. Bone marrow expression of transcription factors involved in megakaryocyte maturation was induced, but megakaryocyte counts were unchanged in bone marrow of SR-BI knockout mice. Interestingly, we did find a striking 62% decrease ($p<0.01$) in proplatelet production by SR-BI knockout megakaryocytes. SR-BI knockout mice displayed an impaired increase in circulating platelet concentrations and bone marrow megakaryocyte numbers upon thrombopoietin challenge. Importantly, megakaryocytes from normolipidemic bone marrow-specific SR-BI knockout mice exhibited a normal ability to produce proplatelets. Moreover, bone marrow-specific deletion of SR-BI did not impair the thrombopoietin response or induce thrombocytopenia, confirming that absence of megakaryocyte SR-BI does not underlie the thrombocytopenic phenotype in total body SR-BI knockout mice. In conclusion, the elevation of plasma unesterified cholesterol levels impairs megakaryopoiesis and platelet production in SR-BI knockout mice. Our findings suggest that, in addition to an increased platelet clearance, a decrease in platelet production may also, in part, explain the thrombocytopenic phenotype associated with SR-BI deficiency in mice.

INTRODUCTION

Platelets are small anucleate cells that play a key role in hemostasis and respond rapidly to changes in the endothelial integrity and exposure of sub-endothelial structures. As such, platelets play a key role in the formation of atherothrombosis [1]. A substantial body of clinical evidence associates platelet characteristics such as density and volume with the risk of acute coronary syndromes [2–5], and implies mean platelet volume, a determinant of platelet reactivity, as both a causal and prognostic factor [6]. Therefore, modification of platelet functionality is a promising therapeutic target in the prevention and treatment of cardiovascular disease.

Platelets are produced by megakaryocytes, which represent the largest cells of the hematopoietic lineage and account for approximately 0.01% of nucleated cells in the bone marrow [7]. Thrombopoietin (TPO) is the primary regulator of platelet production, supporting the formation, proliferation and differentiation of megakaryocytes [8]. Upon stimulation by TPO, megakaryocytes develop from pluripotent hematopoietic stem cells and subsequently mature, i.e. they become polyploid and grow in size. During maturation, megakaryocytes also develop an internal demarcation membrane system (DMS), granules, and organelles that are packaged in bulk for platelet production [9]. DMS formation enables the megakaryocytes to produce long, branching extensions called proplatelets. Proplatelets extend into the vascular sinusoids of the bone marrow, from which eventually platelets are released into the bloodstream [10]. Once released, human platelets stay in the blood circulation for 7-10 days [11],, while rodent platelets circulate for only 4-5 days [12]. Aged platelets, recognized by the Ashwell-Morrell receptor through the loss of sialic acid on their surface-expressed proteins are cleared by hepatocytes [13]. Binding of desialylated platelets to the Ashwell-Morrell receptor stimulates hepatic TPO production, thereby maintaining overall platelet homeostasis [14].

Previous studies have shown that genetic disruption of scavenger receptor BI (SR-BI) function is associated with thrombocytopenia in mice [15–17]. SR-BI is a cell surface receptor that mediates the selective uptake of cholesteryl esters from lipoproteins and is mostly known for its role in reverse cholesterol transport as the functional high-density lipoprotein (HDL) receptor [18]. In mice, disruption of SR-BI functionality results in a 3.2-fold increase in circulating unesterified cholesterol (UC) levels due to substrate inhibition of the plasma enzyme Lecithin:Cholesterol Acyltransferase (LCAT), resulting in a high plasma unesterified-to-total cholesterol ratio (UC:TC) [18–20]. As a result, in the SR-BI knockout mice, platelets accumulate UC and circulate in an activated state [15–17]. Importantly, platelet UC accumulation was also observed in humans carrying a functional mutation in *SCARB1*, the gene encoding SR-BI [21]. However, heterozygous SR-BI deficiency in humans does not lead to thrombocytopenia [21]. Dole and colleagues have suggested that

the platelet UC accumulation in mice is the cause of the thrombocytopenia associated with SR-BI deficiency, as the platelet clearance in SR-BI knockout mice is enhanced [15].

Interestingly, SR-BI is expressed on megakaryocytes [22]. Recent studies have suggested that HDL can directly impact on megakaryopoiesis and platelet production. More specifically, Murphy and colleagues have demonstrated that infusion of reconstituted HDL in mice decreases platelet counts by inhibiting bone marrow megakaryocyte progenitor proliferation [23]. The relevance of the interaction of HDL with SR-BI on megakaryocytes in this context is currently unknown. In the study by Dole et al., SR-BI deficiency did not affect bone marrow megakaryocyte counts [15]. However, Dole et al. focused on platelet clearance and did not execute an in-depth investigation into the impact of SR-BI deficiency on megakaryocyte function, leaving the question regarding a potential impact of SR-BI on megakaryopoiesis and platelet production unanswered. In this study, we therefore examined the effect of SR-BI deficiency on megakaryopoiesis and platelet production to uncover a possible contribution to the thrombocytopenic phenotype.

MATERIALS AND METHODS

Mice

Wild-type and *Scarb1*$^{-/-}$ (SR-BI knockout) mice on a C57Bl6/J background 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.

For all measurements comparing non-treated wild-type and SR-BI knockout mice, 9-14 weeks old female mice were used. For the TPO challenge, male wild-type and SR-BI knockout mice of 42-47 weeks old were used. For bone marrow transplantation experiments, 16 weeks old female wild-type and SR-BI knockout donor mice and 11-20 weeks old female wild-type recipients were used.

Bone marrow transplantation

Donor bone marrow of wild-type and SR-BI knockout mice was isolated by flushing the femurs and tibias with PBS. Recipient wild-type mice were irradiated with 2x4.5 Gy total body radiation using a 225 Smart Röntgen source (YXLON international, Copenhagen, Denmark) one day before transplantation, and received a single cell suspension of $5x10^6$ bone marrow cells in PBS by intravenous injection into the tail vein. The drinking water of the recipient mice was supplemented with antibiotics (83 mg/L ciprofloxacin and 67 mg/L polymixin B sulfate) and 6.5 g/L sucrose from 7 days prior to 4 weeks after

transplantation. At 13-15 weeks post transplantation, mice were anesthetized with a mix of xylazine (70 mg/kg), ketamine (350 mg/kg) and atropine (1.8 mg/kg) and sacrificed by orbital exsanguination, after which the organs, femurs and tibias were collected for further analysis.

TPO challenge

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

Platelet count

Blood obtained from the tail vein was collected in EDTA-coated tubes and diluted 1:4 in PBS for analysis of platelet count. Blood obtained from orbital exsanguination was collected in EDTA-coated tubes and was analyzed non-diluted. Platelet counts were analyzed using an automated XT-2000iV veterinary hematology analyzer (Sysmex Europe GMBH, Norderstedt, Germany).

Plasma cholesterol analysis

Plasma from orbital bleeding-obtained blood was attained by centrifugation (10 minutes, 6000 RPM, room temperature) and stored at −20°C until further use. Plasma UC and TC levels were measured by enzymatic colorimetric assays as described by Out et al. [24].

Bone marrow explants and proplatelet formation

Ex vivo megakaryocyte maturation and proplatelet production in the native bone marrow environment was measured using the bone marrow explant method originally developed by Eckly and colleagues [25]. Bone marrow, isolated by flushing femurs and tibias 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. Images of proplatelet formation were made using a Bio-Rad ZOE™ Fluorescent Cell Imager (Bio-Rad Laboratories, Veenendaal, Netherlands).

Bone marrow megakaryocyte counts

Bone marrow was obtained by flushing the femurs and tibias 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). Four fields of 300x300µm were analyzed in each of four sections per mouse.

GENE EXPRESSION ANALYSIS

Total RNA was isolated from bone marrow and livers 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. Primer sequences are available upon request.

FLOW CYTOMETRY

A single cell suspension of bone marrow cells was obtained by straining flushed bone marrow from the femurs and tibias through a 70 µm nylon mesh (Greiner Bio-One, Kremsmünster, Austria) with PBS. 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 ploidy, $5x10^6$ cells per sample were stained with Brilliant Violet 421™-conjugated anti-CD41 (clone MWReg30, BioLegend) and fixed with ice-cold methanol. Fixed cells were subjected to RNAse treatment (Sigma-Aldrich, Zwijndrecht, The Netherlands) and propidium iodide staining (Sigma-Aldrich) for DNA content measurement. To determine megakaryocyte UC content, $5x10^6$ cells per sample were stained with APC-conjugated anti-CD41 (clone eBioMWReg30, eBioscience, San Diego, California, USA) and filipin III (Cayman Chemical, Ann Arbor, Michigan, US), and fixed with 0.5% paraformaldehyde. 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 Instat software and/or 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) in experiments with multiple variables. Probability values (p) <0.05 were considered significant.

RESULTS

To study the role of SR-BI in megakaryopoiesis and platelet production, we used wild-type and SR-BI knockout mice. We confirmed a strong increase in plasma UC and TC levels in SR-BI knockout mice (2.9- and 2-fold, respectively; p<0.0001 for both; Supplemental figure 1A and B), and a 1.5-fold increase in the UC:TC ratio (p<0.0001; Supplemental figure 1C). Moreover, a marked decrease (-47%; p<0.0001) in blood platelet levels was detected (Supplemental figure 1D), confirming the thrombocytopenia phenotype found in earlier studies [15–17]. Furthermore, we also replicated the SR-BI deficiency-associated

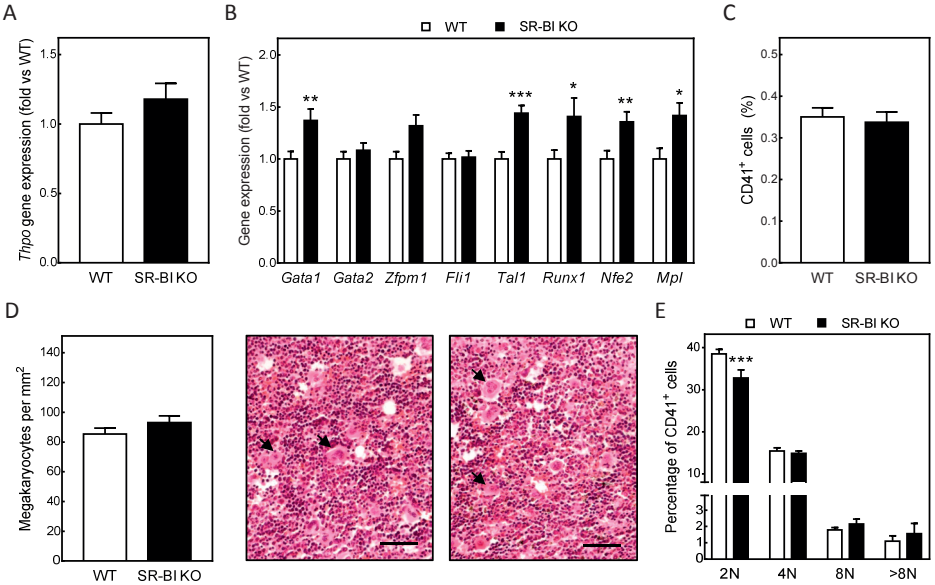

Figure 1: SR-BI deficiency is associated with a minor increase in megakaryocyte maturation, without a change in bone marrow megakaryocyte count. (A) Hepatic gene expression of *Thpo* in wild-type (WT) and SR-BI knockout (SR-BI KO) mice. (B) Relative expression of genes associated with megakaryocyte maturation in bone marrow of wild-type and SR-BI knockout mice. (C) The percentage of $CD41^+$ megakaryocytes in wild-type and SR-BI knockout bone marrow. (D) Quantification of bone marrow megakaryocytes and representative pictures of hematoxylin/eosin-stained bone marrow sections from wild-type and SR-BI knockout mice showing the presence of megakaryocytes (black arrows). Bar represents 50 µm. (E) Ploidy of $CD41^+$ bone marrow megakaryocytes from wild-type and SR-BI knockout mice. Data represent means+SEM of 11 mice per group. *p<0.05, ***p<0.001.

increase in mean platelet volume ($p<0.001$; Supplemental figure 1E) and platelet volume distribution ($p<0.05$; Supplemental figure 1F).

Hepatic *Thpo* expression did not differ between the two genotypes (Figure 1A). In contrast, relative gene expression levels of several key transcription factors that aid in the generation of mature megakaryocytes from bone marrow stem cells as well as that of the TPO receptor c-MPL were mildly increased in bone marrow of SR-BI knockout mice as compared to that of wild-type mice (Figure 1B). This increase in gene expression did not translate into an increase in bone marrow megakaryocyte counts. Both the amount of $CD41^+$ cells as measured by flow cytometry (Figure 1C), as well as histological quantification showed similar levels of bone marrow megakaryocyte numbers (Figure 1D). SR-BI deficiency did not lead to apparent differences in in megakaryocyte ploidy (Figure 1E).

Mature megakaryocytes in the bone marrow migrate to the vascular niche to produce proplatelets and eventually release platelets into the bloodstream. To determine if the minor increase in ploidy in megakaryocytes from SR-BI knockout mice coincided with differences in the final step of platelet production, we measured proplatelet production by primary megakaryocytes from wild-type and SR-BI knockout mice. Interestingly, there was a striking decrease in proplatelet production by SR-BI knockout megakaryocytes (two-way ANOVA: $p<0.01$ for genotype), which translated in a 62% decrease ($p<0.05$) in the amount of proplatelets at the final 6-hour time point (Figure 2A). Plasma crossover experiments indicated that the overall SR-BI knockout genotype effect could not be fully replicated by incubation of wild-type bone marrow explants with hypercholesterolemic SR-BI knockout serum. Importantly, we did note a clear trend towards a decrease in the amount of proplatelet forming megakaryocytes as compared to wild-type explants exposed to wild-type serum (6 hours: 12.4±4.6% vs. 16.3±2.5; Two-way ANOVA: $p=0.16$ for serum type). However, given the limited exposure time of 6 hours, it cannot be excluded that hypercholesterolemia is the driving force in the effects of SR-BI deficiency on proplatelet formation. No evident changes in megakaryocyte or proplatelet morphology were observed (Figure 2B). However, as evident from Figure 2C, megakaryocytes from SR-BI knockout mice exhibited UC accumulation while no accumulation of UC was detectable in wild-type megakaryocytes. These combined findings suggest that an increase in cellular UC levels may underlie the impaired ability of SR-BI knockout megakaryocytes to produce proplatelets.

To confirm that the diminished megakaryocyte functioning ex vivo translated into functional differences in the ability to produce platelets in vivo, we administered TPO to wild-type and SR-BI knockout mice. Given the similar number of megakaryocytes in wild-type and SR-BI knockout mice, mice from both genotypes were anticipated to produce a similar increase in absolute numbers of platelets upon stimulation with TPO. As expected, wild-type mice challenged with TPO displayed a strong increase ($+774x10^9$/L; $p<0.01$) in blood platelet numbers at 5 days post injection (Figure 3A). In contrast, ad-

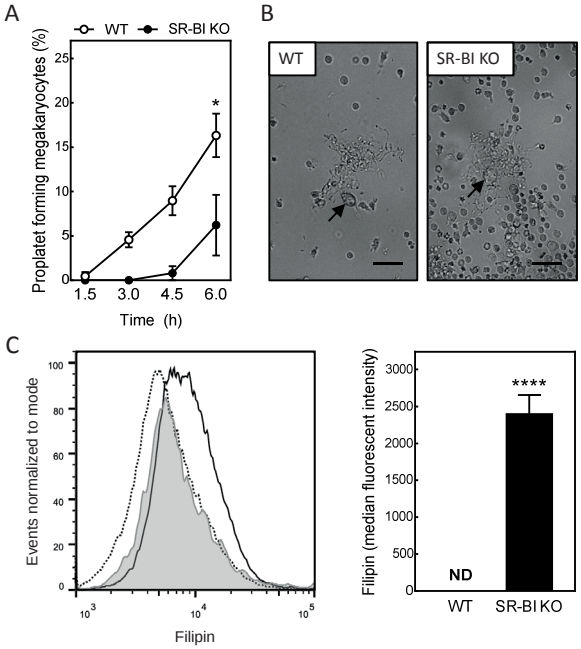

Figure 2: SR-BI knockout megakaryocytes have a decreased ability to form proplatelets and an increased UC content. (A) Percentage of megakaryocytes producing proplatelets in an ex vivo native bone marrow environment of wild-type (WT; n=15) and SR-BI knockout (SR-BI KO; n=3) mice. (B) Representative pictures of proplatelet forming megakaryocytes from wild-type and SR-BI knockout mice in an ex vivo native bone marrow environment. Black arrows: body of a proplatelet-forming megakaryocyte. Bar represents 30 µm. (C) Flow cytometry histogram of filipin fluorescent intensity of wild-type (dotted black line), SR-BI knockout (solid black line), and FMO control (grey shading), and quantification of filipin median fluorescent intensity of $CD41^+$ cells of wild-type and SR-BI knockout mice (n=11 per group), corrected for FMO controls. Data represent means±SEM. *p<0.05; ****p<0.0001.

ministration of TPO to SR-BI knockout mice led to a much smaller, non-significant rise in blood platelet counts (+301x10^9/L; Figure 3A). The difference in the response to generate platelets coincided with a significant difference in the bone marrow megakaryocyte numbers after the TPO challenge. Bone marrow megakaryocyte numbers were 32% percent lower (p<0.001) in TPO-stimulated SR-BI knockout mice as compared to TPO-stimulated wild-type mice (Figure 3B). It thus appears that SR-BI knockout mice exhibit a limited ability to upregulate platelet production in response to TPO stimulation.

Previous studies have shown that SR-BI is present on megakaryocytes and platelets [15,21,22]. To determine whether the effect of total body SR-BI deficiency on megakaryocyte functioning was due to megakaryocyte-specific SR-BI deficiency, we applied the bone marrow transplantation technique to specifically delete SR-BI in all bone marrow-derived cells, including megakaryocytes. As previously reported [16], wild-type mice reconstituted with either wild-type or SR-BI knockout bone marrow had plasma UC and TC levels

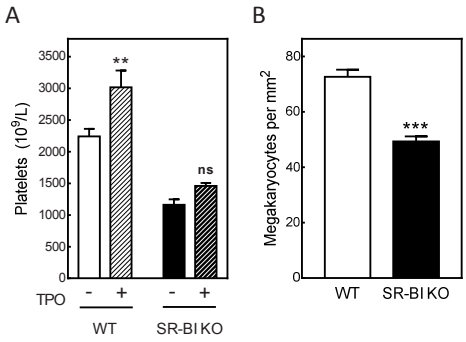

Figure 3: SR-BI knockout mice display a decreased responsiveness to TPO. (A) Blood platelet levels of wild-type (WT) and SR-BI knockout (SR-BI KO) mice before and after treatment with TPO (n=7 per group; 2-way ANOVA: $p<0.0001$ for genotype). (B) Histological quantification of bone marrow megakaryocytes after TPO administration (wild-type: n=3; SR-BI knockout : n=4). Data represent means±SEM. ns = $p>0.05$, **$p<0.01$, ***$p<0.001$.

within the normolipidemic range (data not shown). Moreover, specific deletion of SR-BI in bone marrow was not associated with the thrombocytopenia found in total body knockout mice. Platelet levels were virtually identical in the two groups of bone marrow recipients (Figure 4A). In accordance with the unchanged basal platelet concentrations, investigation of the megakaryocyte function revealed that SR-BI knockout megakaryocytes had a normal ability to produce proplatelets (Figure 4B) and normal capacity to increase platelet levels in response to TPO stimulation in vivo (Figure 4C). Furthermore, megakaryocyte counts were similar between mice reconstituted with wild-type and SR-BI knockout bone marrow under non-stimulated as well as under TPO-stimulated conditions (Figure 4D).

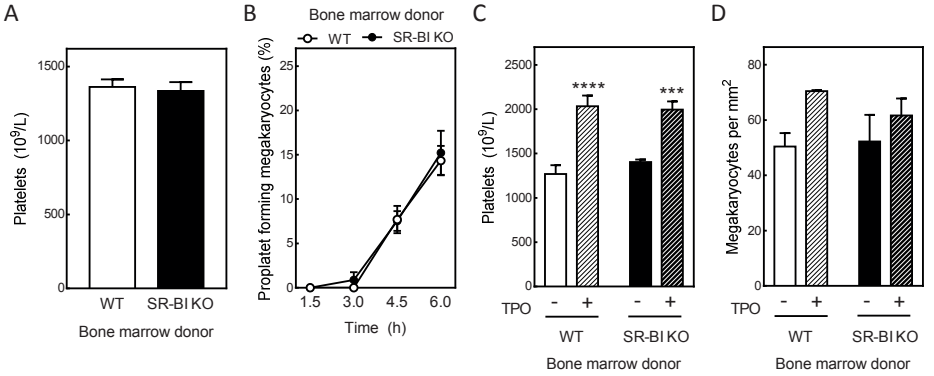

Figure 4: Bone marrow-specific SR-BI deficiency in normolipidemic C57BL/6 mice does not alter platelet count, megakaryopoiesis, or megakaryocyte function under basal and TPO-stimulated conditions. (A) Blood platelet counts in mice that received wild-type (WT; n=19) or SR-BI knockout (SR-BI KO; n=18) bone marrow. (B) Ex vivo proplatelet formation of megakaryocytes in a native bone marrow environment from mice transplanted with wild-type and SR-BI knockout bone marrow (n=3 per group). (C) Blood platelet counts of wild-type (n=7) and SR-BI knockout bone marrow transplanted mice (n=6) before and after treatment with TPO. (D) Quantification of bone marrow megakaryocytes in wild-type and SR-BI knockout bone marrow transplanted mice that have (wild-type: n=2; SR-BI knockout : n=5) or have not (n=3 per group) received an injection with TPO 5 days prior to sacrifice. Data represent means+SEM. ***p<0.001, ****p<0.0001.

DISCUSSION

In the current study we investigated if SR-BI deficiency impacts on megakaryopoiesis and platelet production and in that way contributes to the thrombocytopenia found in SR-BI knockout mice. We show that SR-BI knockout mice exhibit an increased megakaryocyte UC content and minor changes in megakaryopoiesis. The ability of megakaryocytes to form proplatelets, however, was markedly decreased in SR-BI knockout mice. Importantly, these effects are not a direct effect of the absence of megakaryocyte SR-BI but is rather due to an indirect effect of the SR-BI deficiency associated UC accumulation.

Previously, Murphy et al. have shown that reconstituted HDL decreases platelet levels due to a decrease in bone marrow megakaryocyte progenitor proliferation [23]. Based on these studies, we hypothesized that possibly a direct interaction of HDL and its receptor SR-BI would affect megakaryopoiesis and platelet production. Interestingly, although megakaryopoiesis and proplatelet production were altered in hypercholesterolemic total body SR-BI knockout mice, normolipidemic, bone marrow-specific SR-BI knockout mice displayed a normal ability to produce proplatelets. Moreover, specific deletion of SR-BI in bone marrow did not result in thrombocytopenia and showed a normal response to TPO stimulation. Therefore, it is suggested that the elevation of plasma UC levels and not the disruption of the megakaryocyte SR-BI / HDL interaction, impairs megakaryopoiesis and platelet production in total body SR-BI knockout mice.

The increase in plasma UC:TC ratio observed in SR-BI deficient mice is related to the reduced activity of LCAT [18–20]. Altered platelet number and function have been observed in LCAT-deficient mice, especially on a SR-BI-deficient background [26]. Deletion of LCAT rescues the thrombocytopenic phenotype in SR-BI knockout mice, despite increasing the plasma UC:TC ratio [26]. However, reduced LCAT activity lowers plasma lysophosphatidylcholine levels, which is associated with increased megakaryocyte number and function in rats [27]. It can therefore be suggested that the increased platelet number in LCAT / SR-BI double knockout mice can be attributed to increased megakaryopoiesis. However, we did not observe increased megakaryocyte numbers in SR-BI KO mice, suggesting that the influence of the reduced LCAT activity and subsequent lysophosphatidylcholine levels in our experimental setting is minimal.

Mature megakaryocytes produce the DMS to serve as a membrane reservoir for the generation of proplatelets, which extend into the vascular nice to release platelets into the bloodstream [28,29]. It has long been known that membrane cholesterol affects membrane fluidity [30]. In erythrocytes, an increased cholesterol:phospholipid ratio increases cell rigidity and microviscosity [30,31]. Accordingly, in SR-BI knockout mice, a 2-fold increase in the cholesterol:phospholipid ratio causes an increase in cell rigidity and a decrease in deformability of erythrocytes [32]. In the present study we have shown that UC accumulates in the megakaryocytes in SR-BI knockout mice. Moreover, the amount

of proplatelet forming cells was decreased in SR-BI knockout bone marrow. Possibly, the high membrane cholesterol content inhibits the process of DMS and proplatelet production due to an increase in membrane rigidity. In accordance with this hypothesis, transplantation of SR-BI knockout bone marrow into a normolipidemic environment did not replicate the phenotype of decreased proplatelet formation observed in total body SR-BI knockout mice. Further studies on the effect of cholesterol loading of megakaryocyte membranes on DMS functionality and proplatelet formation are warranted to provide further mechanistic support for this hypothesis.

Our findings in hypercholesterolemic SR-BI knockout mice complement previous in vivo observations that hypercholesterolemia changes megakaryocyte and platelet characteristics. Two groups have independently shown that rabbits and guinea pigs fed a high cholesterol diet develop megakaryocytes with increased ploidy and size [33,34]. Hypercholesterolemia in humans is also associated with higher megakaryocyte ploidy, indicative of altered megakaryocyte maturation [35]. Notably, the change in megakaryocyte maturation in hypercholesterolemic human subjects coincided with a higher mean platelet volume [35].

Platelets in SR-BI knockout mice are 1.2- to 1.4-fold larger than wild-type platelets [15,16], which was confirmed by our findings. Dole et al., as well as our own group, have suggested that this larger size can be attributed to cholesterol accumulation in platelets acquired via non-specific lipid exchange from UC-rich HDL particles in the circulation. In support, infused platelets - regardless of donor and recipient genotype - obtain UC levels comparable to resident platelets within 24 hours [15]. Notably, cholesterol in platelets can also be derived from cholesterol taken up by megakaryocytes as these cells can transfer cellular cholesterol to their platelet progeny [36]. In support of the latter, the increase in cholesterol in megakaryocytes in response to a high cholesterol diet challenge appears to precede that in platelets (4-5 day lag time from the start of cholesterol feeding, consistent with the turnover time of platelets). In this study, we have shown that megakaryocytes of SR-BI knockout mice are loaded with excess UC. When taking the aforementioned findings in account, we anticipate that the accumulation of cholesterol in platelets and the observed overall change in platelet count, phenotype and function in SR-BI knockout mice is not only the result of increased passive transfer from the plasma compartment but may - in part - also be inherited from cholesterol accumulation in their megakaryocyte progenitors.

In conclusion, elevation of plasma UC levels impairs megakaryopoiesis and platelet production in SR-BI knockout mice. Our findings suggest that impaired platelet production, in addition to the previously reported augmented platelet clearance, may explain part of the thrombocytopenic phenotype associated with SR-BI deficiency. Hence, our studies highlight megakaryocyte cholesterol homeostasis regulation as a potential therapeutic target in the prevention and treatment of cardiovascular disease.

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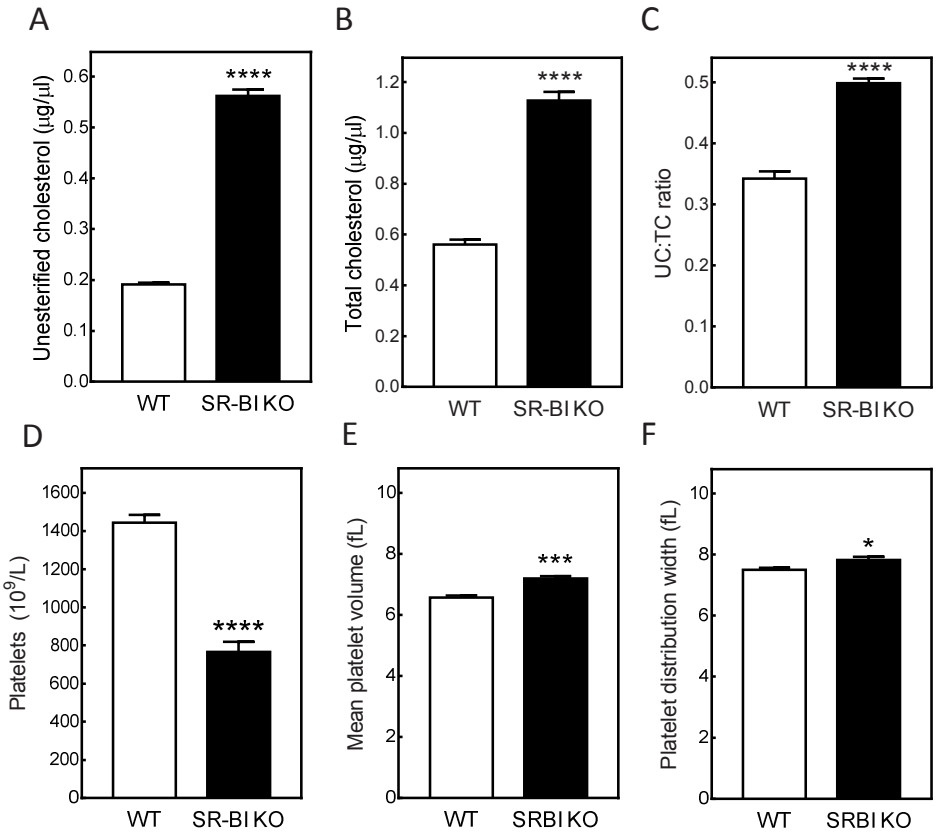
REFERENCES

1. Davì G, Patrono C. Mechanisms of disease: Platelet activation and atherothrombosis. *N Engl J Med*. 2007;357(24):2482-2494. doi:10.1056/NEJMra071014.
2. Martin JF, Plumb J, Kilbey RS, Kishk YT. Changes in volume and density of platelets in myocardial infarction. *Br Med J (Clin Res Ed)*. 1983;287(6390):456-459. doi:10.1136/bmj.287.6390.456.
3. Cameron HA, Phillips R, Ibbotson RM, Carson PH. Platelet size in myocardial infarction. *Br Med J (Clin Res Ed)*. 1983;287(6390):449-451. doi:10.1136/bmj.287.6390.449.
4. Pizzulli L, Yang A, Martin JF, Lüderitz B. Changes in platelet size and count in unstable angina compared to stable angina or non-cardiac chest pain. *Eur Heart J*. 1998;19(1):80-84. doi:10.1053/euhj.1997.0747.
5. Endler G, Klimesch A, Sunder-Plassmann H, Schillinger M, Exner M, Mannhalter C, Jordanova N, Christ G, Thalhammer R, Huber K, Sunder-Plassmann R. Mean platelet volume is an independent risk factor for myocardial infarction but not for coronary artery disease. *Br J Haematol*. 2002;117(2):399-404.
6. Martin J, Bath P, Burr ML. Influence of Platelet Size on Outcome after Myocardial Infarction. *Lancet*. 1991;338(8780):1409-1411. doi:0140-6736(91)92719-I [pii].
7. Nakeff A, Maat B. Separation of megakaryocytes from mouse bone marrow by velocity sedimentation. *Blood*. 1974;43(4):591-595.
8. Hitchcock IS, Kaushansky K. Thrombopoietin from beginning to end. *Br J Haematol*. 2014;165(2):259-268. doi:10.1111/bjh.12772.
9. Machlus KR, Italiano JE. The incredible journey: From megakaryocyte development to platelet formation. *J Cell Biol*. 2013;201(6):785-796. doi:10.1083/jcb.201304054.
10. Richardson JL, Shivdasani RA, Boers C, Hartwig JH, Italiano JEJ. Mechanisms of organelle transport and capture along proplatelets during platelet production. *Blood*. 2005;106(13):4066-4075. doi:10.1182/blood-2005-06-2206.
11. Harker LA, Finch CA. Thrombokinetics in man. *J Clin Invest*. 1969;48(6):963-974. doi:10.1172/JCI106077.
12. Aster RH. Studies of the mechanism of "hypersplenic" thrombocytopenia in rats. *J Lab Clin Med*. 1967;70(5):736-751.
13. Li R, Hoffmeister KM, Falet H. Glycans and the platelet life cycle. *Platelets*. 2016;27(6):505-511. doi:10.3109/09537104.2016.1171304.
14. Grozovsky R, Begonja AJ, Liu K, Visner G, Hartwig JH, Falet H, Hoffmeister KM. The Ashwell-Morell receptor regulates hepatic thrombopoietin production via JAK2-STAT3 signaling. *Nat Med*. 2015;21(1):47-54. doi:10.1038/nm.3770.
15. Dole VS, Matuskova J, Vasile E, Yesilaltay A, Bergmeier W, Bernimoulin M, Wagner DD, Krieger M. Thrombocytopenia and Platelet Abnormalities in High-Density Lipoprotein Receptor – Deficient Mice. 2008. doi:10.1161/ATVBAHA.108.162347.
16. Korporaal SJA, Meurs I, Hauer AD, Hildebrand RB, Hoekstra M, Cate HT, Pratico D, Akkerman J-WN, Van Berkel TJC, Kuiper J, Van Eck M. Deletion of the High-Density Lipoprotein Receptor Scavenger Receptor BI in Mice Modulates Thrombosis Susceptibility and Indirectly Affects Platelet Function by Elevation of Plasma Free Cholesterol. *Arterioscler Thromb Vasc Biol*. 2011;31(1):34-42. doi:10.1161/ATVBAHA.110.210252.
17. Ma Y, Ashraf MZ, Podrez EA. Scavenger receptor BI modulates platelet reactivity and thrombosis in dyslipidemia. 2017;116(11):1932-1942. doi:10.1182/blood-2010-02-268508.An.

18. Rigotti A, Trigatti BL, Penman M, Rayburn H, Herz J, Krieger M. A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. *Proc Natl Acad Sci U S A*. 1997;94(23):12610-12615.
19. Out R, Hoekstra M, Spijkers JAA, Kruijt JK, van Eck M, Bos IST, Twisk J, Van Berkel TJC. Scavenger receptor class B type I is solely responsible for the selective uptake of cholesteryl esters from HDL by the liver and the adrenals in mice. *J Lipid Res*. 2004;45(11):2088-2095. doi:10.1194/jlr.M400191-JLR200.
20. Van Eck M, Twisk J, Hoekstra M, Van Rij BT, Van der Lans CAC, Bos IST, Kruijt JK, Kuipers F, Van Berkel TJC. Differential effects of scavenger receptor BI deficiency on lipid metabolism in cells of the arterial wall and in the liver. *J Biol Chem*. 2003;278(26):23699-23705. doi:10.1074/jbc.M211233200.
21. Vergeer M, Korporaal SJA, Franssen R, Meurs I, Out R, Hovingh GK, Hoekstra M, Sierts JA, Dallinga-Thie GM, Motazacker MM, Holleboom AG, Van Berkel TJC, Kastelein JJP, Van Eck M, Kuivenhoven JA. Genetic variant of the scavenger receptor BI in humans. *N Engl J Med*. 2011;364(2):136-145. doi:10.1056/NEJMoa0907687.
22. Imachi H, Murao K, Cao W, Tada S, Taminato T, Wong NCW, Takahara J, Ishida T. Expression of Human Scavenger Receptor B1 on and in Human Platelets. 2003:898-905. doi:10.1161/01.ATV.0000067429.46333.7B.
23. Murphy AJ, Bijl N, Yvan-Charvet L, Welch CB, Bhagwat N, Reheman A, Wang Y, Shaw JA, Levine RL, Ni H, Tall AR, Wang N. Cholesterol efflux in megakaryocyte progenitors suppresses platelet production and thrombocytosis. *Nat Med*. 2013;19(5):586-594. doi:10.1038/nm.3150.
24. Out R, Hoekstra M, Hildebrand RB, Kruit JK, Meurs I, Li Z, Kuipers F, Van Berkel TJC, Van Eck M. Macrophage ABCG1 deletion disrupts lipid homeostasis in alveolar macrophages and moderately influences atherosclerotic lesion development in LDL receptor-deficient mice. *Arterioscler Thromb Vasc Biol*. 2006;26(10):2295-2300. doi:10.1161/01.ATV.0000237629.29842.4c.
25. Eckly A, Strassel C, Cazenave J-P, Lanza F, Leon C, Gachet C. Characterization of megakaryocyte development in the native bone marrow environment. *Methods Mol Biol*. 2012;788:175-192. doi:10.1007/978-1-61779-307-3_13.
26. Thacker SG, Rousset X, Esmail S, Zarzour A, Jin X, Collins HL, Sampson M, Stonik J, Demosky S, Malide DA, Freeman L, Vaisman BL, Kruth HS, Adelman SJ, Remaley AT. Increased plasma cholesterol esterification by LCAT reduces diet-induced atherosclerosis in SR-BI knockout mice. *J Lipid Res*. 2015;56(7):1282-1295. doi:10.1194/jlr.M048629.
27. Dalal KB, Leven RM, Yee T. Lipid and membrane fluidity abnormalities in platelets and megakaryocytes of the hereditary macrothrombocytopenic Wistar Furth rat. *Biochim Biophys Acta*. 1991;1070(1):253-258.
28. Eckly A, Heijnen H, Pertuy F, Geerts W, Proamer F, Rinckel J-Y, Leon C, Lanza F, Gachet C. Biogenesis of the demarcation membrane system (DMS) in megakaryocytes. *Blood*. 2014;123(6):921-930. doi:10.1182/blood-2013-03-492330.
29. Machlus KR, Italiano JE. The incredible journey: From megakaryocyte development to platelet formation. *J Cell Biol*. 2013;201(6):785-796. doi:10.1083/jcb.201304054.
30. Quinn PJ. The fluidity of cell membranes and its regulation. *Prog Biophys Mol Biol*. 1981;38(1):1-104.
31. Suda T, Shimizu D, Maeda N, Shiga T. Decreased viscosity of human erythrocyte suspension induced by chlorpromazine and isoxsuprine. *Biochem Pharmacol*. 1981;30(15):2057-2064.

32. Meurs I, Hoekstra M, van Wanrooij EJA, Hildebrand RB, Kuiper J, Kuipers F, Hardeman MR, Van Berkel TJC, Van Eck M. HDL cholesterol levels are an important factor for determining the lifespan of erythrocytes. *Exp Hematol*. 2005;33(11):1309-1319. doi:10.1016/j.exphem.2005.07.004.
33. Martin JF, Slater DN, Kishk YT, Trowbridge EA. Platelet and megakaryocyte changes in cholesterol-induced experimental atherosclerosis. *Arteriosclerosis*. 1985;5(6):604-612.
34. Schick BP, Schick PK. The effect of hypercholesterolemia on guinea pig platelets, erythrocytes and megakaryocytes. *Biochim Biophys Acta*. 1985;833(2):291-302. http://www.ncbi.nlm.nih.gov/pubmed/3970955.
35. Pathansali R, Smith N, Bath P. Altered megakaryocyte–platelet haemostatic axis in hypercholesterolaemia. *Platelets*. 2001;12(5):292-297. doi:10.1080/09537100120058810.
36. Schick BP, Schick PK. Cholesterol exchange in platelets, erythrocytes and megakaryocytes. *Biochim Biophys Acta*. 1985;833(2):281-290.

SUPPLEMENTARY DATA



Supplemental figure 1: Total body SR-BI deficiency is associated with hypercholesterolemia and thrombocytopenia. Plasma UC (A) and TC (B) levels, the plasma UC:TC ratio (C), and blood platelet concentrations (D) in wild-type (WT) and SR-BI knockout (SR-BI KO) mice. Data represent means+SEM of 4 mice per group. ****p<0.0001.