Thrombocytopenia and Platelet Abnormalities in High-Density Lipoprotein Receptor–Deficient Mice

Vandana S. Dole, Jana Matuskova, Eliza Vasile, Ayce Yesilaltay, Wolfgang Bergmeier, Michael Bernimoulin, Denis A. Wagner, Monty Krieger

Objective—High-density lipoprotein (HDL) receptor, scavenger receptor class B, type I (SR-BI), mediates cellular uptake of lipoprotein cholesterol controls HDL structure and plasma HDL and biliary cholesterol levels. In SR-BI knockout (KO) mice, an unusually high plasma unesterified-to-total cholesterol ratio (UC/TC) and abnormally large HDL particles apparently contribute to pathology, including female infertility, susceptibility to atherosclerosis and coronary heart disease, and anemia. Here we examined the influence of SR-BI deficiency on platelets.

Methods and Results—The high plasma UC:TC ratio in SR-BI KO mice was correlated with platelet abnormalities, including high cholesterol content, abnormal morphologies, high clearance rates, and thrombocytopenia. One day after platelets from wild-type mice were infused into SR-BI KO mice, they exhibited abnormally high cholesterol content and clearance rates similar to those of endogenous platelets. Platelets from SR-BI KO mice exhibited in vitro a blunted aggregation response to the agonist ADP but a normal response to PAR4.


Key Words: HDL receptor  ■  SR-BI  ■  platelet  ■  thrombocytopenia  ■  clearance

The high-density lipoprotein (HDL) receptor scavenger receptor class B type I (SR-BI) mediates cellular uptake of lipids, especially cholesteryl esters, from lipoproteins and the bidirectional flux of unesterified cholesterol between cells and lipoprotein (see reviews2,3). Hepatic SR-BI controls plasma lipoprotein metabolism, biliary cholesterol secretion, and the structure and composition of plasma HDL particles. The cytoplasmic, SR-BI–binding adaptor protein PDZK1 plays a key role in tissue-specific posttranscriptional control of hepatic SR-BI protein expression.4 SR-BI deficiency in homozygous null knockout (SR-BI KO) mice results in hypercholesterolemia (≈2.2-fold increase in total plasma cholesterol, much of which is carried in abnormally large HDL particles).5 The ratio of plasma unesterified cholesterol (UC)-to-total cholesterol (TC, unesterified plus esterified cholesterol) in SR-BI KO mice is about double that in WT mice.6,7 SR-BI KO mice exhibit enhanced susceptibility to atherosclerosis,8 female infertility,9,10 and red blood cell (RBC) defects.10,11 (see review2). In SR-BI KO mice, essentially liver-specific expression of an SR-BI transgene (KO-Tg) restores plasma cholesterol levels (TC and UC:TC ratio) and the structure of HDL to those seen in WT mice and corrects several SR-BI deficiency–related pathologies.12

In the current study, we examined the effects of SR-BI deficiency on platelets, which in WT mice circulate in the blood with a lifespan of ≈5 days13 followed by clearance via the reticuloendothelial system.14 We found that SR-BI KO mice are thrombocytopenic (reduced platelet count). Their platelets exhibited abnormal morphologies, abnormally high unesterified cholesterol, elevated rates of clearance from the circulation, and a minor defect in ADP-induced aggregation. These abnormalities appear to be a consequence of the high UC:TC ratio.

Materials and Methods

Four-week- to 6-month-old male and female mice fed standard laboratory chow were used with appropriate institutional approvals. Genotypes (determined by polymerase chain reaction [PCR]): SR-BI+/+ (WT), SR-BI−/−, SR-BI−/+ (SR-BI KO), and SR-BI−/+ expressing an essentially liver-specific SR-BI transgene (KO-Tg); the SR-BI transgene is not expressed in bone marrow monocytes/granulocytes (A.Y., M.K., Ching-Hung Shen, and Jianzhu Chen, unpublished results, 2007), all on mixed 50:50 C57BL/6:129-S4 backgrounds.6,12 The 50:50 ratio represents the average percent of genes contributed by the indicated pure background (eg, C57BL/6); however, animal-to-animal variation in the source of any particular gene (C57BL/6 or 129-S4) is possible. It was necessary to use SR-BI KO animals (and their wild-type controls) on mixed genetic backgrounds because we
were unable to generate sufficient numbers of viable SR-BI KO mice on a pure C57BL/6 background using standard husbandry conditions (unpublished). Thus, unless indicated otherwise, WT refers to control mice on the mixed 50:50 C57BL/6:129-S4 background. We also studied PDZK1−/− (on either a mixed 75:25 129Sv:C57Bl/6 or pure 129Sv background, generously provided by Olivier Kocher) and appropriate wild-type controls (50:50 129Sv:C57Bl/6 or pure 129Sv background), SR-BI−/−/apoE−/− (dKO, on a mixed 75:25 C57Bl/6:129Sv background, WT controls),5,15 and apoE−/− and SR-BI−/−/apoE−/− mice (on a pure C57Bl/6 background, Jackson Laboratory, Bar Harbor, Me). All procedures (eg, platelet labeling and determination of platelet lifetimes) were performed as described previously or in the online Data Supplement (see http://atvb.ahajournals.org).

Results

SR-BI Deficiency Causes Thrombocytopenia

Figure 1A shows that there was no difference in the blood platelet count between wild-type (WT, also SR-BI+/+) and heterozygous null SR-BI (SR-BI+/−) mice, whereas there was a substantial reduction (38% of WT) in homozygous null (SR-BI−/−, SR-BI KO or simply KO) mice. This thrombocytopenia suggested an abnormally high rate of platelet clearance or an abnormally low rate of platelet production, or both.

Mechanism Underlying Thrombocytopenia in SR-BI KO Mice

To determine if reduced platelet survival times contributed to the thrombocytopenia, we biotinylated platelets in vivo and determined the percentage of surviving labeled platelets as a function of time. Figure 1B shows that there was a dramatically reduced half-life of platelets in SR-BI KO mice (~27 hours) compared to WT mice (~46 hours). Reduced platelet production did not appear to contribute to the thrombocytopenia, because in SR-BI KO mice there was no decrease in either the number of young platelets in circulation or the number of splenic and bone marrow megakaryocytes. Indeed, there was an increase in splenic megakaryocytes (see supplemental material). These results suggest that thrombocytopenia was likely attributable to abnormally rapid platelet clearance.

Mechanism Underlying the Reduced Lifespan of Circulating Platelets in SR-BI KO Mice

Next we determined if the reduced lifespan of platelets in SR-BI KO mice were a platelet cell autonomous property or if it was primarily dependent on the external environment of the platelets (ie, plasma conditions). We isolated platelets from either WT or SR-BI KO donor mice, labeled them ex vivo with biotin, infused the labeled platelets into recipient WT or SR-BI KO mice, and then determined clearance rates. Figure 2 shows the differences in the survival times of the platelets were primarily dependent on the genotype of the recipients, not the donors. Platelets from either WT or SR-BI KO mice exhibited almost identical, short (~20 hour) lifetimes when infused into SR-BI KO recipients (squares), whereas the lifetimes for platelets from either donor were similar and longer (~43 hour) in WT recipients (diamonds). For the WT recipients, there was a small, but reproducibly, longer lifetime observed for platelets from SR-BI KO donors than from WT donors. It is not clear whether the somewhat larger fraction of “younger” platelets from SR-BI KO mice...
(supplemental Figure IC) contributed to this somewhat longer survival. Control experiments (supplemental Materials) showed that severe thrombocytopenia does not per se reduce the half-life of infused platelets. Taken together these data indicate that the short lifespan of platelets in SR-BI KO mice, and their associated thrombocytopenia, appears to be a consequence primarily of the extracellular environment of the platelets rather than a platelet intrinsic phenomenon.

Mechanism by Which the Extracellular Environment Controls Murine Platelet Survival Time

The most obvious difference in the environments of the platelets in WT and SR-BI KO mice that might influence platelet survival is the characteristic dyslipidemia in SR-BI KO mice [eg, high TC and UC:TC ratio].

Figure 3A shows that, in all of the recipient mice regardless of genotype, the survival time (compare Figures 2 and 4), suggesting that accumulation of excess unesterified cholesterol in the platelets may have contributed to their more rapid clearance from the circulation.

Role of Total and Unesterified Plasma Lipoprotein Cholesterol on Platelet Composition and Metabolism

Hypercholesterolemia per se does not appear to be responsible for platelet abnormalities in SR-BI KO mice. For example, the platelet counts for wild-type control and apoE knockout (apoE−/−) mice, both on a pure C57Bl/6 background, did not differ (6.89±0.31x10^8 versus 6.46±0.42x10^8 platelets/mL, respectively, P>0.05), despite severe hypercholesterolemia in apoE−/− mice (450±11 mg/dL versus 230±9 mg/dL for SR-BI KO; also see5,6,17,18). The filipin staining of the infused platelets (white bars) was virtually identical to that of the resident platelets (gray bars). There was relatively low staining in WT recipients (Figure 4, left) and high staining in SR-BI KO recipients (Figure 4, right), regardless of the genotype of the donor animals. Thus, in the circulation platelets relatively rapidly acquire from or release to lipoproteins or cells, unesterified cholesterol. The extent of filipin staining was inversely correlated with the survival time (compare Figures 2 and 4), suggesting that accumulation of excess unesterified cholesterol in the platelets may have contributed to their more rapid clearance from the circulation.
UC:TC ratio in apoE/H11002 mice is essentially identical to that of WT mice (about half that in SR-BI KO mice), raising the possibility that a high UC:TC ratio may alter platelet structure and metabolism.

To explore this possibility, we examined platelets from 3 additional mouse strains with varying UC:TC ratios (Figure 5A): KO-Tg mice (normocholesterolemic, UC:TC=0.21 [similar to the ratio of 0.23 for WT, also see12]; PDZK1/H11002/H11002 mice (hatched bars), and SR-BI KO/apoE/H11002 double knockout mice (dKO, gray bars) and plasma unesterified-to-total cholesterol ratios (UC:TC; A, n=7 to 18), platelet cholesterol contents (B, filipin staining, n=7), and platelet counts (C, n=7 to 13) were determined (**P<0.001).

KO-Tg, PDZK1/H11002/H11002 and WT mice (all on mixed C57BL/6:129 genetic backgrounds) were not different (P>0.05). Similarly, there was no significant difference for PDZK1/H11002/H11002 and matched wild-type control mice on a pure Sv 129 genetic background ([1.17±0.08]×10⁶ and [1.35±0.07]×10⁶ respectively, n=3, P>0.05). In contrast, the count for SR-BI/apoE dKO mice ([1.09±0.10]×10⁶ platelets/mL) was 3.2-fold less than that of SR-BI single KO mice (Figure 5C, P<0.001). The platelet survival curves for KO-Tg and PDZK1/H11002/H11002 mice were similar to that of WT mice (see supplemental Figure IV).

These data show a strong correlation of platelet counts, platelet unesterified cholesterol levels, and platelet survival times with the plasma lipoprotein UC:TC ratios and are consistent with the hypothesis that the abnormal UC:TC ratio in SR-BI KO mice is responsible, at least in part, for their thrombocytopenia.

Analysis of Platelet Function In Vitro

The kinetics and extent of aggregation of platelet-rich plasma from WT and SR-BI KO mice were similar after stimulation by the thrombin receptor agonist PAR4 peptide (1 mmol/L, A) or ADP (1 μmol/L, B) was measured as increased light transmission in an aggregometer as described in Methods. Aggregation traces from 1 of 3 independent experiments are shown.

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Analysis of Platelet Function In Vitro

The kinetics and extent of aggregation of platelet-rich plasma from WT and SR-BI KO mice were similar after stimulation by the thrombin receptor agonist PAR4 peptide (1 mmol/L) (Figure 6A). However, abnormally low aggregation was reproducibly observed in SR-BI KO platelets stimulated with either 1 μmol/L (Figure 6B) or 5 μmol/L (supplemental Figure V) of the weak agonist ADP, although the extent of reduction varied in different experiments (~30% to 100% reduction). Thus, SR-BI deficiency also led to a modest defect in in vitro platelet function.

Discussion

A distinctive feature of the SR-BI KO mice is that they are hypercholesterolemic with abnormally large circulating HDL particles and an abnormally high unesterified-to-total plasma cholesterol ratio (UC:TC).5–7 In the current study we found that murine hypercholesterolemia per se (eg, in apoE/H11002/H11002 mice) does not induce thrombocytopenia. However, our analysis of SR-BI KO and other related transgenic animals shows a
correlation of an abnormally high plasma UC:TC ratio with abnormally high platelet cholesterol content, rapid platelet clearance, and thrombocytopenia. We also found that 1 day after platelets from either SR-BI KO or WT donor mice were infused into recipient mice, the cholesterol content of the infused platelets, as well as the platelet clearance rates, mirror those of the resident endogenous platelets (either WT or SR-BI KO recipients), regardless of the genotype of the donor. We conclude that the high UC:TC ratio in the plasma lipoproteins of SR-BI KO mice is probably responsible for the rapid platelet clearance and thrombocytopenia. Although it is possible that the high plasma UC:TC ratio, independently of its effects on cholesterol accumulation in platelets, induces increased activity of the reticuloendothelial system responsible for platelet clearance, it seems likely that the accumulation of high unesterified cholesterol in the platelets themselves is the principle cause of their rapid clearance.

Platelets can acquire cholesterol from plasma through exchange with plasma lipoproteins.20,22 Several dyslipidemic states, such as spur cell anemia,22 sitosterolemia,23,24 acanthocytosis,21 and Tangier disease25,26 are associated with concurrent defects in platelets and RBCs. Hyperlipidemia and dyslipidemia have been linked with reduced platelet survival time and thrombocytopenia (eg, inverse correlation of plasma total cholesterol and platelet survival time),23,27,28 although the mechanism by which circulating lipids induce thrombocytopenia remains uncertain. Additionally, several cholesterol-independent factors,29–31 including shedding32 or clustering33 of cell surface GP1bα, are known to influence platelet lifespan. Elevated shedding of GP1bα in SR-BI KO mice is unlikely to cause thrombocytopenia, because GP1bα levels on their platelets were ≈1.44±0.04-fold higher than those from WT mice (see supplemental material).

There are striking similarities of the influence of SR-BI deficiency on platelets (this study) and RBCs30,10,11 with respect to cellular composition and morphology (eg, excess cholesterol), clearance rate (accelerated), and steady state levels in the blood (thrombocytopenia and anemia). These similarities are particularly noteworthy given the substantial differences in the mechanisms governing the production and clearance of these 2 blood cell types.13,29,34–36 It is possible that neither RBCs or their immediate precursors nor platelets have adequate capacity to process the large amounts of intracellular unesterified cholesterol that accumulate in SR-BI KO mice. These cells might not be able either to effectively convert the excess cholesterol to cholesteryl esters for storage or to maintain sufficient levels of cholesteryl ester stores in stable cytoplasmic lipid droplets. Indeed, almost all of the cholesterol in platelets is normally unesterified cholesterol.38 As a consequence, these cells might be especially susceptible to toxic effects of excess unesterified cholesterol accumulation.

Because of the cholesterol accumulation and morphological abnormalities in the platelets in SR-BI KO mice, it was not surprising to find that these platelets exhibited a subtle, but reproducible, functional defect in vitro—reduced responsiveness to the agonist ADP in aggregation assays. It is noteworthy that Shattil and colleagues have shown that in vitro loading of platelets with unesterified cholesterol can increase intracellular cAMP levels.39 Although increased intracellular cAMP has been shown to reduce platelet sensitivity to ADP,40 Shattil et al and others reported increased sensitivity to ADP and epinephrine of platelets loaded with cholesterol in vitro,41 as well as platelets from hypercholesterolemic patients.42,43 Furthermore, treatment of hypercholesterolemic patients with lipid lowering therapy has been reported to reduce response of platelets to ADP.27,44 The effects of cholesterol loading of platelets, whether in vitro or in vivo, are clearly complex, possibly depending on the precise mechanism of loading (eg, during or after platelet formation from megakaryocytes), and other features of lipoprotein metabolism (eg, elevated plasma apoE,5,44,45) also may impact platelets in SR-BI KO mice. The abnormal response of SR-BI KO platelet aggregation to ADP and normal response to the PAR4 peptide were observed in all experiments and clearly show that many key signaling and response pathways in these cells remained intact. Because the mice are on mixed genetic background, some of the observed variation in relative responses to ADP (30% to 100% reduction in induced aggregation) may have been attributable to the mixed genetic backgrounds. The mechanism underlying the reduced responsiveness to ADP and physiological consequences, if any, remains to be determined.

The pathophysiologic consequences, if any, of the platelet abnormalities in SR-BI KO mice remain to be identified. We and others have shown that SR-BI is atheroprotective in a variety of murine models of atherosclerosis (eg, enhanced atherosclerosis in mice with homozygous null mutations in the SR-BI gene8 (reviewed in2,3). Platelets have been shown to contribute substantially to atherosclerosis,6,47 and unesterified cholesterol in RBCs (and possibly platelets) may contribute to cholesterol deposition in atherosclerosis.48 Thus, it is possible that the platelet abnormalities in SR-BI KO mice described here may contribute to the enhanced atherosclerosis observed in SR-BI–deficient mice.

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Disclosures
None.

References


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Supplemental materials

Methods

Blood collection and preparation of washed platelets

Mice were anesthetized with Isofluorane and blood was collected by retro-orbital venous plexus sampling into polypropylene tubes containing heparin (approximate final concentration of 7.5 U/ml). Platelet-rich plasma (PRP) was prepared by centrifugation at 200xg for 5 minutes. The plasma and the buffy coat were gently transferred to a fresh tube, and the centrifugation was repeated (200xg, 2 minutes). The PRP was incubated for 2 minutes with prostacyclin PGI$_2$ (0.1 µg/mL) and platelets isolated by centrifugation (850xg, 4 minutes). The platelet pellet was washed twice with modified Tyrode-HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) buffer (137 mM NaCl, 0.3 mM Na$_2$HPO$_4$, 2 mM KCL, 12 mM NaHCO$_3$, 5 mM HEPES, 5 mM glucose, pH 7.4) containing 0.35% bovine serum albumin (BSA) with PGI$_2$ (0.1 µg/mL) and resuspended in modified Tyrodes-HEPES buffer.

In vivo biotinylation

Biotin-NHS (16 µg/g body weight)$^1$ was slowly infused intravenously into mice to biotinylate platelets in vivo. Blood was collected each day, and labeled platelets were analyzed by flow cytometry as described below.
In vivo survival studies of ex vivo labeled platelets

Washed platelets were isolated as described above and biotinylated with 0.1 mM biotin-NHS for 10 minutes in modified Tyrodes-HEPES buffer at 37°C. Approximately, $10^8$ platelets were infused into recipient mice that were 4-8 weeks old. Platelets were analyzed by flow cytometry as described below.

Flow cytometry

The percentage of biotinylated platelets in an individual blood sample was determined using two-color flow cytometry. Whole blood (1 µl) collected as described above was incubated for 10 minutes with 5 µl of platelet specific rat anti-mouse GPIIbIIIa-[FITC (fluorescein isothiocyanate) or APC (allophycocyanin)] antibody (MoAb Leo.D2 from Emfret, Germany)$^2$ and 10 µl of phycoerytherin conjugated streptavidin$^3$ (PE-SA, 10 µg/ml; BD Biosciences, Franklin Lakes, NJ) to stain biotinylated platelets. Fluorescently labeled beads ($10^5$, Spherotech, Illinois) and 1 ml PBS were added to the stained platelet samples to determine absolute platelet counts. Flow cytometric analysis of the relative and absolute amounts of biotinylated and unlabeled platelets was performed on a Becton-Dickinson FACS LSR II (BD Biosciences). Where indicated, platelets in whole blood pre-stained with GPIIbIIIa were further incubated for 15 minutes with filipin (50 µg/ml in PBS, Sigma, St Louis, MO)$^4$ or thiazole orange (100 ng/ml in PBS, Sigma, St Louis, MO)$^5$ followed immediately by flow cytometric analysis. The forward light scatter, a relative measure of cell size, was also determined by flow cytometry.

Electron Microscopy

Supplemental Material
Platelets separated from plasma (washed platelets) from WT and KO mice (three animals each) were fixed with 2.5% glutaraldehyde and 2.5% formaldehyde in 0.1 M sodium cacodylate-HCl, pH 7.2, for 60–90 minutes at 4° C. The samples were then postfixed with 2% osmium tetroxide for 60 minutes at 4° C, followed by dehydration in graded alcohol up to 70% and en bloc staining with 0.2% uranyl acetate in 70% alcohol (60 minutes, 4°C). The samples were then processed for epon embedding. Thin epon sections (~40 nm) were cut with a diamond knife and post-stained with uranyl acetate and Reynold’s lead citrate and viewed with a FEI -Tecnai™ G² Spirit BioTWIN microscope operated at 80 kV. Digital images were taken with an AMT 2k CCD camera. Multiple fields were imaged at low magnification (4800x) and high magnification (11,000x and 13,000x) for each sample. The total areas of more than 100 platelets were measured on low magnification fields for each sample using ImageJ and OpenLab software, and the mean area and standard deviation were calculated for the pooled data from the three mice in each group.

**Histology**

Mice were anesthetized with 1.25% tribromoethanol infused intraperitoneally. Spleens and livers were excised and immediately fixed in 10% neutral buffered formalin. The bones (tibia) were fixed for 1 week in Bouin’s fixative followed by decalcification prior to paraffin embedding. Sections (4 µm thick) were stained with Harris Hematoxylin & Eosin-Phloxine (Polyscientific) and the density of megakaryocytes (cells/mm²) was determined in randomly selected sections by microscopic examination.
Analysis of plasma cholesterol

Total cholesterol and unesterified cholesterol levels in plasma samples were determined using commercial kits (Wako Chemical USA, Inc., Richmond, VA) or as described previously.  

Aggregometry

Mice were bled and platelet rich plasma (PRP) was prepared as described above. PRP samples from multiple mice of the same genotype were pooled. Platelet poor plasma (PPP) was prepared by further spinning an aliquot of PRP at 850xg, 4 minutes. PRP was adjusted to approximately 2-3 x 10^8 platelets per ml with PPP of the same genotype. The pooled PRP preparation was then split to study ADP and PAR4 mediated aggregation. Platelet rich plasma (100 µL) was then mixed with 160 µl of modified Tyrode buffer containing 1 mM CaCl_2 in a pre-warmed (37 °C) aggregometer cuvette, and stirred while light transmission through the plasma was measured on a Chrono-log 4-channel optical aggregation system (Chrono-log Corp.). After baseline measurements were obtained, platelet agonists (ADP or PAR4 peptide) were added at the indicated concentrations and transmission was recorded for at least 6 minutes.  

Statistics

All values are reported as mean ± SEM. Statistical significance was calculated using the two-tailed unpaired Student’s t-Test or one-way ANOVA with Tukey's multiple comparisons post-test where appropriate. P<0.05 (*) was considered significant.
Supplemental Results

Megakaryocyte density and reticulated platelets: surrogate markers of platelet production rates

Platelets are produced by budding from megakaryocyte precursors primarily in the bone marrow and spleen. Younger, or more recently generated circulating platelets are reticulated and are characterized by staining with the dye thiazole orange (a marker of reticulated, ‘younger’ platelets). To explore the possible role of altered platelet production contributing to the thrombocytopenia in SR-BI KO mice, we compared the megakaryocyte densities in the bone marrow, spleen and liver of WT and SR-BI KO mice as a surrogate marker for platelet production capacity. Supplemental Figure IA shows that there was no difference in the megakaryocyte density (cells/mm²) in the bone marrow of WT and SR-BI KO mice, but that in the spleens there was a significant, 1.86-fold, higher density in SR-BI KO mice (14.15±1.59 vs 7.62±1.88 cells/mm², P<0.05). It is noteworthy that the spleens of SR-BI KO mice, which also exhibit increased erythropoiesis, are significantly larger (1.6 times) than those of WT mice (and our unpublished observations). Thus, the number of splenic megakaryocytes in SR-BI KO mice may be as much as 3-fold greater than in WT mice. We found no megakaryocytes in the livers of WT and SR-BI KO mice. These results suggest that the abnormally low numbers of circulating platelets in SR-BI KO mice are not due to lower numbers of megakaryocytes and unlikely to be caused by a decrease in platelet production. Rather, increases in splenic megakaryocyte density suggests that in SR-BI KO mice there may be slightly increased megakaryopoiesis and platelet production, which is a typical compensatory process observed in many cases of thrombocytopenia.
This conclusion was supported by analysis of the absolute and relative numbers of ‘younger’ reticulated, thiazole orange-positive platelets in WT and SR-BI KO mice. Supplemental Figure IB shows that the number of platelets/ml that stained with the dye thiazole orange (a marker of reticulated, ‘younger’ platelets)\textsuperscript{5,12} was virtually identical in WT and SR-BI KO mice. Because of the lower total platelet count in SR-BI KO mice, the percentage of platelets that are ‘younger’ was significantly greater (2.25-fold) in the SR-BI KO mice (Supplemental Figure IC). If in WT and SR-BI KO mice the rates of production of younger platelets from megakaryocytes and clearance of younger platelets were similar, then it would be likely that these mice would have similar rates of platelet production. However, it is possible that the rate of clearance of younger platelets in SR-BI KO mice is higher than in WT mice (as is the case for the whole platelet population). Given apparently greater number of splenic megakaryocytes in SR-BI KO mice (see above), it seems possible that platelet production in SR-BI KO mice exceeds that in WT mice. Taken together, these data suggest that decreased platelet production is unlikely to account for the thrombocytopenia in SR-BI KO mice.

\textbf{Role of platelet pool size on platelet clearance rates.} Having observed faster rates of clearance of ex vivo biotinylated platelets from both WT and SR-BI KO donors in SR-BI KO recipients relative to those in WT recipient (Figure 2), we were concerned that the differences in endogenous platelet pool sizes in WT and SR-BI KO mice per se (greater in WT mice) might have contributed to the observed, abnormally short, half-lives of platelets in SR-BI KO mice. For example, if a rate controlling step in clearance involved
a process that was saturable with respect to the platelet count (e.g., a process dependent on a limited number of receptors for those platelets to be cleared), then the observed rate of clearance for platelets infused into mice might depend on the endogenous platelet pool size. A large endogenous pool might compete for clearance with the infused platelets and lead to an apparently low clearance rate for the infused platelets, whereas a very low endogenous pool (thrombocytopenia) would provide less competition and result in an apparently higher clearance rate for the infused platelets.

To address the general question of whether or not endogenous platelet pool size can substantially influence the clearance rate of labeled infused platelets, we performed a control experiment in transgenic mice in which the platelet pool could be either left unaltered or specifically and dramatically immunodepleted prior to infusion of the biotinylated donor platelets. GP1bα/IL4R transgenic mice (provided by J. Ware) are ideally suited for such an experiment. GP1bα/IL4R transgenic mice express in platelets, a transgene encoding a chimeric GP1bα protein in which the extracellular domain of the platelet surface protein GP1bα has been replaced with a portion of the IL-4 receptor (IL-4R). Infusion of anti-IL-4R antibody in GP1bα/IL4R transgenic mice induces profound thrombocytopenia within 30 minutes of injection. Therefore, on day 0 we intravenously infused GP1bα/IL4R transgenic mice with either 2 μg/g mouse weight of anti-IL-4R antibody (to immunodeplete the platelets) or an IgG2a isotype control antibody (R&D Systems, Minneapolis, MN). Thirty minutes later, the mice were infused with in vitro biotinylated platelets isolated from WT mice that are not recognized by the anti-IL-4R antibody. The numbers of circulating biotinylated platelets were then determined.
immediately and then daily during the subsequent four days. In addition, the platelet counts for the endogenous, non-biotinylated GP1bα/IL4R platelets were also determined daily and found throughout the entire experiment to be ~1% of the value for the mice injected with the control antibody. On day 3, the mice were re-infused with anti-IL-4R antibody or IgG2a isotype control to ensure a continued thrombocytopenic state. Supplemental Figure II shows that the severe thrombocytopenia induced by immunodepletion did not reduce the platelet half-life, indicating that thrombocytopenia per se does not cause an increase in observed platelet clearance rates. Indeed, the clearance rate appeared to be slightly slower for those mice with severe thrombocytopenia, although the mechanism underlying this observation is unknown.
Supplement References:


Supplemental Figure Legends

Supplemental Figure I. Effects of SR-BI deficiency on tissue megakaryocytes and levels of reticulate, ‘young’ platelets.

A. Bone and spleen samples harvested from wild-type (black bars) and SR-BI KO (white bars) mice were processed for histological analysis as described under Supplemental Methods. Megakaryocytes were identified by size and multinucleated morphology, and their densities (cells per mm² tissue) were determined (n=9-11 animals/group, * P<0.05). No megakaryopoiesis was seen in similarly-processed liver samples (not shown). B and C. Platelets in whole blood were stained with anti-GPIIbIIIa antibody, and 100 ng/ml thiazole orange and the absolute amounts (B, n=8, P =0.34) and the percentage (C) of reticulated ‘young’ platelets (C, n=6-7; ** P<0.001) were determined by flow cytometry.

Supplemental Figure II. Effects of endogenous platelet pool size on platelet survival in a murine model of inducible thrombocytopenia.

On day 0, platelets from wild type (WT) mice were isolated, washed, biotinylated and intravenously infused into recipient GP1bα/IL4R transgenic mice that had been infused 30 minutes earlier with anti-IL4 antibody to deplete platelets (white circles) or an isotype control antibody (black circles). The percentage of labeled platelets remaining in the circulation relative to that on day 0 immediately after infusion (100% of control) was determined. Results represent the average of two independent experiments (n=4-5 mice). No significant difference in platelet clearance between the two groups was observed.
Supplemental Figure III. Effects of SR-BI deficiency on platelet size determined by light scattering.

Platelets in whole blood from WT (black bar) or SR-BI KO (white bar) mice were stained with anti-GPIIbIIIa antibody and the amount of forward light scattering, which increases with increasing cell size, was determined using flow cytometry. Platelets from SR-BI KO mice exhibited 1.28±0.04-fold greater forward light scattering than those from WT mice (n=6, **P<0.001). The apparent increase in cell size in platelets from SR-BI KO mice detected by forward light scattering was also reflected in an increase in per cell levels of several cell surface markers determined by flow cytometric analysis. The relative increases in cell surface expression in SR-BI KO vs WT platelets (n=4-7) for three such markers were: GPIbα, 1.44±0.04 (P<0.001); GPIIbIIIa, 1.48±0.09 (P<0.05); and CD9, 1.22±0.05(P<0.05).

Supplemental Figure IV. Effects of altering SR-BI and PDZK1 expression on platelet survival.

Wild type mice (WT, dashed line), SR-BI KO mice (KO, dotted line), SR-BI KO mice expressing a primarily liver specific SR-BI transgene (KO-Tg, circles), and PDZK1−/− mice (triangles) were intravenously infused with biotin to label platelets in vivo. Mice were then bled each day for five days and the fractions of platelets labeled with biotin in each sample were determined by flow cytometry (n=4-5).

Supplemental Figure V. Effects of platelet agonist ADP (5 µM) on platelet aggregation.
Platelet-rich plasma from WT (black line) and SR-BI KO (gray line) were isolated and platelet aggregation as a function of time after adding the agonists ADP (5 µM) was measured as increased light transmission in an aggregometer. Aggregation traces from one of two independent experiments are shown.
Supplemental Figure I. Effects of SR-BI deficiency on tissue megakaryocytes and levels of reticulate, ‘young’ platelets.

A. Bone and spleen samples harvested from wild-type (black bars) and SR-BI KO (white bars) mice were processed for histological analysis as described under Supplemental Methods. Megakaryocytes were identified by size and multinucleated morphology, and their densities (cells per mm² tissue) were determined (n=9-11 animals/group, * P<0.05). No megakaryopoiesis was seen in similarly-processed liver samples (not shown).  

B and C. Platelets in whole blood were stained with anti-GPIIbIIIa antibody, and 100 ng/ml thiazole orange and the absolute amounts (B, n=8,  P =0.34) and the percentage (C) of reticulated ‘young’ platelets (C, n=6-7; ** P<0.001) were determined by flow cytometry.
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