ABC transporters and scavenger receptor BI : important mediators of lipid metabolism and atherosclerosis
Meurs, I.

Chapter 8

HDL cholesterol levels are an important factor for determining the life-span of erythrocytes

Illiana Meurs[1], Menno Hoekstra[1], Eva J.A. van Wanrooij[1], Reeni B. Hildebrand[1], Johan Kuiper[1], Folkert Kuipers[2], Max R. Hardeman[3], Theo J.C. Van Berkel[1], and Miranda Van Eck[1]

1 Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, Gorlaeus Laboratories, Leiden University, Leiden, The Netherlands
2 Center for Liver, Digestive and Metabolic Disease, Department of Pediatrics, University Hospital Groningen, Groningen, The Netherlands
3 Laboratory for Clinical Hemorheology, Academic Medical Center, Amsterdam, The Netherlands

ABSTRACT

Objective- SR-BI is a multifunctional receptor that promotes the selective uptake of cholesteryl esters from HDL. Disruption of SR-BI in mice results in a dramatic increase in HDL cholesterol. Interestingly, mice lacking SR-BI also develop anaemia, as evidenced by the accumulation of reticulocytes in the circulation. The objective of the current study was to delineate the mechanism underlying the development of anaemia in absence of SR-BI.

Methods- The expression of important mediators of erythropoiesis as well as key enzymes in the degradation of erythrocytes were analysed using real-time PCR in SR-BI wild-type and SR-BI knockout mice. In addition, in vivo studies were performed using biotinylated erythrocytes to determine erythrocyte survival.

Results- The mRNA expression of TAL-1, GATA-1, FOG-1, erythropoietin receptor, and ferrochelatase, important mediators of erythropoiesis was increased in spleens of SR-BI-deficient mice. In addition, the relative amount of early Ter119^{high}CD71^{high} expressing erythroblasts was increased in SR-BI deficient spleens. Interestingly, also the expression of hemeoxygenase 1 and biliverdin reductase, enzymes involved in the degradation of erythrocytes was increased. Furthermore, an elevated amount of conjugated bilirubin, the breakdown product of hemoglobin, was found in bile. Using biotinylated erythrocytes, we show that the survival of erythrocytes was decreased in SR-BI deficient mice. Thus, the observed increased erythropoiesis in the SR-BI deficient mice is most likely a direct response to the reduced erythrocyte life-span. Finally, we show that the increased HDL cholesterol levels due to SR-BI deficiency, induce erythrocyte cholesterol:phospholipid ratios, resulting in decreased deformability and increased osmotic fragility, thereby providing an explanation for the observed reduced life-span.

Conclusion- SR-BI is not only essential for HDL cholesterol homeostasis and atherosclerosis susceptibility, but also for maintaining normal erythrocyte life-span.

INTRODUCTION

High-density lipoprotein (HDL) levels are inversely correlated with the risk for the development of atherosclerosis.[1] An important anti-atherogenic function of HDL includes its role in reverse cholesterol transport (RCT), a process in which HDL removes excess cholesterol from peripheral cells and transports it to the liver.[2] In addition, the interaction of HDL with the arterial wall directly protects against vascular oxidative stress and inflammation.[3] A prominent regulator of HDL metabolism is the scavenger receptor class B, type I (SR-BI), which mediates the selective uptake of cholesteryl esters from HDL without internalisation of the HDL particle.[4]

SR-BI deficiency results in highly increased plasma cholesterol levels[5] and is associated with an increased susceptibility to diet-induced atherosclerosis.[6,7] When cross-bred onto the apolipoprotein E (apoE) knockout background, SR-BI deficiency leads to early onset of occlusive atherosclerotic coronary disease, spontaneous myocardial infarctions, and premature death.[8,9] Recently, Holm et al.[10] demonstrated that erythrocytes from SR-BI-deficient mice were immature, had an irregular shape, and contained large autophagolysosomes. From these studies it was proposed that erythroid maturation was impaired in SR-BI deficient mice as a result of inhibited phagolysosome expulsion from reticulocytes in the presence of the markedly increased cellular cholesterol levels.

The erythrocyte membrane is designed for maximum plasticity and deformability, enabling the erythrocyte to survive in the microcirculation. 40% by weight of the erythrocyte membrane is composed of lipids, in which cholesterol (~16%) and phospholipids (~20%) are abundant.[11] As lipids exchange easily between plasma lipoproteins and the erythrocyte membrane by a continuous non-specific exchange mechanism[12], changes in lipid composition of the erythrocyte membrane easily occur in response to changes in serum cholesterol levels, which may alter the physical properties of the erythrocyte membrane. Altered erythrocyte membrane properties might in turn affect erythrocyte survival.

In this study, erythrocyte turnover was analysed in SR-BI-deficient mice and wild-type littermates. Interestingly, the life-span of erythrocytes in SR-BI-deficient mice with highly elevated HDL cholesterol levels was dramatically reduced. In response to the shortened life-span of the erythrocytes in SR-BI deficient mice, markers of erythropoiesis were enhanced. SR-BI is thus not only essential for HDL cholesterol homeostasis and atherosclerosis susceptibility, but also for maintaining normal erythrocyte life-span.

EXPERIMENTAL PROCEDURES

Animals

SR-BI-deficient mice were provided by Dr. M. Krieger (Massachusetts Institute of Technology, Boston, USA). Heterozygous SR-BI deficient (SR-BI +/-) mice were cross-bred to generate wild-type (SR-BI +/+) and homozygous SR-BI deficient (SR-BI -/-) progeny. The presence of the targeted and wild-type SR-BI alleles was assessed by PCR amplification of DNA extracted from tail biopsies (primers 5'-GAT-GGG-ACA-TGG-GAC-ACG-AAG-CCA-TTC-T-3' and 5'-TCT-GTC-TCC-GTC-TCC-TTC-AGG-TCC-TGA-3'). The mice were fed a

chow diet containing 4.3% (w/w) fat without added cholesterol (RM3, Hope Farms, Woerden, The Netherlands). Animal experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the national laws. All experimental protocols were approved by the Ethics Committee for Animal Experiments of the Leiden University.

Erythrocyte parameters

Blood (200μl) was collected in EDTA coated tubes (Sarstedt, Numbrecht, Germany) by tail bleeding of mice fasted overnight. Subsequently, the blood was analysed using a Sysmex XE-2100 hematology analyzer (Sysmex Corp. Kobe, Japan) for erythrocyte counts, mean cellular volume (MCV), erythrocyte distribution width-standard deviation (RDW-SD), hematocrit, hemoglobin (HGB), mean cellular hemoglobin (MCH), and the mean cellular hemoglobin concentration (MCHC).

Detection of reticulocytes

Blood was drawn by tail bleeding and incubated with a solution of 1% (w/v) new methylene blue, 0.9% (w/v) sodium chloride, and 1.6% (w/v) potassium oxalate (Reticulocyte stain, Sigma) according to manufacturer's instructions. After the incubation, a blood smear was made and the reticulocyte count was examined microscopically.

Erythrocyte turnover analysis

Erythrocytes of SR-BI +/+ and SR-BI -/- mice were biotinylated by tail vein injection of 3 mg EZ-Link Sulfo-NHS-biotin (Pierce; Rockford, III) dissolved in 0.2 ml PBS. After 4 hours circulation, 20μl blood was drawn from the tail vein for 100% biotinylated erythrocyte determination. Subsequently, 20μl blood was drawn weekly for turnover analysis. Erythrocytes were washed in 0.9% NaCl, labeled with phycoerythrin-conjugated streptavidin (1:500) (Molecular Probes, Eugene, Ore) in 0.9% NaCl, 2.5mmol/l $CaCl_2$, and analysed by flow cytometry.[13] Erythrocyte survival was determined by assessment of the number of biotinylated (streptavidin-labeled) erythrocytes relative to the starting level, which was 100% biotinylated.

Gall bladder bile bilirubin analysis

The biliary bilirubin concentrations were assessed using routine clinical procedures.

Iron Quantification in liver and spleen

Liver and spleen, stored in Zinc Formal Fixx (Shandon, U.K.), were frozen in Tissue Tek OCT (Sakura, Torrance, CA) and 10 μM cryostat sections were prepared. Perls Prussian Blue staining for trivalent iron was performed by incubation of the cryostat sections with a mixture of equal parts 2% potassium ferrocyanide and 2% hydrochloric acid for 20 minutes. The amount of trivalent iron in the sections was quantified using a light microscope connected to a full colour video camera and Leica Qwin image analysis software.

Analysis of gene expression by real-time quantitative PCR

Spleens and liver from SR-BI +/+ and SR-BI -/- mice were isolated after whole-body perfusion using phosphate-buffered saline via a cannula in the left ventricle of the heart,

frozen in liquid N_2, and stored at -80°C until RNA isolation. Total RNA was extracted from liver and spleen by the acid guandinium thiocyanate-phenol chloroform extraction method, according to Chomczynski and Sacchi.[14] cDNA was synthesized from 0.5-1µg of total RNA using RevertAid™ M-MuLV reverse transcriptase. Quantitative gene expression analysis was performed on an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) using SYBR-green technology, according to manufacturer's instructions. PCR primers (Table 1) were designed using Primer Express 1.7 software with the manufacturer's default settings (Applied Biosystem). The relative gene expression numbers of the target gene were calculated by subtracting the C_t of the target gene from the C_t of the housekeeping gene (36B4) and raising 2 to the power of this difference. C_t values are defined as the number of PCR cycles at which the fluorescent signal during the PCR reaches a fixed threshold.

Table 1. Primer for real-time PCR analysis

Gene	GenBank™ assession No.	Forward primer	Reverse primer	Amplicon size
GATA-1	NM_008089	TGGAATCCAGACGAGGAACC	CTCCAGCCAGATTCGACCC	71
GATA-2	NM_008090	CCCTAAGCAGAGAAGCAAGGC	TGGCACCACAGTTGACACACT	65
FOG-1	NM_009569	TCCCCTGAGAGAGAAGAACCG	GCAGCATCCTTAGCCAGCA	73
TAL-1	NM_011527	CTAGGCAGTGGGTTCTTTGGG	CCTCTTCACCCGGTTGTTGTT	69
EPOr	BC_046282	CAACAGCGGACACATCGAGTT	TAGCGAGGAGAACCGGACG	70
FECH	NM_007998	CCAGAAGCCCACCATGAAGT	TGCAAAGCACTGGATGAGGA	72
BLVRA	NM_026678	CTTGATGGAAGAGTTCGAATTCCT	GTGAAGCGAAGAGATCCTTTTAGC	75
SR-BI	U76205	GTTGGTCACCATGGGCCA	CGTAGCCCCACAGGATCTCA	65
36B4	NM_007475	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG	85
HO-1	NM_010442	CAACAGTGGCAGTGGGAATTTA	CCAGGCAAGATTCTCCCTTACC	107
UGT1A	NM_145079	CACCACACCTGCGCCC	CCAATCACATCCAAGGAGTGGTA	67

Abbreviations: GATA-1, globin transcription factor 1; GATA-2, globin transcription factor 2; FOG-1, friend of GATA-1; TAL-1, basic helix-loop-helix factor; EPOr, erythropoietin receptor; FECH, ferrochelatase; BLVRA, biliverdin reductase A; SR-BI, scavenger receptor class B1; 36B4, acidic ribosomal phosphoprotein PO; HO-1, hemeoxygenase 1; UGT1A, diphosphoglucuronate glucuronosyltransferase 1A.

Flow cytometric characterization of erythroid-lineage cells in spleen

Spleens from SR-BI +/+ and SR-BI -/- mice were isolated after whole-body perfusion using phosphate-buffered saline via a cannula in the left ventricle of the heart. Subsequently, single-cell suspensions were prepared by passing the spleen through a 70-µm nylon cell strainer (BD Bioscience Europe). Cells were first blocked in PBS/1% BSA for 1 hour. Subsequently, for each sample 3×10^5 cells were incubated with 0.25 µg phycoerythrin (PE) anti-mouse CD71 (eBioscience) and 0.25 µg fluorescein isothiocyanate (FITC) anti-mouse TER-119 (eBioscience) for 45 min at 4°C. Finally, cells were washed and re-suspended in PBS for flow cytometric analysis on a FACSCalibur flow cytometer.

Phosphatidyl serine expression on erythrocytes

Blood (30µl) was collected in EDTA coated tubes (Sarstedt, Numbrecht, Germany) by tail bleeding of mice fasted overnight. The erythrocytes were washed twice with PBS (pH 7.4). The erythrocyte pellet (10µl) was resuspended in 200µl incubation buffer, containing 10mM

Hepes, 145mM NaCl, 5mM KCl, 1mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.4, with 0.5µl Annexin V (Alexa, Molecular Probes). The samples were incubated for 15 minutes in the dark at 4°C and subsequently washed with incubation buffer and redissolved in incubation buffer. Labeled cells were enumerated and characterized on a FACS-Scan flow cytometer.

Lipid analysis

Serum concentrations of cholesterol, cholesteryl ester, and triglycerides were determined using enzymatic colorimetric assays (Roche Applied Science). The distribution of cholesterol over the different lipoproteins in serum was analysed by fractionation of 30µl of serum of each mouse using a Superose 6 column (3.2 x 30 mm, Smart system, Amersham Biosciences). Total cholesterol content of the effluent was determined using enzymatic colorimetric assays (Roche Applied Science).

Erythrocyte lipid analysis

Blood (150µl) was collected in EDTA coated tubes (Sarstedt, Numbrecht, Germany) by tail bleeding of mice fasted overnight. The erythrocytes were washed once with PBS and twice with 0.9% NaCl (pH 7.4). The washed and packed erythrocytes (30µl) were extracted with 100µl methanol and 100µl chloroform, respectively. The extracted supernatants were evaporated under nitrogen and redissolved in 30µl ethanol. Subsequently, the concentrations of cholesterol and phospholipids were determined using enzymatic colorimetric assays (Roche Applied Science).

Erythrocyte osmotic fragility

The osmotic fragility of erythrocytes was determined using solutions, with decreasing concentrations of NaCl. As the NaCl concentration decreases, the solution changes in isotonicity resulting in hemolyses of a fraction of the red blood cells and red coloration of the solution, which is measured spectrophotometrically at 550 nm. Different dilutions of the NaCl solution were prepared to provide the equivalent of 0.9, 0.65, 0.575, 0.55, 0.525, 0.50, and 0.0 g/l of NaCl. Total hemolysis was determined by exposure to distilled water. Five microliters of erythrocytes suspension was added to 500µl of each solution. After gently mixing by inverting the tubes several times, the solutions were allowed to stand at room temperature for 20 min. Subsequently, erythrocytes (remnants) were pelleted by centrifugation at 2000rpm for 10 min and the absorbance of the supernatants was determined at 550 nm. The percent hemolysis was plotted versus the NaCl concentration.

Erythrocyte deformability

Erythrocyte deformability was measured with a rotational viscometer (LORCA, Mechatronics, Zwaag, The Netherlands) as previously described.[15] Briefly, a sample of 10 mL from the erythrocyte suspensions was added to 2.0 mL of 0.14 mol/L polyvinylpyrolidone in PBS. After careful mixing, 1.0 mL of the erythrocyte suspension was transferred into the LORCA. At the start of the experiment, the rotation speed was reduced and then the cells were subjected automatically to varying shear stresses by increasing the rotational speed. Deformation was expressed by the elongation index (EI), derived from the resulting ellipsoid diffraction pattern. Finally, the deformation curve was obtained by plotting the calculated

values of EI versus the corresponding shear stress (Pa).

Statistical analyses

Statistical significant differences were determined with a two-tailed, unpaired Student's *t* test using GraphPad Instat software. P values of less than 0.05 were considered significant. Data are presented as mean ± SEM.

Table 2. Hematological data for SR-BI +/+ and SR-BI -/- mice

Mice	n	Erythrocytes	MCV	RDW-SD	Hematocrit	HGB	MCH	MCHC
		E12/L	fL	fL	%	mmol/L	fmol	mmol/L
SR-BI +/+	7	10.5 ± 0.1	49.8 ± 0.5	24.9 ± 0.6	51.3 ± 0.8	10.0 ± 0.3	0.98 ± 0.02	19.9 ± 0.1
SR-BI -/-	6	9.2 ± 0.4**	52.9 ± 0.5**	34.5 ± 1.2***	50.1 ± 0.8	9.4 ± 0.2	1.00 ± 0.01	18.9 ± 0.1***

Data represent means ± S.E. Statistically significance of ** $p < 0.01$ and *** $p < 0.001$ compared with control mice.

RESULTS

Effect of SR-BI deficiency on erythrocyte morphology

Hematological analysis showed that the amount of erythrocytes in blood of SR-BI deficient mice was decreased 12% as compared to wild-type controls ($p < 0.01$, Table II). Interestingly, the SR-BI -/- erythrocytes were abnormally large (MCV, $p < 0.01$) and more heterogeneous in size (RDW-SD, $p < 0.001$) compared with wild-type erythrocytes. No significant difference in hematocrit values was observed, as the decrease in the amount of erythrocytes was compensated by the increase in MCV in SR-BI deficient mice. In addition, SR-BI deficiency did not affect the hemoglobin content (HGB) and mean cellular hemoglobin content (MCH). However, SR-BI deficient mice did exhibit a small, but significant decrease in mean cellular haemoglobin concentration (MCHC, $p < 0.001$). In agreement with Holm et al.[10], blood smears of SR-BI deficient mice stained with new methylene blue for reticulocytes, immature erythrocytes[16], showed an increased amount of reticulocytes (Fig. 1). Quantification of the reticulocyte counts revealed that the amount of reticulocytes was increased ~4-fold ($p < 0.001$) from 265±36*10^9/L in SR-BI wildtypes to 1037±152*10^9/L in SR-BI deficient mice. Reticulocytes are immature anucleated erythrocytes that still contain mitochondria and ribosomes. During the last step of erythrocyte maturation these remaining organelles are removed by the spleen. Normally, the amount of reticulocytes in the circulation is low. The observed increased reticulocyte counts thus indicate impaired maturation or enhanced erythropoietic activity in the SR-BI deficient mice.
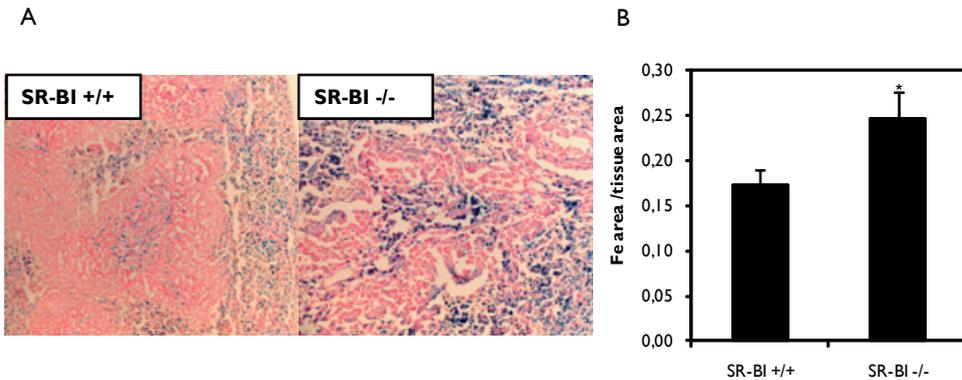
Effect of SR-BI deficiency on erythropoiesis

Hematopoietic compensation in mice takes largely place in the spleen rather than in the bone marrow.[17,18] Wild-type mice exhibited a spleen weight of 97.9 ± 8.5 mg. Interestingly, the spleen weight of SR-BI deficient mice was 1.6-fold increased ($p < 0.01$) to 154.4 ± 16.3 mg. In addition to the enlargement of the spleen also the iron content of the red pulp of spleens of SR-BI deficient mice was 1.4-fold increased ($p < 0.05$) (Fig. 2). Thus, the accumulation of erythrocytes in the spleen of SR-BI -/- mice might be the result of increased hematopoiesis. Therefore, the mRNA expression of genes involved in splenic erythropoiesis was determined

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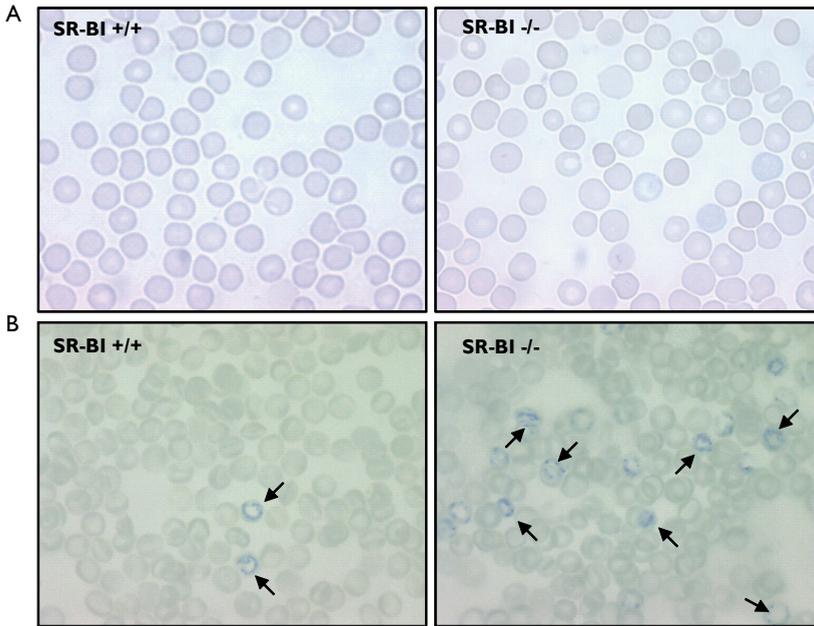
Figure 1. Effect of SR-BI deficiency on erythrocyte morphology.
Photomicrographs of blood smears of the SR-BI +/+ and SR-BI -/- mice stained with May-Grunwald-Giemsa (A) and for reticulocytes (arrows)(B). Original magnification x 100.

Figure 2. Effect of SR-BI deficiency on splenic iron content.
Iron content was determined in the spleens of SR-BI +/+ and SR-BI -/- mice using the leica image analysis system. A) Representative photomicrographs of Perls Prussian blue-stained cross sections of the spleen. Original magnification x10. B) Quantification of the mean iron content. Values represent the mean ± SEM of 5-6 mice. *, statistically significant difference of $p<0.05$ compared with the SR-BI +/+ mice.
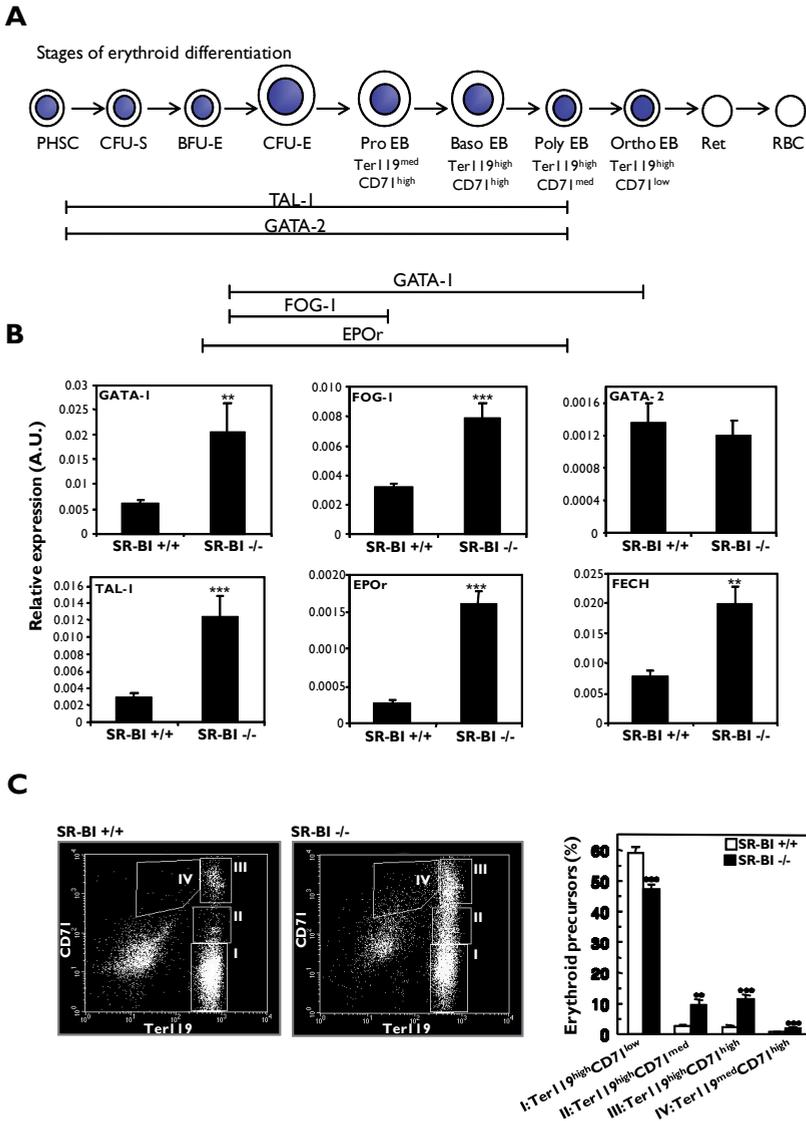
using quantitative real time PCR. The splenic SR-BI expression was determined to confirm the lack of SR-BI expression in SR-BI deficient mice. Indeed, a dramatic decrease in SR-BI expression was observed in SR-BI deficient mice compared to the wild-type mice (p<0.001, data not shown). Genes involved in the commitment of hematopoietic cells to the erythroid lineage are TAL-1 and GATA-2.[19] SR-BI deficiency led to a significant increase in TAL-1 (4-fold, p<0.01, Fig. 3), however, did not affect GATA-2 expression. Furthermore, SR-BI deficient mice exhibited a significant increase in GATA-1 (3-fold, p<0.01), and FOG-1 (2-fold, p<0.001), genes involved in the differentiation of committed erythroid progenitor cells.[19] The expression of the erythropoietin receptor (EPOr), involved in the proliferation and differentiation of the CFU-E and proerythroblasts, was induced (6-fold, p<0.001) in SR-BI deficient mice.[20,21] Furthermore, SR-BI deficiency resulted in a 3-fold (p<0.01) increase in mRNA expression levels of ferrochelatase (FECH), the rate-limiting enzyme for the synthesis of heme. These data indicate that the expression of key regulators in erythropoiesis is affected by SR-BI deficiency.

To determine whether the increased spleen size is indeed the result of expansion of erythropoietic tissue, erythroid precursor differentiation stages were analysed in spleens of SR-BI +/+ and SR-BI -/- mice by FACS analysed, as previously described by Socolovsky et al.[22] Classification is based on the expression of the cell-surface erythroid-specific Ter119 antigen, which is expressed by terminally differentiated erythroblasts and the transferrin receptor CD71. Ter119 is expressed at intermediate levels at the proerythroblast stage, while all erythroid precursors beyond the proerythroblast stage express Ter119 at high levels. Conversely, CD71 is expressed at very high levels by early erythroid precursors, including proerythroblasts and early basophilic erythroblasts. Thus, simultaneous immunostaining for Ter119 and CD71 allows determination of the ratio of early and late erythroid progenitors. The ratio of early (Ter119^{high}CD71^{high}, region II) to late (Ter119^{high}CD71^{low}, region IV) is 1:33 in SR-BI +/+ spleens, while a ratio of 1:5 (p=0.002, Fig. 3) was found in SR-BI -/- spleens. The ratio of early to late erythroblasts is thus dramatically increased in spleens of SR-BI -/- mice.
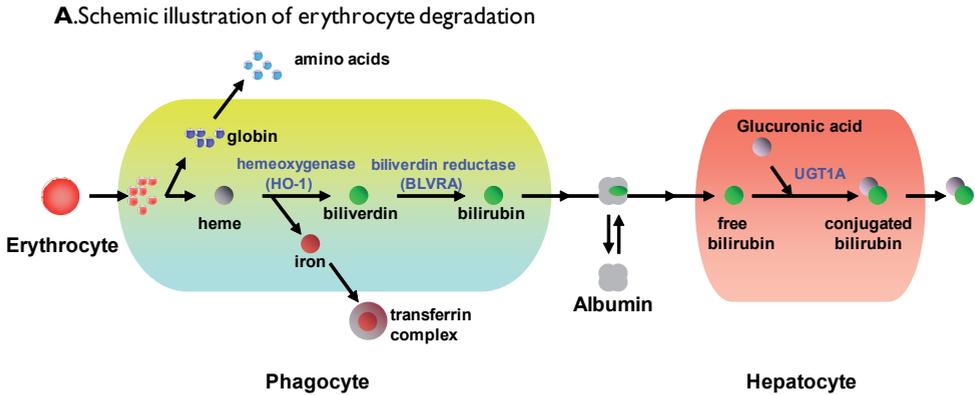
Effect of SR-BI deficiency on erythrocyte degradation

The reticulo-endothelial systems of the spleen, liver, and marrow are involved in the detection and removal of aged erythrocytes. In this process, the spleen is the most discriminating organ.[23,24] Therefore, the mRNA expression levels of genes involved in erythrocyte catabolism by the spleen were determined, including hemeoxygenase (HO-1) and biliverdin reductase A (BLVRA)(Fig.4). In accordance with the observed increase in splenic iron levels, SR-BI deficiency led to a significant increase in HO-1 (57%; p<0.05) and BLVRA (43%; p<0.05), suggesting an increase in erythrocyte catabolism. Also in liver, SR-BI deficiency resulted in a 2.6-fold increase in HO-1. This, however, failed to reach statistical significance due to a wide variation in the SR-BI deficient mice (p=0.099). No effect of SR-BI deficiency on the hepatic mRNA expression levels of BLVRA and UGT1A was observed.

After phagocytosis of the erythrocyte, bilirubin, the breakdown product of hemoglobin, is transported to the liver and excreted into the biliary duct system. Conjugated bilirubin contents of the bile of SR-BI deficient mice was 194 ± 55 μmol/L as compared to only 67 ± 30 μmol/L (p<0.05) in wild-type mice. No accumulation of conjugated of bilirubin was found in plasma (data not shown).

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A

Stages of erythroid differentiation

TAL-1
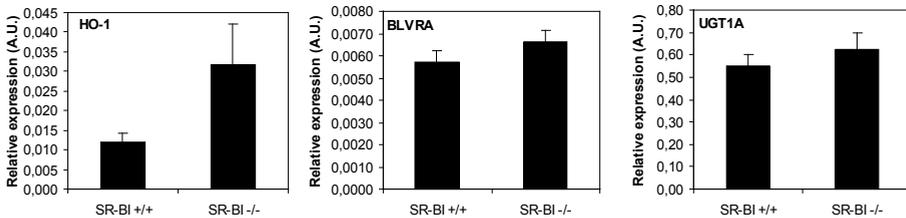
GATA-2

GATA-1

FOG-1

EPOr

B

C

Figure 3. Effect of SR-BI deficiency on splenic mRNA expression of genes involved in erythropoiesis.
A) Schematic illustration of genes involved in different stages of erythropoiesis. Several erythroid precursor differentiation stages can be distinguished: pluripotent hematopoietic stem cell (PHSC), Colony-forming units spleen (CFU-S), burst-forming unit-erythroid (BFU-E), cluster-forming unit-erythroid (CFU-E), proerythroblasts (ProEB), basophilic erythroblasts (BasoEB), polychromatic eryhthroblasts (PolyEB), orthochromatic erythroblasts (OrthoEB), reticulocytes (Ret), and red blood cells (RBC). B) mRNA levels of the indicated genes in spleens of SR-BI +/+ and SR-BI -/- mice were quantified using real-time PCR with SYBR-green detection. Values are expressed relative to 36B4 expression and represent the mean ± S.E of 7-8 mice. C) Flow cytometric assessment of spleen erythroid precursors. Freshly dissociated spleen cells from SR-BI +/+ and SR-BI -/- mice were labelled with FITC-conjugated anti-mouse Ter119 and PE-conjugated anti-mouse CD71 mAbs. The left panels demonstrate density plots with the axes indicating the relative fluorescence units for FITC (x-axis) and PE (Y-axis). Regions I to IV were selected as indicated, with region I representing late OrthoEB, region II late BasoEB and PolyEB, region III Baso EB, and region IV ProEB[22]. Values represent mean ± SEM of 6 mice. Statistically significant difference of **p<0.01 and ***p<0.001 compared with the SR-BI +/+ mice.

A. Schemic illustration of erythrocyte degradation

B. Splenic mRNA expression
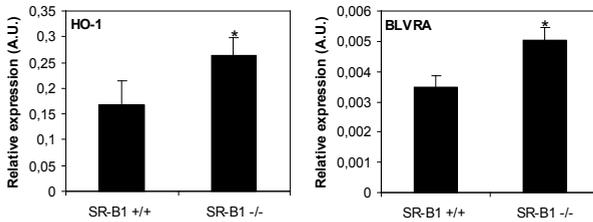

C. Hepatic mRNA expression

Figure 4. Effect of SR-BI deficiency on splenic and hepatic mRNA expression of genes involved erythrocyte degradation.
A) Schematic illustration of genes involved in erythrocyte degradation. B) mRNA levels of the indicated genes in spleens of SR-BI +/+ and SR-BI -/- were quantified using real-time PCR with SYBR-green detection. c) Relative mRNA expression levels of hepatic genes involved in erythrocyte degradation Values are expressed relative to 36B4 expression and represent the mean ± SEM of 6-8 mice. *, statistically significant difference of p<0.05 compared with the control mice.

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Effect of SR-BI deficiency on erythrocyte life-span

To determine if the increased erythropoietic activity in SR-BI deficienct-mice is a direct result of an altered erythrocyte life-span, an erythrocyte survival experiment using biotinylated erythrocytes was performed. SR-BI -/- mice exhibited an accelerated clearance of the erythrocytes as compared to the controls. The erythrocyte half-life was reduced 1.6-fold (p<0.05) from 5.1±0.5 weeks in wild-type animals to only 3.2±0.5 weeks in SR-BI deficient animals. At 5 weeks after biotinylation, approximately 55% of the control erythrocytes were still biotinylated, while only 10% of the biotinylated erythrocytes of the SR-BI deficient mice survived (p<0.01, Fig.5). At week 7 no biotinylated erythrocytes could be detected in SR-BI -/- mice, while in the control mice 25% of biotinylated erythrocytes were still present. These findings show that the erythrocyte life-span in SR-BI -/- mice is indeed shorter than the erythrocyte life-span in the controls.
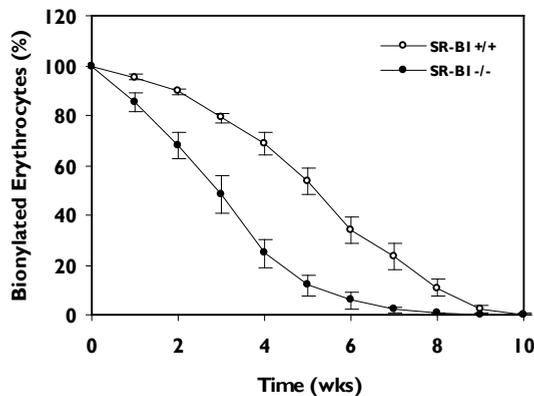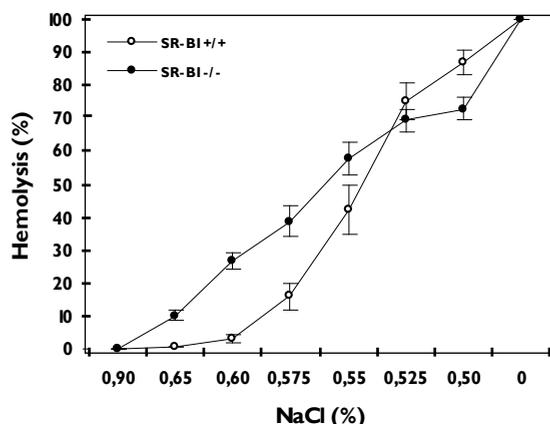

Figure 5. Effect of SR-BI deficiency on erythrocyte survival.
Erythrocytes were biotinylated by tail vein injection of EZ-Link Sulfo-NHS-biotin and blood was drawn weekly. Erythrocytes were labeled with phycoerythrin-conjugated streptavidin and biotinylated erythrocytes were quantified by flow cytometry. Values represent the mean ± SEM of 3 mice.

Effects of SR-BI deficiency on factors influencing erythrocyte survival

Erythrocyte survival is determined by several factors, including phosphatidylserine (PS) exposure on the outer membrane, and membrane fluidity and fragility. Phosphatidylserine normally localizes to the inner leaflet of erythrocyte membranes, but becomes exposed in aged or abnormal erythrocytes. The exposure of PS at the extracellular surface of erythrocytes subsequently stimulates clearance of these erythrocytes from peripheral blood by phagocytosis.[25-27] Annexin V, which binds specifically to PS, was used to assess the exposure of this phospholipid on isolated SR-BI +/+ and SR-BI -/- erythrocytes. No effect of SR-BI deficiency on the PS exposure on the outer membrane of the erythrocytes was observed (data not shown). The failure to show increased PS on SR-BI -/- erythrocytes might be a direct effect of the efficient removal of PS exposing erythrocytes from the circulation. To determine whether the decreased erythrocyte survival in absence of SR-BI was a direct effect of changes in osmotic fragility, the osmotic resistance of the erythrocyte membrane

was analysed by exposure to solutions with decreasing concentrations of NaCl. As indicated in Fig. 6, erythrocytes of SR-BI deficient mice exhibited a significant increase in osmotic fragility for the NaCl concentrations 0.65% to 0.55%.

Figure 6. Effect of SR-BI deficiency on erythrocyte osmotic fragility.
Erythrocyte osmotic fragility was analysed using solutions with decreasing concentrations of NaCl. Osmotic fragility in SR-BI deficient mice was significantly different from the control mice. Values represent the mean ± SEM of 7 mice.

The fluidity of erythrocytes membranes and thus deformability of the erythrocytes is determined by the cellular cholesterol concentration, where increased cholesterol-to-phospholipid ratios of erythrocytes results in loss of membrane fluidity.[28-33] Furthermore, there is a close relation between erythrocyte membrane cholesterol and the amounts of cholesterol transported by lipoproteins in the circulation. Cholesterol transfer between erythrocytes and plasma occurs exclusively by a non-specific exchange mechanism.[12] SR-BI deficiency results in a 1.7-fold increase (p<0.001, Table III) in total plasma cholesterol levels. This increase in total cholesterol was mainly due to a 2.7-fold increase in free cholesterol (p<0.001, Table III). In agreement with previous studies[34], the changes in serum cholesterol were mainly caused by a major increase in the amount of cholesterol transported by HDL (2.1-fold, p<0.01; Table III). An erythrocyte lipid extraction was performed to analyse the effect of the increased plasma HDL levels on the erythrocyte cholesterol content. In agreement with Holm et al.[10], the erythrocyte cholesterol content was positively correlated with the amount of unesterified cholesterol in the plasma. In the absence of SR-BI, the erythrocyte cholesterol content was 1.8-fold increased (p<0.01) from 48 ± 3 mg/dl in SR-BI +/+ mice to 88 ± 9 mg/dl in SR-BI -/- mice, while the phospholipid content was not affected (49 ± 6 mg/dl in SR-BI +/+ mice to 45 ± 5 mg/dl in SR-BI -/- animals). Erythrocytes from SR-BI deficient mice thus display a 2-fold increased cholesterol:phospholipid ratio.

The observed increase in cholesterol-to-phospholipid ratio can result in a decreased deformability of the erythrocytes, thereby leading to an increased risk of damage by shear stress in the microcirculation. Therefore, the deformability as measured by the elongation index (EI) of SR-BI +/+ and SR-BI -/- erythrocytes was determined at increasing shear stress

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using a laser-assisted optical rotational cell analyser (LORCA). At low shear stress of 0.3 Pa, the EI of both SR-BI +/+ and SR-BI -/- erythrocytes is low, indicating perfect circular cells. At shear stresses of 3.0 to 30 Pa, the SR-BI -/- erythrocytes could not deform as well as the SR-BI +/+ erythrocytes. At 30 Pa, the EI of SR-BI -/- erythrocytes was 0.562 ± 0.003, as compared to 0.596 ± 0.004 for SR-BI +/+ erythrocytes (p<0.001).

Table 3. Effect of SR-BI deficiency on serum lipid levels

Mice	n	Triglyceride	Total cholesterol	Free cholesterol	Cholesteryl ester	HDL cholesterol
		mg/dl	mg/dl	mg/dl	mg/dl	mg/dl
SR-BI +/+	8	132 ± 17	93 ± 8	23 ± 3	117 ± 11	48 ± 3
SR-BI -/-	8	222 ± 21*	160 ± 5**	63 ± 4**	158 ± 7*	99 ± 5**

Data represent the means ± S.E. Statistically significance of *p < 0.01 and **p < 0.001 compared with SR-BI +/+ mice.

DISCUSSION

SR-BI plays an important role in HDL metabolism by mediating reverse cholesterol transport.[34] SR-BI deficiency results in highly increased plasma HDL cholesterol levels due to the impaired selective uptake of cholesterol from HDL by the liver and is associated with an increased susceptibility to atherosclerosis.[4-8] In agreement with previously published data from Holm et al.[10], we show that mice lacking SR-BI display abnormal erythrocyte numbers with increased size and cholesterol content as well as reticulocytosis. Cholesterol enrichment of erythrocytes associated with abnormal erythrocyte morphology, reticulocytosis, and hemolytic anemia has been found in several animal models, including guinea pigs[35], rabbits[36], and dogs[37], fed a high cholesterol diet. In addition, Suda et al.[38] have recently observed in a patient with familial lecithin: cholesterol acyltransferase (LCAT) deficiency that cholesterol enrichment of the erythrocyte membrane as a result of LCAT deficiency leads to increased erythrocyte MCV, abnormal erythrocyte shape, anemia, and reticulocytosis. The erythrocyte abnormalities observed in mice lacking SR-BI thus have an equivalent in humans and are most likely related to the observed high cholesterol content of the erythrocytes as a result of increased serum (free) cholesterol levels. This was further supported by Braun et al., who found that erythrocyte abnormalities in SR-BI/apolipoprotein E double knockout mice can be corrected by probucol treatment, a lipid-lowering and antioxidative drug.[39] Holm et al. proposed that the reticulocytosis observed in SR-BI deficient mice was a direct effect of impaired maturation of erythrocytes as a result of autophagocytosis and impaired expulsion of phagolysosomes due to increased cellular cholesterol levels.[10]

In the present study we show that SR-BI deficient mice exhibit splenomegaly with increased iron deposition. Spleen enlargement may be caused by a block in erythrocyte maturation. Takahashi et al. [40] have shown that loss of GATA-1 function in GATA-1.05 heterozygous mutant mice results in stimulated proliferation and suppressed differentiation of erythroid progenitor cells. The impaired erythrocyte differentiation in these mutant mice leads to massive accumulation of immature erythrocytes in the spleen and splenomegaly. In the current study we also found that the relative amount of early Ter119^{high}CD71^{high} expressing erythroblasts was increased in spleens of SR-BI deficient mice. On the other hand, spleen enlargement and the accumulation of early erythroblasts might be the result of massive extramedullar erythropoiesis.[41] In support of this, SR-BI deficiency resulted in increased expression levels of genes involved in the commitment and differentiation of erythroid

progenitors, including TAL-1, GATA-1, FOG-1, and EPOr. No effect was observed on the expression of GATA-2. Tsang *et al.* [42] have previously shown that FOG-1 and GATA-1 synergize in the induction of genes specific for erythropoiesis. The expression of GATA-2 decreases upon induction of GATA-1 expression during erythroid differentiation.[43] SR-BI deficiency also resulted in an upregulation of the expression of FECH, the rate-limiting enzyme for the synthesis of heme. It is thus likely that the observed reticulocytosis and splenomegaly are caused by enhanced erythropoiesis.

Enhanced erythropoiesis might develop as a result of increased catabolism of erythrocytes. The spleen is the principal and most sensitive organ for the detection and removal of erythrocytes.[17, 18] Interestingly, the expression of HO-1 and BLVRA, enzymes involved in the degradation of heme was upregulated. Furthermore, the secretion of bilirubin, the primary degradation product of heme, into the bile was increased, indicating that the catabolism of erythrocytes in SR-BI deficient mice was induced. Using biotinylated erythrocytes, we show that the erythrocyte half-life was decreased from ~5 weeks in wild-type animals to ~3 weeks in SR-BI deficient mice. The accumulation of reticulocytes in the circulation of SR-BI deficient mice is thus most likely a secondary effect of a shortened life-span of the erythrocytes in these mice.

The osmotic fragility of erythrocytes isolated from SR-BI deficient mice was increased, indicating that in absence of SR-BI the physiological properties of the erythrocyte membrane are affected. In addition, we found a two-fold increase in cholesterol:phospholipids (C:P) ratio in erythrocytes from SR-BI deficient mice. This is agreement with recent findings of Kempaiah *et al.* [44,45], who have shown that an increased ratio of erythrocyte membrane C:P ratio in rats fed a diet enriched in cholesterol caused an increased osmotic fragility. In addition, Suda et al. demonstrated increased erythrocyte fragility due to abnormal lipid composition in the membrane of erythrocytes from LCAT deficient patients.[38] Other studies, however, have previously reported a negative correlation between the membrane cholesterol content and erythrocyte osmotic fragility.[46,47] It has been unequivocally established that an increased C:P ratio induces erythrocyte rigidity and microviscosity, leading to a decreased erythrocyte fluidity and deformability and an increased risk of damage by shear stress in the microcirculation.[48,49] In agreement, we found that the increase in C:P ratio in erythrocytes from SR-BI deficient mice was associated with a small, but significant reduction in the deformability of circulating erythrocytes. The spleen is highly efficient in the elimination of pathological rigid erythrocytes from the circulation.[50] The small changes in deformability of SR-BI-/- erythrocytes in the presence of a proper functioning spleen, thus, provide important pathological information. Furthermore, the observed decreased deformability and increased fragility of erythrocytes in SR-BI deficient mice are most likely the underlying cause of the enhanced erythrocyte destruction in absence of SR-BI.

In conclusion, SR-BI deficiency results in reticulocytosis due to a reduced life-span of erythrocytes, apparently caused by erythrocyte cholesterol enrichment, and resulting in an enhanced erythropoietic activity. The expression of SR-BI thus appears not only essential for optimal HDL cholesterol metabolism, but also for maintaining normal erythrocyte life-span.

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ACKNOWLEDGMENTS

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