Regulation of High-Density Lipoprotein on Hematopoietic Stem/Progenitor Cells in Atherosclerosis Requires Scavenger Receptor Type BI Expression

Mingming Gao,* Dong Zhao,* Sarah Schouteden, Mary G. Sorci-Thomas, Paul P. Van Veldhoven, Kristel Eggermont, George Liu, Catherine M. Verfaillie,† Yingmei Feng†

Objective—Recently, we demonstrated that scavenger receptor type BI (SR-BI), a high-density lipoprotein (HDL) receptor, was expressed on murine hematopoietic stem/progenitor cells (HSPC) and infusion of reconstituted HDL and purified human apolipoprotein A-I (apoA-I) suppressed HSPC proliferation. We hypothesized that SR-BI expression is required for the observed antiproliferative effects of HDL on HSPC.

Approach and Results—SR-BI–deficient (SR-BI−/−) mice and wild-type controls were fed on chow or high-fat diet (HFD) for 8 to 10 weeks. Under chow diet, a significant increase in Lin− Sca1+ cKit+ (LSK cells, so-called HSPC) was found in the bone marrow of SR-BI−/− mice when compared with wild-type mice. HFD induced a further expansion of CD150+CD48− LSK cells (HSC), HSPC, and granulocyte monocyte progenitors in SR-BI−/− mice. Injection of reactive oxygen species inhibitor N-acetylcysteine attenuated HFD-induced HSPC expansion, leukocytosis, and atherosclerosis in SR-BI−/− mice. ApoA-I infusion inhibited HSPC cell proliferation, Akt phosphorylation and reactive oxygen species production in HSPC and plaque progression in low-density lipoprotein receptor knockout (LDLr−/−) apoA-I−/− mice on HFD but had no effect on SR-BI−/− mice on HFD. Transplantation of SR-BI−/− bone marrow cells into irradiated LDLr−/− recipients resulted in enhanced white blood cells reconstitution, inflammatory cell production, and plaque development. In patients with coronary heart disease, HDL levels were negatively correlated with white blood cells count and HSPC frequency in the peripheral blood. By flow cytometry, SR-BI expression was detected on human HSPC.

Conclusions—SR-BI plays a critical role in the HDL-mediated regulation HSPC proliferation and differentiation, which is associated with atherosclerosis progression. (Arterioscler Thromb Vasc Biol. 2014;34:1900-1909.)

Key Words: atherosclerosis ■ cholesterol, HDL

High-density lipoprotein (HDL) and its major component, apolipoprotein A-I (apoA-I), are negatively correlated with the incidence of coronary heart disease.† Recently, Yvan-Charvet et al‡ and our group demonstrated that infusion of reconstituted HDL or lipid-poor human apoA-I inhibits hematopoietic stem/progenitor cells (HSPC) proliferation in hypercholesterolemic Abca1−/− Abcg1−/− mice and C57BL/6 mice, therefore, limiting white blood cell (WBC) expansion in the peripheral blood (PB).§ In addition, we demonstrated that the HDL receptor, scavenger receptor type BI (SR-BI), is expressed on murine Lin− Sca1+ cKit+ (LSK cells, so-called HSPC cells),# which led to the hypothesis that HDL and apoA-I may regulate HSPCs by binding to SR-BI.

HSPCs, responsible for all blood cell generation, reside in a hypoxic bone marrow (BM) niche, and are largely quiescent. In general, HSPC are defined as LSK cells that include both hematopoietic stem and progenitor cells. Within HSPC, the most primitive subpopulation that is capable of repopulating the hematopoietic system is named long-term hematopoietic stem cells (LT-HSCs; defined as CD150+CD48− LSK cells). HSCs give rise to multiple progenitor cells (CD150−CD48− MPPs), a mature subset of HSPC, that sequentially generate progenitors, lineage restricted precursors, and finally all blood cells. Therefore, LSK cells are widely used in studying HSPC. The potential for self-renewal, proliferation, and differentiation of HSPCs is finely balanced by different intrinsic and extrinsic signals, which guarantee the continuous blood supply throughout life. Extrinsic factors, such as cytokines, chemokines, growth factors, and extracellular matrix molecules bind to receptors on HSPCs, which then activate downstream events such as proliferation, differentiation, and survival.
signaling pathways, such as phosphoinositide 3-kinase/ AKT and mitogen-activated protein kinase (MAPK) signaling molecules, which regulate cell survival versus apoptosis, or self-renewal versus differentiation. The Akt family of serine threonine kinases are activated by phosphorylation of phosphoinositide 3-kinase. Although Akt1−−/− or Akt2−−/− mice have only mild defects in hematopoiesis, HSPC function, including proliferation and reconstitution capacities, is severely affected in Akt1−−/− Akt2−−/− mice, suggesting an essential role of Akt in HSPC biology.5,7 MAPKs are also a family of serine threonine kinases consisting of ERKs, JNKs, and p38MAPKs.8–10 The role of ERKs in HSPC proliferation differentiation and survival is well established, as are the roles of JNKs on erythropoiesis, and p38MAPK activation in the regulation of erythropoiesis and myeloid differentiation.11

Aside from phospho-Akt (pAkt) and MAPK, another group of molecules that critically regulates HSPC proliferation and differentiation are reactive oxygen species (ROS).12 The hypoxic BM microenvironment is responsible for low ROS production in HSPCs, which is important for the maintenance of HSPC quiescence and self-renewal but not differentiation.11 Comparison between ROShigh HSPCs and ROSlow HSPCs showed that the ROSlow population represents the quiescent HSPC population with self-renewal potential, whereas the ROShigh HSPC subpopulation is activated, undergoes differentiation, and then exhaustion.12,11 Moreover, pAkt acts upstream of ROS production, whereas p38MAPK activation is downstream of ROS production in HSPCs.11

SR-BI, the HDL receptor, is expressed on hepatocytes and facilitates selective cholesterol ester uptake from HDL. In addition, SR-BI also mediates low-density lipoprotein (LDL) and VLDL clearance.14 Others and we have illustrated the atheroprotective function of SR-BI in different mouse models.15–18 Different from mice, SR-BI deficiency in humans leads to multiple pathophysiological phenotypes.19,20 In mice, SR-BI deficiency is associated with impaired HDL function, intracellular cholesterol accumulation, and increased oxidative stress.21 We previously demonstrated that SR-BI is expressed on murine HSPC. This led us to hypothesize that SR-BI deficiency impairs cholesterol homeostasis, modulates Akt and MAPK phosphorylation, and leads to increased ROS production in HSPC, resulting in enhanced HSPC proliferation and differentiation.

We here demonstrate a significant increase in LT-HSC, HSPCs, and granulocyte monocyte progenitors (GMP) in the BM of SR-BI−−/− mice when compared with wild-type (WT) mice on high-fat diet (HFD). Infusion of human apoA-I reduced HSPCs proliferation, Akt phosphorylation, and ROS production in HSPCs and inhibited plaque progression in LDLr−−/− apoA-I−−/− mice on HFD but not in SR-BI−−/− mice on HFD. Third, transplantation of SR-BI−−/− BM cells into irradiated LDLr−−/− recipients resulted in enhanced WBC reconstitution, inflammatory cell production, and plaque development. Fourth, ROS inhibitor treatment reversed leukocytosis and the increased LT-HSC and HSPC frequency and attenuated atherosclerosis progression in SR-BI−−/− mice induced by HFD. Finally, we tested SR-BI expression on human HSPC and assessed the effect of HDL levels on WBC count, as well as HSPC frequency in the blood. As we found in murine models, SR-BI is expressed on human HSPC. In patients with coronary heart disease, HDL levels were negatively correlated with WBC and HSPC frequency in the blood.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

HFD Induces Leukocytosis, Monocytosis, GMP, HSPC, and LT-HSC Expansion and Early Onset of Atherosclerosis in SR-BI−−/− Mice

We have previously demonstrated that SR-BI, a HDL receptor, is expressed on murine HSPCs. As infusion of reconstituted HDL or lipid-poor human apoA-I inhibits HSPC proliferation in hypercholesterolemic Aβc1−−/− Abcg1−−/− mice and C57BL/6 mice,2,3 we here investigated whether and how SR-BI might be involved in these effects. Eight-week-old SR-BI−−/− and SR-BI−−/− mice were fed on chow diet or HFD (1% cholesterol; 34% fat) for 8 to 10 weeks. The lipoprotein profiles are shown in Figure I in the online-only Data Supplement. HFD induced leukocytosis and monocytosis and increased the number of F4/80+ macrophages in the PB of SR-BI−−/− mice when compared with WT mice on HFD (Figure 1A–1C). In addition, we found more extensive atherosclerotic plaques in the aortic roots in SR-BI−−/− when compared with WT mice (24517±10625.1 versus 6489±1881.3 µm²; n=7 for each; P<0.001).

To address the role of SR-BI in the effects of HDL on HSPC, we enumerated the frequency of LT-HSC cells (briefly, HSC), LSK cells (HSPC), and GMP (CD34+ FcR+ Lin− Sca1+ cKit+) in BM of SR-BI−−/− and SR-BI−−/− mice on chow and HFD. In animals maintained on chow diet, we found a 1.7-fold increase in the percentage of LSK cells in the BM of SR-BI−−/− mice when compared with WT controls (LSK%, 0.090% versus 0.054%; P<0.05; n=8–10). After HFD, both HSC and LSK frequency was increased in BM of SR-BI−−/− when compared

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
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<tr>
<td>apoA-I</td>
<td>apolipoprotein A-I</td>
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<td>BM</td>
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<td>BMCs</td>
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<td>DKO</td>
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<td>HFD</td>
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<td>HSPC</td>
<td>hematopoietic stem/progenitor cells</td>
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<td>LDL</td>
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<td>LDLr−−/−</td>
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<td>LT-HSC</td>
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<td>N-acetylcysteine</td>
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<td>PB</td>
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with WT mice (HSC%, 0.014% versus 0.009% at 8 weeks of HFD; 0.017% versus 0.011% at 10 weeks of HFD; n=11 for each; P<0.01 and LSK%, 0.135% versus 0.095% at 8 weeks of HFD; 0.184% versus 0.090%; n=11 for each; P<0.01; Figure 1D and 1E; Figures II and VI in the online-only Data Supplement). Although no difference was seen when mice were maintained on chow diet, the percentage of GMPs in BM cells was 1.2- and 1.5-fold increase in SR-BI−/− mice on HFD after 8 and 10 weeks of HFD when compared with WT mice on HFD (GMP%, 0.633% versus 0.530% at 8 weeks of HFD; 0.816% versus 0.537% at 10 weeks of HFD; n=11 for each; P<0.05; Figure 1F; Figure VI in the online-only Data Supplement). Consistent with this, BM cells (BMCs) from SR-BI−/− mice on HFD contained significantly greater numbers of hematopoietic colony-forming cells when compared with BMCs from WT mice on HFD (n=8; Figure 1G). In addition, when LSK cells were isolated from mice on HFD and cultured in vitro, SR-BI−/− LSK cells produced more F4/80+ macrophages than SR-BI+/+ LSK cells (n=7; Figure 1H). Finally, the frequency of LSK cells in the spleen and PB of SR-BI−/− mice fed on HFD was significantly higher than in WT mice of HFD (n=4–8; Figure 1I).

Transplanted SR-BI−/− BM Caused Enhanced Atherosclerotic Plaques Formation, Wherein Grafted Cells Could Be Detected

To demonstrate the increased frequency of HSPC cells in BM of WT and SR-BI−/− mice on chow diet further, we performed limiting dilution competitive repopulation studies. CD45.2+ WT or SR-BI−/− BM cells were mixed with CD45.1 BM cells and transplanted into irradiated CD45.1 recipients. SR-BI−/− CD45.2 chimerism at 4 and 16 weeks after transplantation was significantly higher, consistent with increased frequency of progenitors and HSPCs in SR-BI−/− murine BM (n=4–6; Figure 2A).

We next determine whether SR-BI−/− HSC/HSPC play a role in atherosclerotic plaque development under HFD conditions. To address this question, we grafted CD45.2 BM cells of SR-BI+/+ or SR-BI−/− mice mixed with equal numbers of CD45.1 BM cells in irradiated LDLr−/− recipients. After
transplantation, recipients were fed on chow diet for the first 8 weeks and then switched to HFD for another 8 weeks. Consistent with the limiting dilution analysis, higher chimerism of SR-BI−/− CD45.2 cells was observed at 4 and 16 weeks (n=6–10; Figure 2B). Moreover, after 16 weeks of BM transplantation, significantly greater numbers of CD45.2+ granulocytes and monocytes were observed in recipients transplanted with SR-BI−/− than WT BM cells (n=6–10; Figure 2C and 2D).

We also found accelerated atherosclerosis in the aortic roots of LDLr−/− recipients transplanted with SR-BI−/− BM cells when compared with that of WT BM cells (n=6–8; Figure 3E and 3F). To explore the contribution of SR-BI−/− and SR-BI+/+ derived inflammatory cells to plaque formation further, cryosections were stained with biotin-CD45.2 and rat antimouse CD45 and then streptavidin 555 and goat antirat Alexa 488. The fraction of CD45.2 cells among the CD45+ cells in the plaques was 1.2-fold higher in recipients transplanted with SR-BI−/− BM cells when compared with those transplanted with WT BMCs (n=6–7; Figure 4G and 4H; Figure III in the online-only Data Supplement).

Infusion of Lipid-Free Human ApoA-I Inhibited HFD-Induced HSPC Proliferation, Akt Phosphorylation, and Plaque Progression in LDLr−/− ApoA-I−/− Mice but Had No Effect on SR-BI−/− Mice

To determine whether the increased HSPC frequency in SR-BI−/− mice on HFD was because of enhanced HSPC proliferation, BrdU was injected intraperitoneally into mice 12 hours before euthanasia and BM cells were stained with anti-LSK and anti-BrdU FITC Abs as described before.3 The percentage of BrdU incorporating LSK cells among LSK population was 12% in WT mice on HFD but increased to 18% in SR-BI−/− mice on HFD (SR-BI+/+, 12.2±3.32%; SR-BI−/−, 18.6±4.33%; n=6 for each; P<0.05; Figure 3A). Apart from enhanced HSPC proliferation, FACS data also demonstrated an increased percentage of pAkt+ LSK cells in SR-BI−/− mice on HFD when compared with WT mice (pAkt+ LSK%, 15.5±5.00% versus 9.2±3.76%; n=8 for each; P<0.05; Figure 3B). To assess the pAkt status in HSPC further, LSK cells were sorted from BM of SR-BI+/+ and SR-BI−/− mice on HFD. After 4 days of culture in SFEM supplemented with
stem cell factor and thrombopoietin, pAkt expression in LSK cells was measured by ELISA (n=11 for each; Figure 3C).

To explore whether SR-BI was required for the HDL-mediated regulation of HSPC further, SR-BI−/− and WT mice were placed on HFD for 11 weeks and 500 µg lipid-free human apoA-I or saline was injected intraperitoneally into mice twice per week for 3 weeks. In parallel, DKO mice on HFD for 9 weeks were injected for the last 3 weeks with saline or apoA-I twice per week for 3 weeks. Mice with deficiency of LDLr and apoA-I developed hypercholesterolemia and accelerated atherosclerosis when fed on atherogenic diet. In addition, SR-BI is expressed in DKO mice. Thus, performing apoA-I infusion on DKO and SR-BI−/− mice would allow us to investigate the effect of SR-BI on cells. Consistent with previous reports, apoA-I did not alter cholesterol levels in the blood (data not shown). However, apoA-I infusion reduced plaque size in DKO mice (4598±1895.1 versus 74878±25510.1 µm²; n=6–9; P<0.05; Figure 3D; Figure IV in the online-only Data Supplement). We found a 1.6-fold reduction in LSK frequency in DKO mice injected with apoA-I when compared with that injected with saline (n=6–7; Figure 3E). Moreover, the percentage of BrdU+LSK cells in the whole LSK population was 37% decreased in DKO mice with apoA-I injection when compared with saline group (n=6–7; Figure 3F). In addition, we also found a 39% reduction in pAkt-positive LSK cells in mice that received apoA-I infusion when compared with control (n=6–7; Figure 3G).

Figure 3. Human apolipoprotein A-I (apoA-I) infusion inhibited hematopoietic stem/progenitor cell (HSPC) proliferation, reduced reactive oxygen species (ROS) production in HSPC and reversed plaque progression. To quantify the proliferative status of HSPC in vivo, BrdU was injected in mice. Brdu-positive Lin− Sca1− cKit− (LSK) cells were quantified by FACS (Figure 4A). Akt phosphorylation (pAkt) in LSK cells was studied by staining bone marrow cells (BMcs) with PE-conjugated anti-pAkt and LSK antibodies for FACS (B) and pAkt level in ex vivo expanded LSK cells were confirmed by ELISA (C).

D. Plaque size in aortic roots of SR-BI+/+ and LDLr−/− apoA-I−/− (double knockout [DKO]) mice that were treated with HFD and injection of saline or apoA-I. G. The percentage of pAkt+ LSK cells in the entire LSK cell population in mice was measured by FACS. H. BMcs were stained with LSK antibodies and then incubated with DCF-DA (diacetyldichlorofluorescein, 2',7'-dichlorofluorescein diacetate). The percentage of ROS+ LSK cells in the LSK population was quantified by FACS. I. Only male SR-BI−/−, SR-BI+/− and LDLr−/−apoA-I−/− mice were used in the apoA-I infusion experiments.

I, ABCA1 expression in LSK cells of male SR-BI+/+ and SR-BI−/− mice on chow and HFD. n=3 to 6. J. After apoA-I injection, LSK frequency in LDLr−/− recipients transplanted with SR-BI+/+ or SR-BI−/− BMC. n=5 to 7. Six male LDLr−/− and 6 LDLr−/− female recipients were used in the BM transplantation experiment. SR-BI indicates scavenger receptor type BI.
Inhibition of Akt and p38MAPK Phosphorylation Maintained HSPC Quiescence in SR-BI−/− Mice on HFD

As mentioned earlier, p38MAPK phosphorylation is an important mediator of HSPC differentiation. To evaluate whether p38MAPK phosphorylation was involved in the regulation of HSPC by HDL and whether apoA-I infusion had any effect on p38MAPK phosphorylation, SR-BI−/− and SR-BI−/− mice were fed on HFD and received saline or apoA-I infusion as described above. BMCs of SR-BI−/− and SR-BI−/− mice were stained with antibodies against LSK markers and phospho-p38MAPK. FACS data demonstrated that the percentage of phospho-p38MAPK+ LSK cells was 1.7-fold higher in SR-BI−/− mice when compared with SR-BI−/− mice (15.2±4.01% versus 8.8±2.37%; n=8 for each; P<0.01). Nevertheless, apoA-I infusion did not affect p38MAPK phosphorylation in LSK cells (Figure 4A) or in LSK cells of DKO mice (data not shown).

To assess the possible roles of pAkt and phospho-p38MAPK in the aberrant behavior of LSK cells from SR-BI−/− mice further, LSK cells from SR-BI−/− and WT mice were selected by FACS and cultured in SFEM with stem cell factor, thrombopoietin, and IL-3 in the presence or absence of inhibitors against pAkt and phospho-p38MAPK. On day 5, cell numbers were enumerated. LSK expansion was determined by comparing cell number with or without inhibition. As shown in Figure 6B, compared with LSK cells without inhibition, addition of phospho-p38MAPK inhibitor SB203580 led to 46% and 28% reduction of LSK expansion in cells from WT mice on HFD and cells from SR-BI−/− mice on chow diet. However, phospho-p38MAPK inhibitor further inhibited LSK expansion in SR-BI−/− cells on HFD (n=5–6; Figure 4B). Likewise, pAkt inhibition LY 294002 resulted in 60% and 64% decrease in LSK cells in cells from WT mice on HFD and cells from SR-BI−/− mice chow diet, respectively, when compared with control. Again, addition of LY 294002 further reduced LSK expansion in SR-BI−/− cells from HFD (n=5–6; Figure 4C).

Inhibition of ROS Production Limited SR-BI−/− HSPC Expansion Induced by HFD

To determine whether the increased ROS levels found in LDLr−/− apoA-I−/− mice on HFD could also be linked to the SR-B1 receptor further, NAC (1 mg/kg daily) was injected in SR-BI−/− and SR-BI−/− mice placed on HFD at 8 weeks for 12 weeks.23 NAC administration did not alter total cholesterol (n=3–7; Figure 5A) and lipoprotein profiles (data not shown). NAC-treated mice developed body weight loss when compared with saline-treated mice (data not shown). However, plasma levels of Serum Amyloid A was not different among the groups, indicating that NAC injection did not induce systemic inflammation (SR-BI−/− mice, 160±13.7 on saline group versus 157±48.7 µg/mL on NAC group; SR-BI−/− mice, 173±9.4 versus 160±57.7 µg/mL on NAC group). Besides reversed HFD-induced leukocytosis (n=5–8; Figure 5B), NAC treatment led to a significantly decreased frequency of LT-HSC, LSK cells, and GMP in the BM of SR-BI−/− mice on HFD (n=7–11; Figure 5C–5E). Furthermore, NAC injection significantly reduced plaque size 3.8-fold in SR-BI−/− mice from chow diet to HFD for 8 weeks. Starting from fifth week of HFD, 500 µg purified human apoA-I was injected subcutaneously to all the recipients twice per week for 3 weeks. Two days after the last injection, mice were euthanized and BMC were stained with an Ab cocktail against LSK cells. FACS data demonstrated that LSK frequency in BMC was lower in LDLr−/− recipients transplanted with SR-BI−/− BMC when compared with that of LDLr−/− recipients transplanted with SR-BI−/− BMC (0.08±0.028% versus 0.13±0.013%; n=5–7; P<0.05; Figure 3J). These data indicate that HDL/apoA-I directly regulates HSPC frequency, proliferation, and ROS production, all of which is mediated via HDL receptor, SR-BI.
HDL Negatively Regulated HSPC Frequency and WBC Count in PB of Human Subjects

To investigate whether the effect of HDL on mouse HSC and HSPC is also found in human, we studied the correlation between HDL and WBC level in patients (n=37) with coronary heart disease. To avoid the influence of acute infection, individuals with WBC counts of >10,000 cells/µL or neutrophil frequency >75% were excluded from the study. The basic characterization of these patients is shown in Table 2. Spearman analysis demonstrated a negative correlation between HDL levels and total WBC count in PB (P<0.05; r=-0.3; n=37; Figure 6A), as well as neutrophils (P<0.05; r=-0.4; n=37; Figure 6B). As we observed that females had higher HDL-cholesterol than males (HDL-cholesterol, 1.07±0.050 versus 0.92±0.040; P<0.05), Multilogistic Regression Analysis was performed to investigate the association between HDL and WBC count by taking age and sex as covariates further. When taking these variables into account, HDL-cholesterol remained negatively correlated with WBC count in these patients (P=0.02). Consistent with previous reports,24,25 patients received statins treatment had higher HDL-cholesterol. Both statins and aspirin treatments did not affect WBC count. Next, we measured HSPC (Lin-CD34+CD38-CD45RA-/low) in the blood of patients with low and high levels of HDL. The percentage of HSPC in mononuclear cells in PB was 0.025% in the patients with low HDL and 0.014% in patients with normal HDL (Figure 6C and 6D; Figure VI in the online-only Data Supplement; n=6–11; P<0.05). We could also detect SR-BI expression on human HSPC (n=6; Figure 7E). Hence, as in mice, in humans HDL levels seem to be correlated with leukocytosis and the presence of higher numbers of HSPC, that also express the HDL receptor SR-BI, in the blood.

Discussion

Accumulated studies have described the antiatherosclerotic properties of HDL. Besides reverse cholesterol transport, HDL promotes endothelium repair to maintain endothelium integrity and inhibits inflammatory cell infiltration to lesion site.26–29 Most of the beneficial regulation of HDL is mediated through HDL receptor Abca1, Abcg1, and SR-BI.26,30–32 Apart from that, SR-BI also plays a major role in the clearance of...
lipopolysaccharide.33 Recently, others and we reported that HDL suppresses HSPC proliferation, resulting in the inhibition of leukocytosis and atherosclerosis progression.2,3,34 Although we described that SR-BI is expressed on murine neutrophils,109/L 4.9±0.27 3.7±0.28 0.004* LDL, mmol/L 2.85±0.184 3.74±0.323 0.014* Total cholesterol, mmol/L 4.54±0.219 5.3±0.315 0.046* Platelet 227±15.4 211±15.6 0.93 Glucose, mmol/L 9.4±1.12 9.5±1.01 0.97 Red blood cells 4.2±0.18 4.1±0.18 0.43 Total triglyceride, mmol/L 2.12±0.271 1.69±0.284 0.30

Patients diagnosed as coronary heart disease were enrolled in the study. Their disease history and medical measurements are characterized in the Table. BMI indicates body mass index; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.

HDL Inhibits Hematopoietic Stem Cells via SR-BI

Figure 6. Negative correlation between high-density lipoprotein (HDL), white blood cell (WBC), and hematopoietic stem/progenitor cells (HSPC) in peripheral blood (PB) of human subjects. In patients with coronary heart disease the correlation between HDL level and WBC and neutrophil count was assessed. Patients whose WBC count exceeded 10×10⁹/L or neutrophil frequency exceeding 75% were excluded to avoid the influence of acute inflammation. A, Correlation between HDL and WBC count. r=−0.339, P=0.04, n=37. B, Correlation between HDL and neutrophil count. r=−0.356, P=0.031, n=37. C, When mononuclear cells in PB (PBMC) were stained with anti-CD34, CD38, CD45a and lineage antibodies, and the number of CD34+CD38−Lin-cells in PBMC of patients with low (<1.04 mmol/L) and normal HDL (>1.04 mmol/L) was analyzed. D, Representative dot plots demonstrate CD34−CD45RA− HSPC when gated on Lin− CD38− cells. BMC indicates bone marrow cells.
(1) higher HSPC frequency and more HSPC proliferation in SR-BI−/− on HFD than SR-BI+/− on HFD; (2) apoA-I infusion prohibited Akt phosphorylation in HSPC of DKO mice on HFD but not in SR-BI−/− on HFD; and correspondingly, (3) infusion of human apoA-I reduced HSPC frequency and inhibited HSPC proliferation in DKO mice on HFD, but not in SR-BI−/− mice on HFD; (4) in vitro inhibition of Akt phosphorylation reduced SR-BI−/− LSK expansion more than that of SR-BI+/− LSK cells when both were isolated from HFD feeding mice. Apart from higher pAkt in HSPC of SR-BI−/− mice on HFD, we also detected more phospho-p38MAPK on HSPC in these mice. Addition of phospho-p38MAPK inhibitor restrained more SR-BI−/− LSK expansion than SR-BI+/− LSK when they were isolated from mice on HFD. Thus, both Akt phosphorylation and p38MAPK phosphorylation may contribute to HSPC activation and proliferation, which are downstream of SR-BI. However, to prove this fully, in vivo Akt and p38MAPK inhibition studies will be needed, which can technically not be performed. Apart from these findings, we noticed that neither apoA-I infusion nor NAC injection altered cholesterol level in the blood of these mice, despite inhibition prohibited Akt phosphorylation in HSPC of DKO mice on HFD than SR-BI +/+ on HFD; (2) apoA-I infusion attenuated HFD-induced ROS production, HSPC proliferation, and leukocytosis in blood, inflammatory cell infiltration in plaque, and plaque progression.

**Acknowledgments**

Hereby, we express our sincere appreciation to Deney Van der Westhuyzen for providing SR-BI−/− mice and the littermates and advice for us to generate the colonies, to Dr Xuhong Wang, Dr Baoyu Zhang, and Dr Caiguo Yu from the Department of Endocrinology in Lu He hospital for providing patients profiles and blood samples, to Evelyn De Schryver and Thomas Vanweltend for their technical assistance, to Jan Stassen from Katholieke Universiteit Leuven, Leuven, for his advice.

**Sources of Funding**

This work was supported by FWO funding (G1508612N) to Y. Feng; Beijing Technology Foundation (Z131100006813018) to D. Zhao; National Institutes of Health (NIH) NHLBI R01HL112270, HL64146, and HL112276 to M.G. Sorci-Thomas; Major National Basic Research Program of the People’s Republic of China (2011CB503900 and 2012CB517505) and National Natural Science Foundation of the People’s Republic of China (30930037 and 81121061) to G. Liu; the Vanwayenberge funds, FWO funding (GO85111N), and NIH-P01-CA-65493-06 funding to C.M. Verfaillie.

**Disclosures**

None.

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Significance
The atheroprotective properties of high-density lipoprotein and scavenger receptor type Bl have been shown in multiple cell types, including hepatocytes, macrophages, endothelial cells, and endothelial progenitors. Hereby, we demonstrated that high-density lipoprotein suppresses hematopoietic stem/progenitor cell (HSPC) proliferation that is mediated via scavenger receptor type Bl. In addition, we highlighted the link between high-density lipoprotein-mediated inhibition of reactive oxygen species content in HSPC, reduced HSPC activation and leukocytosis, and attenuation of atherosclerosis progression, all of which is scavenger receptor type Bl dependent. Providing that inflammatory cells in atherosclerotic plaques are exclusively derived from HSPC, these findings not only enrich our knowledge of atherosclerosis in the context of high-density lipoprotein, scavenger receptor type Bl and HSPC, but also shed lights on therapeutic interventions of atherosclerosis.
Regulation of High-Density Lipoprotein on Hematopoietic Stem/Progenitor Cells in Atherosclerosis Requires Scavenger Receptor Type B1 Expression
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doi: 10.1161/ATVBAHA.114.304006
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplemental Materials

Supplementary figure I
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Supplementary figure I. Cholesterol and lipoprotein profiles of SR-BI<sup>+/+</sup> and SR-BI<sup>-/-</sup> mice on diet. SR-BI<sup>+/+</sup> and SR-BI<sup>-/-</sup> mice were fed on chow of high fat diet (HFD) for 8 weeks. Plasma samples were collected after overnight fasting. Total cholesterol, free cholesterol and cholesterol ester levels are shown in A, B and C, respectively. (D) Plasma of wt and ko mice on chow and HFD was separated by FPLC and cholesterol content in lipoproteins was quantified. CWT: wt mice on chow diet; CSR: ko mice on chow diet; HWT: wt mice on HFD; and HSR: ko mice on HFD. n=5 for each group.
Supplementary figure II. HSPC frequency in SR-BI^{+/+} and SR-BI^{-/-} mice on chow and high fat diet. Representative dot plots demonstrating the analysis of LSK cells in BMC. SR-BI^{+/+} and SR-BI^{-/-} mice were fed on chow or HFD for 8-10 weeks. BMCs were stained with anti-Lineage APC, anti-Sca-1 PerCP-Cy5.5 and anti-cKit APC-H7 antibodies. LSK cells were quantified by FACS. LSK frequency in BMC are illustrated.
Supplementary figure III. Identification of donor-derived inflammatory cells in atherosclerotic plaque. Cryosections were stained with biotin-CD45.2 and rat anti-mouse CD45 overnight and then streptavidin 555 and goat anti-rat Alexa 488. (A). Yellow arrows indicate cells derived from CD45.1 donor, whereas, red arrows indicate cells from CD45.2 donor. (B) CD45+ cells are shown in green and (C) CD45.2+ cells are shown in pink. Scale bar: 20 µm.
Supplementary figure IV. The effect of human apoA-I injection on atherosclerosis. SR-BI<sup>−/−</sup> and LDLr<sup>−/−</sup> apoA-I<sup>−/−</sup> (DKO) mice were fed on HFD and subcutaneously injected with saline or human apoA-I. Representative H&E stained aortic roots of SR-BI<sup>−/−</sup> mice that received saline (i) or human apoA-I injection (ii). As positive controls, aortic roots of DKO mice treated with saline (iii) or human apoA-I (iv). Scale bar: 200 µm.
Supplementary figure V. The effect of NAC injection on atherosclerosis in SR-BI<sup>−/−</sup> mice on high fat diet. Cryosections were performed Oil Red O staining. Positive staining areas are indicated by black arrows. (A) SR-BI<sup>−/−</sup> mice received high fat diet and saline injection; (B) SR-BI<sup>−/−</sup> mice received high fat diet and NAC injection. Scale bar: 400 µm.
**Supplementary figure VI. FACS analysis of murine and human HSPC.** (A) Murine BMC were stained with Ab cocktail against lineage, cKit, Sca-1, CD150 and CD48. From FSC and SSC, living BMC are indicated in the box. Shown by the blue arrow, lineage- cells are obtained when gated on living BMC. Shown by the red arrow, cKit⁺ Sca-1⁺ cells are obtained when gated on lineage- cells, which are called LSK cells. When gated on LSK cells, CD150⁺ CD48⁻ LT HSC are identified and indicated by the green arrow. (B) Murine BMC were stained with Ab cocktail against lineage, cKit, Sca-1, CD34 and FcR. From FSC and SSC, living BMC are indicated in the box. Followed by the blue arrow, lineage- cells are identified in the box. Followed by the red arrow, cKit⁺ Sca-1⁻ progenitors (i.e. lineage- cKit⁺ Sca-1⁻ cells) are shown in the box. Followed by the green arrow, when gated on lineage- cKit⁺ Sca-1⁻ cells, CD34⁺ FcR⁺ cells are granulocyte monocyte progenitors (GMP) and CD34⁺ FcR⁻ cells are megakaryocyte-erythroid progenitors (MEP), whereas CD34⁻ FcR⁻ cells are common myeloid progenitors (CMP). (C) Mononuclear cells in peripheral blood (PBMC) were isolated from human blood. PBMC were stained with Ab cocktail against lineage, CD38, CD45RA and CD34. From FSC and SSC, living PBMC are shown in the box. Indicated by the blue arrow, lineage- CD38⁻ cells are identified and shown in the box. When gated on lineage- CD38⁻ cells, CD45RA⁻/low/CD34⁺ cells are indicated by the red arrow.
Materials and Methods:

Human studies

Subjects: Patients (n=37) diagnosed with coronary heart disease in the Department of LuHe hospital (Beijing, China) were included in the study. General information including medical history, medication use, total cholesterol, LDL cholesterol, HDL cholesterol, triglyceride, glucose, blood pressure and white blood cell count were obtained. We excluded individuals with white blood cell (WBC) count ≥ 10,000cells/µl or ≥ 75% granulocytes, suggestive of acute inflammation. All patients signed an informed consent, approved by the Ethics Committee in LuHe hospital.

HSPC measurement in patients: Peripheral blood (PB) (2-5 ml) was collected from patients with low HDL (< 1.04 mmol/l) or normal HDL (1.04 ~ 1.53 mmol/l). Mononuclear cells in the PB (PBMNC) were isolated by Ficoll (GE Healthcare, Belgium) and stained with an anti-human Lineage cocktail APC (BD), anti-human CD34 APC (eBioscience), anti-human CD34PE (BD) and anti-human CD45RA PerCP-Cy5.5 (eBioscience). The frequency of HSPCs (Lin CD34+CD38+CD45RAlow cells) was determined on a Gallios apparatus (Beckman Coulter).

SR-BI expression on human HSPC: hPBMC were stained with rabbit anti-mouse SR-BI (1 µg/1x10^6 cells, Clone Ab3, Abcam) for 20 min and then goat anti-rabbit Alexa 488 1/400 (Invitrogen), anti-human Lineage APC, anti-human CD38 APC, and anti-human CD34 PE for 20 min. SR-BI expression on HSPC was quantified by FACS.

Murine studies

Mice, diet and treatments: Wild type C57BL/6J (CD45.2) and B.6SJL-PTPRCA (CD45.1) were used at the age of 8-12 weeks. SR-BI knockout (SR-BI−/−) mice and SR-BI+/− littermates were kindly provided by Prof. Deneys van der Westhuyzen from Kentucky University. Homozygous LDL receptor knockout (LDLr−/−) and apoAItm1/unc mice were purchased from Jackson Laboratory (Bar Harbor, Maine, U.S.A.). Mice homozygous for the apoAItm1/unc targeted mutation are in C57BL/6 background and have no apoA-I protein in the plasma. LDLr−/− mice were backcrossed with C57BL/6J mice for at least 6 generations to achieve 98.44% C57BL/6J background and then crossed with apoAItm1/unc mice for at least 9 generations to yield LDLr−/− apoAI−/− double knockout (DKO) mice, as previously described.1 At the age of 8-12 weeks, SR-BI−/− mice and control littermates were fed on HFD (34% fat, 1% cholesterol, Catalog no. D12492 mod, BioServices, the Netherlands) or chow diet for 8-10 weeks. For apoA-I studies, SR-BI−/− and SR-BI+/− mice, or DKO mice, fed with HFD for 11 and 9 weeks respectively, received during the last 3 weeks subcutaneous saline or lipid-free human apoA-I injection (500 µg per injection, 2 injections per week).2 For ROS inhibitor experiments, SR-BI+/− and SR-BI−/− mice were fed with a HFD and received i.p. injection of saline or N-acetylcysteine (NAC, Sigma-Aldrich, 1 mg/kg, daily) for 12 weeks. In total, 287 mice including 243 males and 44 females were used in this study. All the mice used were males except the following experiments: (1) the frequency of LT HSC, LSK and GMP in BM in Figure 1, in which 8 of 10 SR-BI+/− mice on chow diet were females and 8 of10 SR-BI−/− mice were females; (2) NAC injection experiments in Figure 5, in which 9 of 13 were SR-BI+/− females and 10 of 18 SR-BI−/− were females; and (3) the effect of apoA-I injection on irradiated LDLr−/− mice that were transplanted with SR-BI+/− BMC or SR-BI−/− BMC, in which 6 LDLr−/− mice were males and 6 LDLr−/− recipients were females. All mice were maintained in the animal facility of the KU Leuven or Beijing University. All experiments were carried out with approval of the ethics committees of the KU Leuven and of the Beijing University.

Lipoprotein separation by gel filtration: Murine plasma (50 µl) was separated on fast performance liquid chromatography (FPLC) as described before.3 Cholesterol and cholesterol esters content of plasma and gel filtration fractions were quantified.3
**Lipoprotein fractionation:** Plasma lipoproteins were fractionated and cholesterol content was measured as described before.\(^4\)

**White blood cell count:** Leukocytes, lymphocytes, monocytes and granulocytes in the PB were enumerated with an Ac-Tdiff hematology analyzer as described before (Beckman Coulter; Brea, CA, U.S.A.).

**Lineage negative cell and LSK cell isolation:** Lin- cells were isolated from BM cells using the Lineage cell depletion Kit (Miltenyi Biotech, Bergisch Gladbach, Germany). Lin- cells were stained with an anti-Sca-1, anti-Lin and anti-cKit antibodies (Abs) (see table xx) and LSK cells were isolated on a FACS Aria III (Becton Dickinson, NJ, U.S.A.).

**ELISA for pAkt:** To study the phosphorylation status of Akt in LSK cells, BM LSK cell from SR-BI\(^{+/+}\) and SR-BI\(^{-/-}\) mice on HFD were isolated by FACS. LSK cells were maintained in SFEM (Stem Cell Technologies, Vancouver, Canada) supplemented with SCF (50 ng/ml, Oxon, UK), TPO (100 ng/ml, R&D Systems) and IL-3 (10 ng/ml, R&D Systems) for 4 days. Cells were collected and enumerated. Equal amount of cells were added to a 96-well plate, fixed and stained with a primary Ab against pAkt and a secondary Ab according to the instruction. pAkt levels were measured by optical density (OD). Data are expressed as optical density (OD)/1x10^6 LSK cells.

**ELISA for Serum Amyloid A (SAA):** Plasma samples were subjected to SAA ELISA kit (Invitrogen) and SAA was measured according to the manual.

**Flow cytometry:** Details regarding antibodies used are as in Table 1. After red blood cell lysis, WBCs were stained with anti-mouse CD11b PE, anti-mouse Gr-1 APC and anti-mouse F4/80 APC-Cy7 and analysed by FACS. To quantify different HSPC populations in BM, spleen and PB, splenocytes and PB cells were stained with anti-Lineage cocktail, anti-Sca-1, anti-cKit Abs with or without anti-CD150 and anti-CD48 Abs, or anti-FcR Abs as described before.\(^3\) Granulocyte monocyte progenitors (GMP) (CD34\(^+\) FcR\(^-\) Lin\(^-\) Sca-1\(^-\) cKit\(^+\) cells), HSPC (or LSK cells; Lin\(^-\) Sca-1\(^-\) cKit\(^+\) cells) and LT-HSC (briefly HSCs; CD150\(^+\) CD48\(^-\) LSK cells) were quantified by FACS.

To study LSK cell proliferation, BrdU was injected i.p. in mice 12 hours before sacrifice. SR-BI\(^{+/+}\) and SR-BI\(^{-/-}\) BMCs were stained with antibodies against BrdU, cKit, Sca-1 and Lineage antigens, and BrdU incorporating LSK cells were quantified by FACS.\(^3\), \(^5\)

To assess Akt and p38 MAPK phosphorylation in LSK cells, BM cells were fixed, permeabilized and then stained with anti-Sca-1, anti-Lin and anti-cKit Abs combined with anti-pAkt or anti-p38MAPK, Abs.\(^3\) The percentage of pAkt+ LSK or p38MAPK+ LSK cells in LSK population was quantified by FACS.

To measure reactive oxygen species (ROS) content in LSK cells, BM cells were first stained with anti-Sca-1, anti-Lin and anti-cKit Abs for 20 min. After washing with PBS, cells were incubated with 2’-7’-dichlorofluorescein diacetate (DCF-DA) 10 µM at 37°C for 10 min. ROS\(^{high}\) LSK cells were quantified by FACS.

To evaluate chimerism, red blood cells in PB were lysed. White blood cells were stained with combinations of anti-CD45.1, anti-CD45.2, anti-CD4, anti-CD8, anti-B220, anti-CD11b PE, and anti-Gr-1 Abs.

FACS antibodies list is shown in Table 1.

All FACS analyses were performed using appropriate isotype control Abs. To obtain reliable quantifications, at least 100,000 events were acquired for BM cells and 10,000 events were acquired for Lin\(^{low}\) cells. Data were acquired using a FACSCanto (BD), FACS Aria III (BD) or Gallios (Beckman Coulter) apparatus and analyzed by FlowJo.
Table 1. FACS antibodies list. Fluorescent-conjugated primary antibodies that were used in flow cytometry are summarized below:

<table>
<thead>
<tr>
<th>antibodies</th>
<th>clones/catalogue number</th>
<th>company</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-mouse Sca-1 PerCP-Cy5.5, &amp; FITC</td>
<td>D7</td>
<td>eBioscience</td>
</tr>
<tr>
<td>anti-mouse Sca-1 PE</td>
<td>D7</td>
<td>BD</td>
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<tr>
<td>anti-mouse cKit PE</td>
<td>2B8</td>
<td>eBioscience</td>
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<td>anti-mouse cKit APC-H7</td>
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<td>BD</td>
</tr>
<tr>
<td>anti-mouse lineage cocktail APC</td>
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<td>BD</td>
</tr>
<tr>
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<td>M1/70</td>
<td>BD</td>
</tr>
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<td>anti-mouse CD45.1 FITC</td>
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<td>BD</td>
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<td>BD</td>
</tr>
<tr>
<td>anti-mouse CD45R/B220 APC</td>
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<td>BD</td>
</tr>
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<td>GK1.5</td>
<td>BD</td>
</tr>
<tr>
<td>anti-mouse CD8 APC-Cy7</td>
<td>53-6.7</td>
<td>eBioscience</td>
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<tr>
<td>Anti-mouse F4/80 APC-Cy7</td>
<td>BM8</td>
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<td>Cell Signaling technology (Bioké, Leiden, the Netherlands)</td>
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<tr>
<td>Phospho-p38 MAPK Alexa Fluor 555 conjugated</td>
<td>3D7</td>
<td>Cell Signaling technology (Bioké, Leiden, the Netherlands)</td>
</tr>
</tbody>
</table>

HSPC cultures:
LSK cells from SR-BI<sup>+/+</sup> and SR-BI<sup>-/-</sup> mice on HFD were cultured in SFEM medium supplemented with 50 ng/ml SCF and 100 ng/ml TPO with PBS or 10 µM SB203580 (pP38MAPK inhibitor, Calbiochem) or 10 µM Ly 204950 (pAkt inhibitor, Calbiochem) for 5 days and the cell number numerated. Data are expressed as fold reduction (inhibition vs. no inhibition).

LSK cells were isolated from the BM of SR-BI<sup>+/+</sup> and SR-BI<sup>-/-</sup> mice on HFD by FACS and seeded at 1000 LSK cells per well in 96-well plate, and cultured in SFEM containing 50...
ng/ml SCF, 100 ng/ml TPO and 10 ng/ml IL-3. Cells were collected and stained with anti-CD11b and anti-F4/80 Abs for FACS analysis.

Colony forming cell (CFC) frequency in BM of SR-BI+/+ and SR-BI−/− mice on chow or HFD were enumerated by seeding 1x10^4 BMC in methylcellulose assays and CFCs were scored 10 days after seeding.

**qRT-PCR:** Total RNA from sorted LSK cells were extracted using RNAeasy microkit (Qiagen, Valencia, CA). mRNA was reverse transcribed to cDNA using Ovation RNA Amplification System V2 (Nugen, The Netherlands). Primers used in this study are as following:

ABCA1: forward 5'-TAGCAGCACCCTGGTTGTC3' and reverse: 5'-TACGCGACACATAGGTACG3';

β-actin: forward 5'-CGTGGGCCGCCCTGGGCACCA3' and reverse:5'-TGGCCTTAGGGTTCAGGGGGG3'.

**Transplantation studies:**
To compare the number of HSPC in BM of SR-BI+/+ and SR-BI−/− mice on chow diet, SR-BI+/+ or SR-BI−/− BM cells (CD45.2+) were mixed in limiting dilution studies with CD45.1 BM cells in the ratios of 1:3, 1:1 or 3:1 and injected into irradiated CD45.1 recipients via tail vein. Four and sixteen weeks after transplantation, blood cells were stained with anti-CD45.2 and anti-CD45.1 to evaluate the chimerism. Data were expressed as the ratio between CD45.2 and CD45.1.

In other studies, SR-BI+/+ or SR-BI−/− BM cells mixed with equal amounts of CD45.1 BM cells were injected into irradiated LDLr−/− recipients. Following transplantation, LDLr−/− recipients were first fed on chow diet for 8 weeks and switched to HFD for another 8 weeks, and chimerism, expressed as the ratio of CD45.2 and CD45.1, was assessed at 16 weeks.

LDLr−/− recipients were lethally irradiated and then received 7 x 10^6 SR-BI+/+ or SR-BI−/− BMC via tail vein. Five days after transplantation, mice were placed on HFD for 8 weeks. Starting from 5th weeks of HFD, mice were subcutaneously injected with 500 µg purified human apoA-I twice per week for three weeks. After sacrifice, BMC were stained with an Ab cocktail containing anti-mouse Lineage APC, anti-mouse Sca-1 FITC and anti-mouse cKit PE. LSK cells were quantified by FACS.

**Histology and immunostaining of hearts and aortas:** After perfusion with saline and 4% paraformaldehyde, hearts and aortas were dissected. Cryosections of 7µm thickness were obtained. Morphometric analysis was performed on H&E stained slides using KS300 software (Carl Zeiss, Oberkochen, Germany). Alternatively, to quantify plaque area, cryosections were stained with oil red O (ORO) staining (counterstained with hematoxylin) as described before. Cryosections were incubated with rat anti-mouse CD45 (5 µg/ml, Biolegend, San Diego, U.S.A.) and biotin CD45.2 (5 µg/ml, BD) overnight and then labeled with goat anti-rat Alexa 488 1/500 (Invitrogen) and streptavidin 555 1/500. For negative controls, slides were probed with rat anti-mouse IgG (5 µg/ml, DAKO) overnight and then labeled with goat anti-rat Alexa 488 1/500 and streptavidin 555 1/500. CD45.2-derived inflammatory cells in the atherosclerotic plaques were identified and quantified using the Axiovision software (Axioimager Z1 microscope, Carl Zeiss) on Z-Stack images after extended focus computation (Carl Zeiss).

**Statistics:** Data were expressed as mean ± SD. The mouse data were first tested for normal distribution by D'Agostino-Pearson Omnibus test (when there were no less than 8 values per group) or Shapiro-Wilk test (when there were no less than 7 values per group). For data confirmed normal distribution, in the experiment where there 2
experimental groups, unpaired, 2-tailed Student’s t test was used; In the experiments where there were no less than 3 groups, One-way ANOVA with Dunnett was used when comparing treated groups against control and ANOVA with Bonferroni was applied when comparing all groups. The mouse data that did not pass Normality test or less than 7 values per group, nonparametric Mann Whitney analysis was used to compare 2 groups and nonparametric Kruskal-Wallis analysis was used to compare 3 or more than 3 groups. For human study, nonparametric Spearman analysis was performed by SAS. A P value less than 0.05 was considered significant.

References:


