

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

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**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<sup>-/-</sup>) 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<sup>-</sup> Sca1<sup>+</sup> cKit<sup>+</sup> cells (LSK cells, so-called HSPC) was found in the bone marrow of SR-BI<sup>-/-</sup> mice when compared with wild-type mice. HFD induced a further expansion of CD150<sup>+</sup>CD48<sup>-</sup> LSK cells (HSC), HSPC, and granulocyte monocyte progenitors in SR-BI<sup>-/-</sup> mice. Injection of reactive oxygen species inhibitor N-acetylcysteine attenuated HFD-induced HSPC expansion, leukocytosis, and atherosclerosis in SR-BI<sup>-/-</sup> 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<sup>-/-</sup>) apoA-I<sup>-/-</sup> mice on HFD but had no effect on SR-BI<sup>-/-</sup> mice on HFD. Transplantation of SR-BI<sup>-/-</sup> bone marrow cells into irradiated LDLR<sup>-/-</sup> 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.<sup>1</sup> Recently, Yvan-Charvet et al<sup>2</sup> 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*<sup>-/-</sup> *Abcg1*<sup>-/-</sup> mice and C57BL/6 mice, therefore, limiting white blood cell (WBC) expansion in the peripheral blood (PB).<sup>2,3</sup> In addition, we demonstrated that the HDL receptor, scavenger receptor type BI (SR-BI), is expressed on murine Lin<sup>-</sup> Sca1<sup>+</sup> cKit<sup>+</sup> (LSK cells, so-called HSPC cells),<sup>3</sup> 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<sup>4,5</sup> 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<sup>+</sup>CD48<sup>-</sup>LSK cells). HSCs give rise to multiple progenitor cells (CD150<sup>+</sup>CD48<sup>-</sup>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

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Nonstandard Abbreviations and Acronyms	
<b>apoA-I</b>	apolipoprotein A-I
<b>BM</b>	bone marrow
<b>BMCs</b>	bone marrow cells
<b>DKO</b>	LDL <sup>-/-</sup> apoA-I <sup>-/-</sup>
<b>GMP</b>	granulocyte monocyte progenitors
<b>HDL</b>	high-density lipoprotein
<b>HFD</b>	high-fat diet
<b>HSPC</b>	hematopoietic stem/progenitor cells
<b>LDL</b>	low-density lipoprotein
<b>LDL<sup>-/-</sup></b>	low density lipoprotein receptor knockout
<b>LSK cells</b>	Lin <sup>-</sup> Sca1 <sup>+</sup> cKit <sup>+</sup> cells
<b>LT-HSC</b>	long-term hematopoietic stem cells
<b>NAC</b>	N-acetylcysteine
<b>pAkt</b>	phospho-Akt
<b>PB</b>	peripheral blood
<b>ROS</b>	reactive oxygen species
<b>SR-BI</b>	scavenger receptor type BI
<b>WBC</b>	white blood cells
<b>WT</b>	wild-type

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<sup>-/-</sup> or Akt2<sup>-/-</sup> mice have only mild defects in hematopoiesis, HSPC function, including proliferation and reconstitution capacities, is severely affected in Akt1<sup>-/-</sup> Akt2<sup>-/-</sup> mice, suggesting an essential role of Akt in HSPC biology.<sup>6,7</sup> MAPKs are also a family of serine threonine kinases consisting of ERKs, JNKs, and p38MAPKs.<sup>8-10</sup> The role of ERKs in HSPC proliferation differentiation and survival is well established, as are the roles of JNKs on erythropoiesis, and p38MPAK activation in the regulation of erythropoiesis and myeloid differentiation.<sup>11</sup>

Aside from phospho-Akt (pAkt) and MAPK, another group of molecules that critically regulates HSPC proliferation and differentiation are reactive oxygen species (ROS).<sup>12,13</sup> 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.<sup>11</sup> Comparison between ROS<sup>high</sup> HSPCs and ROS<sup>low</sup> HSPCs showed that the ROS<sup>low</sup> population represents the quiescent HSPC population with self-renewal potential, whereas the ROS<sup>high</sup> HSPC subpopulation is activated, undergoes differentiation, and then exhaustion.<sup>12,13</sup> Moreover, pAkt acts upstream of ROS production,<sup>6</sup> whereas p38MAPK activation is downstream of ROS production in HSPCs.<sup>11</sup>

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.<sup>14</sup> Others and we have illustrated the antiatherosclerotic function of SR-BI in different mouse models.<sup>15-18</sup> Different from mice, SR-BI deficiency in humans leads to multiple pathophysiological phenotypes.<sup>19,20</sup> In mice, SR-BI

deficiency is associated with impaired HDL function, intracellular cholesterol accumulation, and increased oxidative stress.<sup>21</sup> We previously demonstrated that SR-BI is expressed on murine HSPC.<sup>3</sup> 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<sup>-/-</sup> 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 LDL<sup>-/-</sup> apoA-I<sup>-/-</sup> mice on HFD but not in SR-BI<sup>-/-</sup> mice on HFD. Third, transplantation of SR-BI<sup>-/-</sup> BM cells into irradiated LDL<sup>-/-</sup> 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<sup>-/-</sup> 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

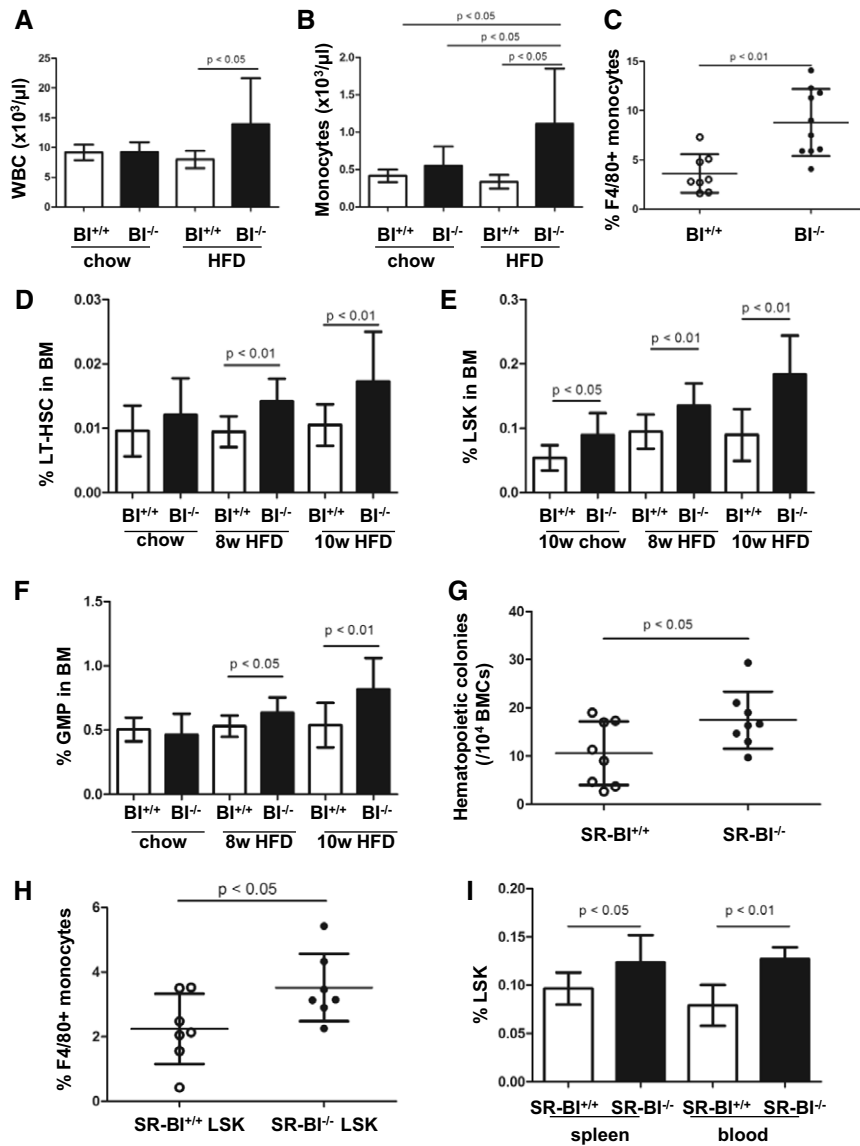
Materials and Methods are available in the online-only Supplement.

## Results

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

We have previously demonstrated that SR-BI, a HDL receptor, is expressed on murine HSPCs.<sup>3</sup> As infusion of reconstituted HDL or lipid-poor human apoA-I inhibits HSPC proliferation in hypercholesterolemic *Abca1*<sup>-/-</sup> *Abcg1*<sup>-/-</sup> mice and C57BL/6 mice,<sup>2,3</sup> we here investigated whether and how SR-BI might be involved in these effects. Eight-week-old SR-BI<sup>-/-</sup> and SR-BI<sup>+/+</sup> mice were fed on chow 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<sup>-/-</sup> 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<sup>-/-</sup> when compared with WT mice (24517±10625.1 versus 6489±1881.3 μm<sup>2</sup>; 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<sup>+</sup> FcR<sup>+</sup> Lin<sup>-</sup> Sca1<sup>+</sup> cKit<sup>+</sup>) in BM of SR-BI<sup>-/-</sup> and SR-BI<sup>+/+</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> when compared



**Figure 1.** High-fat diet (HFD) promoted leukocytosis, monocytosis in peripheral blood (PB) and hematopoietic stem/progenitor cell (HSPC) expansion in bone marrow (BM), which was associated with atherosclerosis development. SR-BI<sup>+/+</sup> and SR-BI<sup>-/-</sup> mice were placed on chow or HFD at 3 months onward for 8 or 10 weeks. Peripheral white blood cell (WBC) and monocyte counts are shown in **A** and **B**, respectively. **C**, To assess the components of monocytes in PB, blood cells of mice on HFD were stained with anti-CD11b PE and anti-F4/80 APC-Cy7, and macrophages were quantified by FACS. **D**, BM cells (BMCs) were stained with anti-CD150 FITC, anti-CD48 PE and Lin<sup>-</sup> Sca1<sup>+</sup> cKit<sup>+</sup> (LSK) markers and long-term hematopoietic stem cells (LT-HSC; CD150<sup>+</sup> CD48<sup>-</sup> LSK cells) was quantified by FACS. **E**, Quantification of LSK frequency in mice on chow and HFD. **F**, Quantification of GMP (CD34<sup>+</sup> FcR<sup>+</sup> Lin<sup>-</sup> cKit<sup>+</sup> Sca-1 cells) in BMCs. n=8 to 10. **G**, BMCs (1×10<sup>4</sup>) from SR-BI<sup>+/+</sup> and SR-BI<sup>-/-</sup> mice on HFD were seeded on methylcellulose and hematopoietic colonies were enumerated at day 10 to 14. **H**, LSK cells of SR-BI<sup>+/+</sup> and SR-BI<sup>-/-</sup> mice on HFD were sorted out by FACS and cultured in vitro for 10 days. Cells were stained with anti-CD11b PE and anti-F4/80 APC-Cy7 to study macrophage production. **I**, Quantification of LSK frequency in splenocytes and mononuclear cells in PB. To achieve a comparable analysis of HSPC frequency, 8 of 10 SR-BI<sup>+/+</sup> mice on chow diet were females and 8 of 10 SR-BI<sup>-/-</sup> mice on chow diet were females. The mice on HFD were all males. SR-BI indicates scavenger receptor type BI.

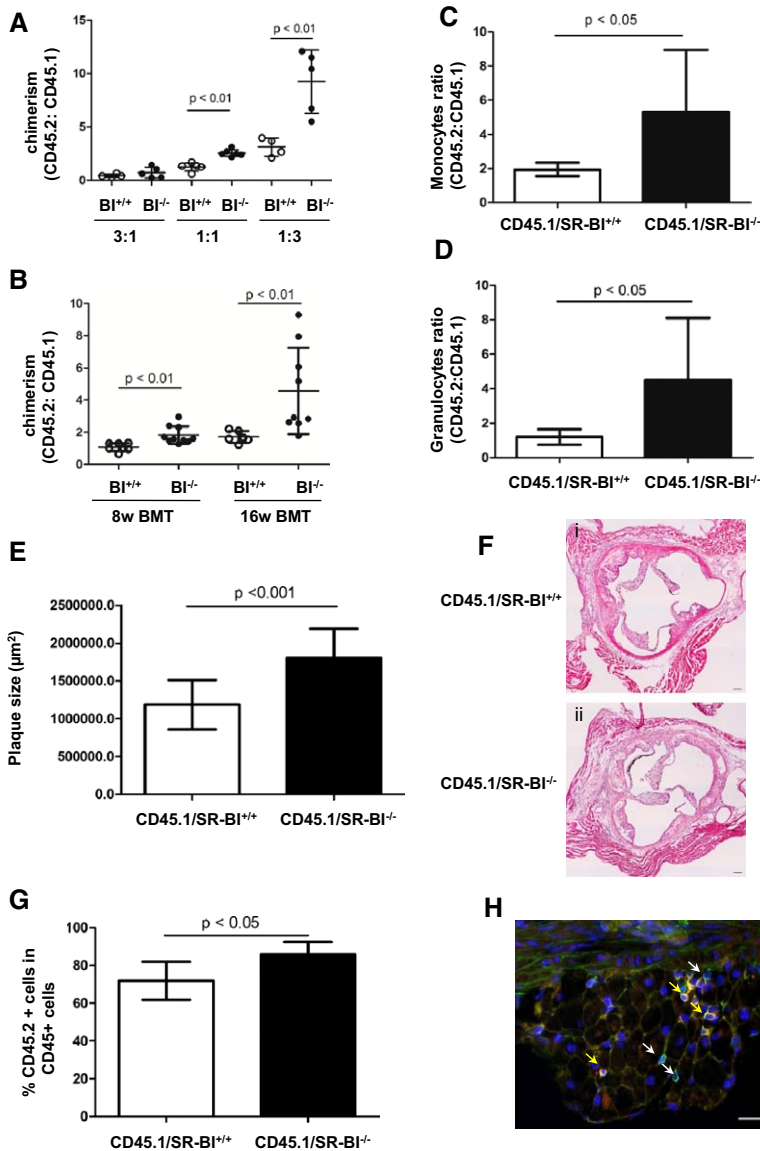
with WT mice (HSC%, 0.014% versus 0.009% at 8 weeks of HFD; 0.017% versus 0.011% at 10 weeks of HFD; n=11 for each, *P*<0.01 and LSK%, 0.135% versus 0.095% at 8 weeks of HFD; 0.184% versus 0.090%; n=11 for each; *P*<0.01; Figure 1D and 1E; Figures II and VI in the online-only Data Supplement). Although 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<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> LSK cells produced more F4/80<sup>+</sup> macrophages than SR-BI<sup>+/+</sup> LSK cells (n=7; Figure 1H). Finally, the frequency of LSK cells in the spleen and PB of

SR-BI<sup>-/-</sup> mice fed on HFD was significantly higher than in WT mice of HFD (n=4–8; Figure 1I).

### Transplanted SR-BI<sup>-/-</sup> 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<sup>-/-</sup> mice on chow diet further, we performed limiting dilution competitive repopulation studies. CD45.2<sup>+</sup> WT or SR-BI<sup>-/-</sup> BM cells were mixed with CD45.1 BM cells at ratios of 1:3, or 1:1 or 3:1, and injected in irradiated CD45.1 recipients. SR-BI<sup>-/-</sup> CD45.2 chimerism at 4 and 16 weeks after transplantation was significantly higher, consistent with increased frequency of progenitors and HSPCs in SR-BI<sup>-/-</sup> murine BM (n=4–6; Figure 2A).

We next determine whether SR-BI-deficient 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<sup>+/+</sup> or SR-BI<sup>-/-</sup> mice mixed with equal numbers of CD45.1 BM cells in irradiated LDLr<sup>-/-</sup> recipients. After

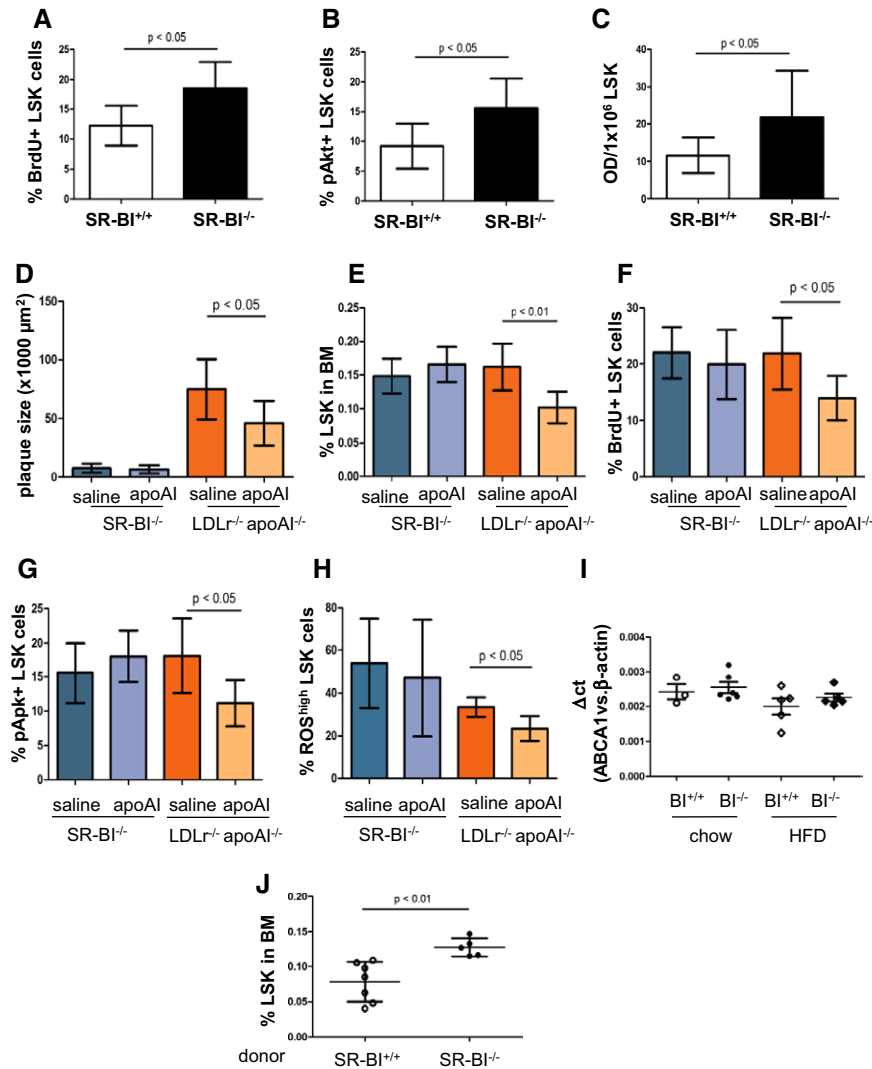


**Figure 2.** The effect of scavenger receptor type BI (SR-BI) deficiency on bone marrow (BM) reconstitution and plaque progression in hypercholesterolemic mice. **A**, To compare the Lin<sup>-</sup> Sca1<sup>+</sup> cKit<sup>+</sup> (LSK) frequency in mice on chow diet, SR-BI<sup>+/+</sup> or SR-BI<sup>-/-</sup> CD45.2 BM cells (BMCs) were mixed with CD45.1 BMCs at ratios of 3:1, 1:1, and 1:3 and injected in irradiated CD45.1 recipients. **B**, Equal numbers of SR-BI<sup>+/+</sup> or SR-BI<sup>-/-</sup> BMCs were mixed with CD45.1 BMCs and injected in irradiated LDLr<sup>-/-</sup> recipients. After BM transplantation (BMT), the recipients were first fed on chow diet for 8 weeks and then switched to high-fat diet (HFD) for another 8 weeks. Blood cells were stained with antimouse CD45.1 and antimouse CD45.2 to assess chimerism by FACS. **C** and **D**, The contribution of SR-BI<sup>+/+</sup> and SR-BI<sup>-/-</sup> HSPC to monocytes and granulocytes is shown in **C** and **D**, respectively. **E**, Quantification of atheroma in aortic roots of LDLr<sup>-/-</sup> mice transplanted with SR-BI<sup>+/+</sup> or SR-BI<sup>-/-</sup> BMCs. **F**, Representative H&E pictures of LDLr<sup>-/-</sup> mice received SR-BI<sup>+/+</sup> or SR-BI<sup>-/-</sup> BMCs. Scale bar, 200  $\mu\text{m}$ . **G**, Cryosections were stained with rat antimouse CD45 and biotin CD45.2 antibodies overnight and then goat antirat Alexa 488 and Streptavidin 555. CD45.1- and CD45.2-derived cells were quantified. Data are expressed as the percentage of CD45.2+CD45+ cells in CD45+ cells. **H**, Representative image demonstrating CD45 cells in the plaques. CD45.1-derived cells are indicated by white arrows, whereas CD45.2-derived cells are indicated by yellow arrows. Scale bar, 20  $\mu\text{m}$ . Male donors and recipients were used in both BMT experiments.

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<sup>-/-</sup> 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<sup>-/-</sup> than WT BM cells (n=6–10; Figure 2C and 2D). We also found accelerated atherosclerosis in the aortic roots of LDLr<sup>-/-</sup> recipients transplanted with SR-BI<sup>-/-</sup> BM cells when compared with that of WT BM cells (n=6–8; Figure 3E and 3F). To explore the contribution of SR-BI<sup>-/-</sup> and SR-BI<sup>+/+</sup>-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<sup>+</sup> cells in the plaques was 1.2-fold higher in recipients transplanted with SR-BI<sup>-/-</sup> 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<sup>-/-</sup> ApoA-I<sup>-/-</sup> Mice but Had No Effect on SR-BI<sup>-/-</sup> Mice

To determine whether the increased HSPC frequency in SR-BI<sup>-/-</sup> 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.<sup>3</sup> The percentage of BrdU incorporating LSK cells among LSK population was 12% in WT mice on HFD but increased to 18% in SR-BI<sup>-/-</sup> mice on HFD (SR-BI<sup>+/+</sup>, 12.2±3.32%; SR-BI<sup>-/-</sup>, 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<sup>-/-</sup> 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<sup>+/+</sup> and SR-BI<sup>-/-</sup> mice on HFD. After 4 days of culture in SFEM supplemented with

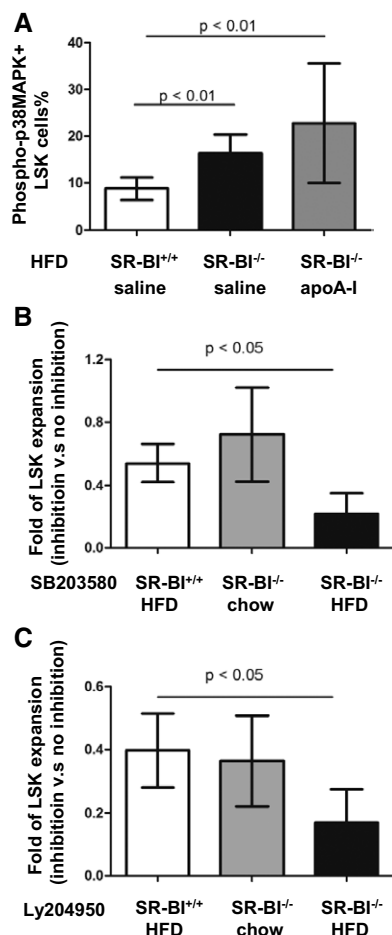


**Figure 3.** Human apolipoprotein A-I (apoA-I) infusion inhibited hematopoietic stem/progenitor cell (HSPC) proliferation, reduced reactive oxygen species (ROS) production in HSPC and reversed plaque progression. To quantify the proliferative status of HSPC in vivo, BrdU was injected in mice. BrdU-positive Lin<sup>-</sup> Sca1<sup>+</sup> cKit<sup>+</sup> (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<sup>-/-</sup> and LDLr<sup>-/-</sup> apoA-I<sup>-/-</sup> (double knockout [DKO]) mice that were placed on high-fat diet (HFD) and received saline or human apoA-I injection. Quantification of LSK frequency (E) and LSK proliferation (F) in BMC of SR-BI<sup>-/-</sup> and 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 (diacetylchlorofluorescein 2',7'-dichlorofluorescein diacetate). The percentage of ROS<sup>high</sup> LSK cells in the LSK population was quantified by FACS. Only male SR-BI<sup>+/+</sup>, SR-BI<sup>-/-</sup> and LDLr<sup>-/-</sup> apoA-I<sup>-/-</sup> mice were used in the apoA-I infusion experiments. I, ABCA1 expression in LSK cells of male SR-BI<sup>+/+</sup> and SR-BI<sup>-/-</sup> mice on chow and HFD. n=3 to 6. J, After apoA-I injection, LSK frequency in LDLr<sup>-/-</sup> recipients transplanted with SR-BI<sup>+/+</sup> or SR-BI<sup>-/-</sup> BMC. n=5 to 7. Six male LDLr<sup>-/-</sup> and 6 LDLr<sup>-/-</sup> female recipients were used in the BM transplantation experiment. SR-BI indicates scavenger receptor type BI.

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<sup>-/-</sup> 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 weekly for 3 weeks. Mice with deficiency of LDLr and apoA-I developed hypercholesterolemia and accelerated atherosclerosis when fed on atherogenic diet.<sup>22</sup> In addition, SR-BI is expressed in DKO mice. Thus, performing apoA-I infusion on DKO and SR-BI<sup>-/-</sup> mice would allow us to investigate the effect of SR-BI on cells. Consistent with previous reports,<sup>22</sup> apoA-I did not alter cholesterol levels in the blood (data not shown). However, apoA-I infusion reduced plaque size in DKO mice (45985±18951.1 versus 74878±25510.1 μm<sup>2</sup>; n=6–9; P<0.05; Figure 3D; Figure IV in the online-only Data Supplement).<sup>22</sup> 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). Interestingly, apoA-I infusion also reversed the increased ROS content in LSK cells of DKO mice on HFD (% ROS<sup>high</sup> LSK cells in LSK population: 33.5±4.50 versus 23.4±5.78; P<0.05; n=5–6; Figure 4H). In contrast to DKO mice, apoA-I infusion had no effect on plaque size, LSK cell proliferation, or Akt phosphorylation of HSPC in SR-BI<sup>-/-</sup> mice on HFD (n=4–7; Figure 3D–3H). To investigate whether regulation of apoA-I on HSPC requires SR-BI further, LSK cells were obtained from SR-BI<sup>+/+</sup> and SR-BI<sup>-/-</sup> mice for 8 weeks on chow diet or HFD and transcripts for ABCA1 and β-actin expression measured in LSK cells by qRT-PCR (Figure 3I). We performed another head-to-head comparison to confirm that SR-BI is required for apoA-I-mediated modulation of HSPC number. LDLr<sup>-/-</sup> recipients were lethally irradiated and then transplanted with 7×10<sup>6</sup> SR-BI<sup>+/+</sup> or SR-BI<sup>-/-</sup> BMC. Five days after BM transplantation, the recipients were switched



**Figure 4.** The roles of p38MAPK and Akt phosphorylation on Lin<sup>-</sup> Sca1<sup>+</sup> cKit<sup>+</sup> (LSK) quiescence. SR-BI<sup>+/+</sup> and SR-BI<sup>-/-</sup> mice were fed on high-fat diet (HFD) for 8 weeks and then injected with saline or apolipoprotein A-I (apoA-I) while keeping the mice on HFD. **A**, BMCs were stained with PE-conjugated antibody against phospho-p38MAPK and LSK antibodies. The percentage of phospho-p38MAPK<sup>+</sup> LSK cells in the LSK population was obtained by FACS. **B** and **C**, LSK cells from SR-BI<sup>+/+</sup> on HFD, SR-BI<sup>-/-</sup> on chow and HFD mice were isolated by FACS and cultured *in vitro* in the presence or absence of the phospho-p38MAPK inhibitor SB 203580 (**B**) and phospho-Akt inhibitor Ly 204950 (**C**). Four days after culture, the cell number was enumerated. Data are expressed as fold reduction when compared with cells cultured without inhibitor. Male SR-BI<sup>+/+</sup>, SR-BI<sup>-/-</sup> mice were used in the apoA-I infusion experiments *in vivo* and hematopoietic stem/progenitor cells expansion *in vitro*. SR-BI indicates scavenger receptor type BI.

from chow diet to HFD for 8 weeks. Starting from fifth week of HFD, 500  $\mu$ g 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<sup>-/-</sup> recipients transplanted with SR-BI<sup>+/+</sup> BMC when compared with that of LDLr<sup>-/-</sup> recipients transplanted with SR-BI<sup>-/-</sup> BMC (0.08 $\pm$ 0.028% versus 0.13 $\pm$ 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.

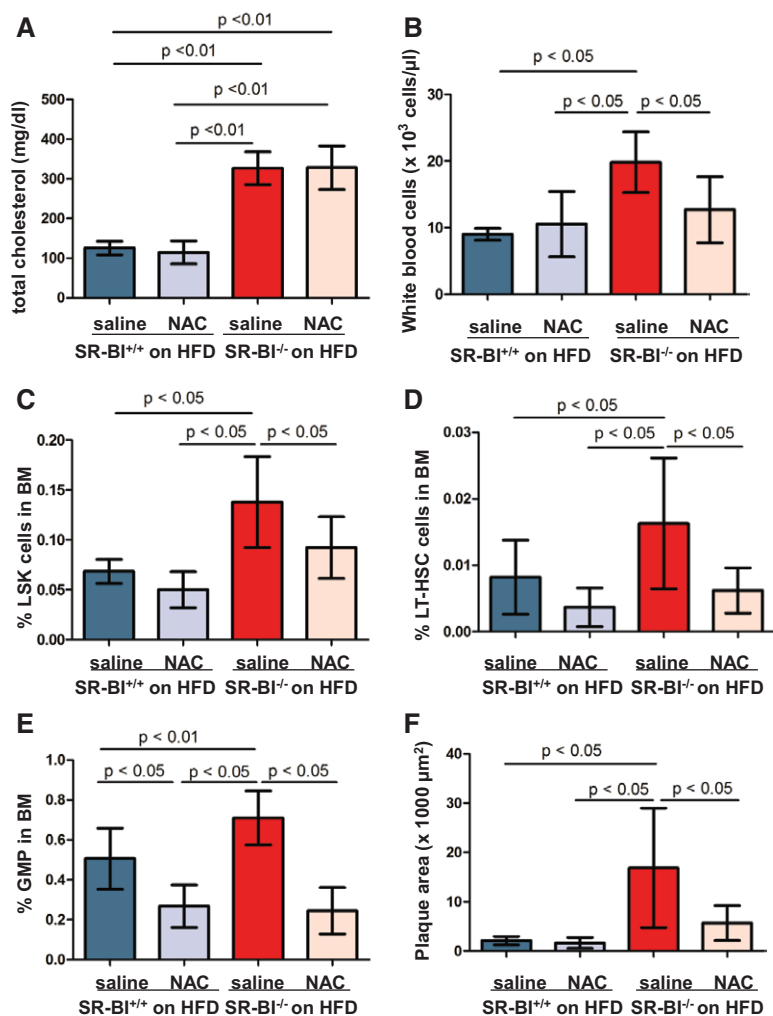
### Inhibition of Akt and p38MAPK Phosphorylation Maintained HSPC Quiescence in SR-BI<sup>-/-</sup> Mice on HFD

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<sup>+/+</sup> and SR-BI<sup>-/-</sup> mice were fed on HFD and received saline or apoA-I infusion as described above. BMCs of SR-BI<sup>+/+</sup> and SR-BI<sup>-/-</sup> mice were stained with antibodies against LSK markers and phospho-p38MAPK. FACS data demonstrated that the percentage of phospho-p38MAPK<sup>+</sup> LSK cells was 1.7-fold higher in SR-BI<sup>-/-</sup> mice when compared with SR-BI<sup>+/+</sup> mice (15.2 $\pm$ 4.01% versus 8.8 $\pm$ 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<sup>-/-</sup> mice further, LSK cells from SR-BI<sup>-/-</sup> 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<sup>-/-</sup> mice on chow diet. However, phospho-p38MAPK inhibitor further inhibited LSK expansion in SR-BI<sup>-/-</sup> 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<sup>-/-</sup> mice chow diet, respectively, when compared with control. Again, addition of LY 294002 further reduced LSK expansion in SR-BI<sup>-/-</sup> cells from HFD (n=5-6; Figure 4C).

### Inhibition of ROS Production Limited SR-BI<sup>-/-</sup> HSPC Expansion Induced by HFD

To determine whether the increased ROS levels found in LDLr<sup>-/-</sup> apoA-I<sup>-/-</sup> mice on HFD could also be linked to the SR-BI receptor further, NAC (1 mg/kg daily) was injected in SR-BI<sup>+/+</sup> and SR-BI<sup>-/-</sup> mice placed on HFD at 8 weeks for 12 weeks.<sup>23</sup> 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<sup>+/+</sup> mice, 160 $\pm$ 13.7 on saline group versus 157 $\pm$ 48.7  $\mu$ g/mL on NAC group; SR-BI<sup>-/-</sup> mice, 173 $\pm$ 9.4 versus 160 $\pm$ 57.7  $\mu$ g/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<sup>-/-</sup> mice on HFD (n=7-11; Figure 5C-5E). Furthermore, NAC injection significantly reduced plaque size 3.8-fold in SR-BI<sup>-/-</sup> mice



**Figure 5.** The effect of reactive oxygen species inhibition on hematopoietic stem/progenitor cell frequency and plaque progression. SR-BI<sup>+/+</sup> and SR-BI<sup>-/-</sup> mice were fed on high-fat diet (HFD), with or without N-acetyl-L-cysteine (NAC) injection for 12 weeks. Total cholesterol is shown in **A**. White blood cell count is displayed in **B**. Bone marrow cells (BMCs) were stained with anti-CD150 FITC, anti-CD48 PE, and Lin<sup>-</sup> Sca1<sup>+</sup> cKit<sup>+</sup> (LSK) antibodies to quantify the percentage of LSK cells (**C**) and CD150+CD48-LSK cells (**D**) in BMC. **E**, Quantification of granulocyte monocyte progenitor (GMP) frequency in BMCs of mice on HFD with or without NAC treatment. **F**, Plaque size in aortic roots of mice on HFD with or without NAC treatment. n=7 to 11 per group. In the entire NAC injection experiments, 9 of 13 SR-BI<sup>+/+</sup> mice were females and 10 of 18 SR-BI<sup>-/-</sup> mice were females. LT-HSC indicates long-term hematopoietic stem cells; and SR-BI, scavenger receptor type BI.

on HFD when compared with HFD-fed SR-BI<sup>-/-</sup> mice with saline injection (SR-BI<sup>+/+</sup> on HFD, 2112±849.4 μm<sup>2</sup>; SR-BI<sup>+/+</sup> on HFD and NAC injection, 1643±1096.5 μm<sup>2</sup>; SR-BI<sup>-/-</sup> on HFD, 17237±12131.6 μm<sup>2</sup>; SR-BI<sup>-/-</sup> on HFD and NAC treatment, 5691±3538.9 μm<sup>2</sup>; n=6–11; Figure 5F; Figure V in the online-only Data Supplement).

### 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,<sup>24,25</sup> 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<sup>-low</sup>) 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.<sup>26–29</sup> Most of the beneficial regulation of HDL is mediated through HDL receptor *Abca1*, *Abcg1*, and SR-BI.<sup>26,30–32</sup> Apart from that, SR-BI also plays a major role in the clearance of

**Table. Basic Characteristics of Patients Enrolled in the Correlation and Hematopoietic Stem/Progenitor Cell Studies**

Parameters	HDL<1.04 mmol/L	HDL>1.04 mmol/L	P Value
n	22	15	
Age, y	64±3.0	60±2.7	0.33
BMI, kg/m <sup>2</sup>	24.2±1.23	23.0±0.79	0.44
Systolic pressure, mm Hg	124.2±4.34	125.9±2.22	0.75
Diastolic pressure, mm Hg	78.33±3.66	75±3.59	0.52
White blood cells, 10 <sup>9</sup> /L	7.6±0.28	6.2±0.33	0.03*
Neutrophils, 10 <sup>9</sup> /L	4.9±0.27	3.7±0.28	0.004*
Red blood cells	4.2±0.18	4.4±0.18	0.43
Glucose, mmol/L	9.4±1.12	9.5±1.01	0.97
Platelet	227±15.4	211±15.6	0.93
Total cholesterol, mmol/L	4.54±0.219	5.3±0.315	0.046*
LDL, mmol/L	2.85±0.184	3.74±0.323	0.014*
Total triglyceride, mmol/L	2.12±0.271	1.69±0.284	0.30

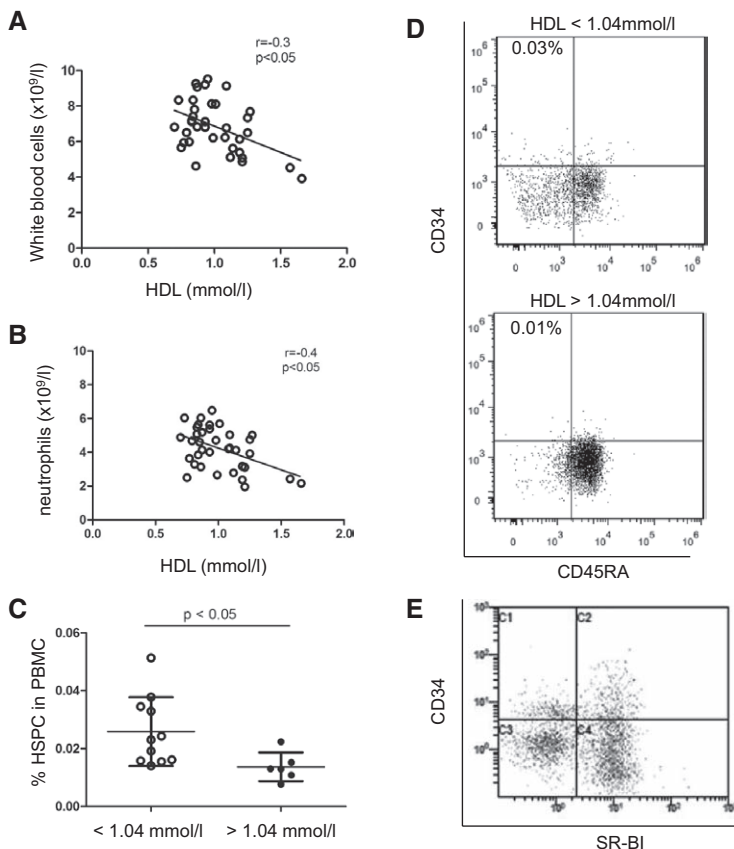
Patients diagnosed as coronary heart disease were enrolled in the study. Their disease history and medical measurements are characterized in the Table. BMI indicates body mass index; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.

\*Compared with patients with low HDL.

lipopolysaccharide.<sup>33</sup> Recently, others and we reported that HDL suppresses HSPC proliferation, resulting in the inhibition of leukocytosis and atherosclerosis progression.<sup>2,3,34</sup> Although we described that SR-BI is expressed on murine HSPC and that HSPC proliferation is inhibited by apoA-I

infusion,<sup>3</sup> whether and how SR-BI is involved in the HDL-mediated regulation of HSPC is unknown. Here, we demonstrate that SR-BI is required for the HDL-mediated regulation of HSPC quiescence, proliferation, and differentiation. The signaling pathways underlying the regulation of HDL on HSPC are Akt phosphorylation, p38 MAPK phosphorylation, and ROS production downstream of SR-BI.

Hypercholesterolemia stimulates monocyte and HSPC proliferation,<sup>2,3,35</sup> leading to leukocytosis, monocytosis, and atherosclerosis development. Like macrophages, HSPCs express *Abca1*, *Abcg1*, and apoE,<sup>2,34</sup> all of which regulate intracellular cholesterol homeostasis. Both *Abca1/Abcg1* and apoE deficiency impair cholesterol efflux, leading to cholesterol accumulation in lipid rafts in cell membranes. This change of lipid rafts leads to increased localization of receptors, such as IL-3/GM-CSF receptor on the surface, and modulates phosphorylation cascades originating from membrane-bound proteins.<sup>2,34</sup> As a result, proliferation and differentiation of HSPC are triggered, resulting in an enhanced WBC pool in the PB. In this study, we identified a role for SR-BI in HSPC quiescence versus proliferation in response to apoA-I. The phenotype of SR-BI<sup>-/-</sup> mice shared some features in common with *Abca1*<sup>-/-</sup> *Abcg1*<sup>-/-</sup> and apoE<sup>-/-</sup> mice in the aspect of HSPC proliferation, leukocytosis, and accelerated atherosclerosis induced by hypercholesterolemia. On the basis of the nature of SR-BI in cholesterol transport and our findings, we assumed that both impaired cholesterol homeostasis and activated phosphorylation cascades may be involved in SR-BI<sup>-/-</sup> HSPC proliferation. This assumption is supported by several lines of observations:



**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 10×10<sup>9</sup>/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<sup>+</sup>CD38<sup>-</sup>CD45A<sup>-</sup>Lin<sup>-</sup> 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<sup>+</sup>CD45RA<sup>-</sup> HSPC when gated on Lin<sup>-</sup>CD38<sup>-</sup> cells. HSPC frequency is indicated in the left. **E**, PBMC were stained with rabbit antimouse scavenger receptor type BI (SR-BI) and then goat antirabbit Alexa 488 and anti-CD34, CD38, and lineage Abs, SR-BI expression was detected on CD34<sup>+</sup>CD38<sup>-</sup>Lin<sup>-</sup> cells by FACS. The representative dot plots showed SR-BI expression in CD34<sup>+</sup> cells when gated on Lin<sup>-</sup> and CD38<sup>-</sup> cells. BMC indicates bone marrow cells.



(1) higher HSPC frequency and more HSPC proliferation in SR-BI<sup>-/-</sup> on HFD than SR-BI<sup>+/+</sup> on HFD; (2) apoA-I infusion prohibited Akt phosphorylation in HSPC of DKO mice on HFD but not in SR-BI<sup>-/-</sup> 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<sup>-/-</sup> mice on HFD; (4) in vitro inhibition of Akt phosphorylation reduced SR-BI<sup>-/-</sup> LSK expansion more than that of SR-BI<sup>+/+</sup> LSK cells when both were isolated from HFD feeding mice. Apart from higher pAkt in HSPC of SR-BI<sup>-/-</sup> mice on HFD, we also detected more phospho-p38MAPK on HSPC in these mice. Addition of phospho-p38MAPK inhibitor restrained more SR-BI<sup>-/-</sup> LSK expansion than SR-BI<sup>+/+</sup> 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 they suppressed HSPC proliferation. These data suggest that intracellular signaling pathways may contribute more substantially than lipid raft to the determination of HSPC cell fate.

As described earlier, HSPC reside in low-oxygen BM niche and have low intracellular level of ROS, which help maintain their quiescence and self-renewal capacity.<sup>6,13</sup> In Ataxia telangiectasia (A-T) mutated (*Atm*<sup>-/-</sup>) mice, *Atm*<sup>-/-</sup> HSPC cycle more active than *Atm*<sup>+/+</sup> HSPC, resulting in HSPC exhaustion and loss of BM reconstitution capacity.<sup>11</sup> In *Mdm2*<sup>-/-</sup> mice, because of *Mdm2* deficiency, p53 expression was upregulated and ROS production was increased in HSPC, leading to enhanced cell death.<sup>36</sup> Prohibition of ROS production by NAC treatment rescued ROS-induced HSPC defects in *Atm*<sup>-/-</sup> and *Mdm2*<sup>-/-</sup> mice.<sup>11,36</sup> In parallel, the adverse effects of ROS in atherosclerosis development have been extensively studied. All the findings consistently delineated that enhanced ROS production induced endothelial cell death and dysfunction,<sup>37,38</sup> plaque instability by ROS-induced activation of matrix metalloproteinases,<sup>39</sup> and inflammation.<sup>40,41</sup> In this study, we investigated the physiological and pathological meaning of ROS in the context of HSPC, HDL, SR-BI, and atherosclerosis. We demonstrate that hypercholesterolemia stimulated HSPC proliferation and enhanced ROS production, which was associated with plaque development in SR-BI<sup>-/-</sup> mice. ApoA-I infusion attenuated HFD-induced ROS production, HSPC expansion, and atherosclerosis progression in DKO mice. Similarly, NAC treatment reversed HFD-induced HSPC proliferation and leukocytosis and inhibited atherosclerosis progression in SR-BI<sup>-/-</sup> mice. Although the contribution of pAkt and phospho-p38MAPK on ROS production in HSPC is yet to be defined, suppression of ROS production in HSPC seemed to have a beneficial role in atherosclerosis. To our knowledge, this is the first study demonstrating a link between hypercholesterolemia, ROS, HSPC activation, and atherosclerosis.

By FACS, SR-BI was detected on human and murine HSPC. Furthermore, the number of WBC and HSPC in blood was intimately and negatively associated with HDL level in individuals.

Taken the mice findings together, we speculated that HDL could regulate human HSPC via SR-BI. Perspective, it would be interesting to investigate the relationship of HSPC and atherosclerosis in patients with SR-BI mutation and deficiency.

Overall, in line with the previous reports, this study confirmed the link between HSPC proliferation, leukocytosis, and atherosclerosis progression. In addition, we demonstrated that the HDL receptor, SR-BI is expressed on HSPC, as well as *Abca1* and *Abcg1*. SR-BI could play a critical role in the control of HSPC proliferation and differentiation, therefore, limiting HFD-induced leukocytosis in blood, inflammatory cell infiltration in plaque, and plaque progression.

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## Disclosures

None.

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## Significance

The atheroprotective properties of high-density lipoprotein and scavenger receptor type BI 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 BI. 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 BI dependent. Providing that inflammatory cells in atherosclerotic plaque are exclusively derived from HSPC, these findings not only enrich our knowledge of atherosclerosis in the context of high-density lipoprotein, scavenger receptor type BI and HSPC, but also shed lights on therapeutic interventions of atherosclerosis.