Hypercholesterolemia-induced priming of hematopoietic stem and progenitor cells aggravates atherosclerosis

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ABSTRACT Modulation of hematopoietic stem and progenitor cells (HSPCs) determines immune cell function. In this study, we investigated how hypercholesterolemia affects HSPC biology and atherosclerosis. Hypercholesterolemia induced loss of HSPC quiescence, characterized by increased proliferation and expression of cyclin B1, C1, and D1, and a decreased expression of Rb, resulting in a 3.6-fold increase in the number of HSPCs in hypercholesterolemic Ldbr−/− mice. Competitive bone marrow (BM) transplantsations showed that a hypercholesterolemic BM microenvironment activates HSPCs and skews their development toward myeloid lineages. Conversely, hypercholesterolemia-primed HSPCs acquired an enhanced propensity to generate myeloid cells, especially granulocytes and Ly6C+ monocytes, even in a normocholesterolemic BM microenvironment. In conformity, macrophages differentiated from hypercholesterolemia-primed HSPCs produced 17.0% more TNF-α, 21.3% more IL-6, and 10.5% more MCP1 than did their normocholesterolemic counterparts. Hypercholesterolemia-induced priming of HSPCs generated leukocytes that more readily migrated into the artery, which resulted in a 2.1-fold increase in atherosclerotic plaque size. In addition, these plaques had a more advanced phenotype and exhibited a 1.2-fold increase in macrophages and 1.8-fold increase in granulocytes. These results identify hypercholesterolemia-induced activation and priming of HSPCs as a novel pathway in the development of atherosclerosis. Inhibition of this proinflammatory differentiation pathway on the HSPC level has the potential to reduce atherosclerosis.—Seijkens, T., Hoeksema, M. A., Beckers, L., Smeets, E., Meiler, S., Levels, J., Tjwa, M., de Winther, M. P. J., Lutgens, E. Hypercholesterolemia-induced priming of hematopoietic stem and progenitor cells aggravates atherosclerosis. FASEB J. 28, 2202–2213 (2014). www.fasebj.org

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During homeostasis, hematopoiesis is balanced between the myeloid, lymphoid, and erythroid–megakaryocytic lineages. Hematopoietic stressors disturb this balance and skew hematopoietic stem and progenitor cell (HSPC) development. For example, severe hemorrhage or hemolytic anemia skew HSPC development toward the erythroid lineage, and bacterial infection rapidly induces granulopoiesis and increases the number of immature granulocytes in the peripheral blood (1–4). Besides these acute hematopoietic stressors, chronic inflammatory conditions alter HSPC homeostasis. Animals subjected to autoimmune arthritis or systemic lupus erythematosus exhibit increased myeloid output of the bone marrow (BM; refs. 5, 6). These

Abbreviations: αSMA, α-smooth muscle actin; ApoE, apolipoprotein E; BM, bone marrow; BMC, bone marrow cell; BMDM, bone marrow-derived macrophage; BrdU, bromodeoxyuridine; BMT, bone marrow transplantation; cBMT, competitive bone marrow transplantation; CFU, colony-forming unit; CFU-G, colony-forming unit granulocyte; CFU-M, colony-forming unit megakaryocyte; ChIP, chromatin immunoprecipitation; CMP, common myeloid progenitor; CVD, cardiovascular disease; EDTA, ethylenediaminetetraacetic acid; E-MPP, early multipotent progenitor; GMP, granulocyte–monocyte progenitor; H&E, hematoxylin and eosin; HDL, high-density lipoprotein; HDF, high-fat diet; HSPC, hematopoietic stem and progenitor cell; IL, interleukin; LDL, low-density lipoprotein; Ldbr−/−, low-density lipoprotein receptor knockout; Lin−, lineage negative; L-MMP, late multipotent progenitor; LSK, Lin− Sca1+ cKit+; LT-HSC, long-term hematopoietic stem cell; MCP1, monocyte chemotactic protein 1; PBS, phosphate-buffered saline; qPCR, quantitative PCR; Rb, retinoblastoma; SCF, stem cell factor; ST-HSC, short-term hematopoietic stem cell; TNF-α, tumor necrosis factor α; VLDL, very low density lipoprotein

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myeloid cells subsequently participate in the ongoing inflammatory response. Hence, inflammation-associated alterations in HSPC biology may have a decisive role in the persistence and/or progression of chronic inflammatory diseases.

Atherosclerosis is a chronic, hypercholesterolemia-driven inflammatory disease that results in the formation of plaques in the arterial wall (7). In early stages, immune cells, especially monocytes and granulocytes, are actively recruited to sites of vascular inflammation and promote plaque development by recruiting more inflammatory cells (8, 9). Subsequently, these immune cells produce proinflammatory chemokines and cytokines, thereby decisively influencing the propensity of a given atherosclerotic plaque to rupture and cause clinical manifestations (e.g., myocardial infarction and ischemic stroke; refs. 7, 8). An increased number of peripheral blood leukocytes, especially monocytes and granulocytes, is associated with occurrence and outcome of cardiovascular disease (CVD; refs. 10, 11). Hypercholesterolemia, a common risk factor in patients with CVD, promotes leukocytosis (12–14). Hypercholesterolemia is characterized by increased levels of low-density lipoprotein (LDL) and very low density lipoprotein (VLDL) and decreased levels of high-density lipoprotein (HDL; refs. 7, 13–16). As shown by other laboratories, lipoproteins regulate HSPC biology (17–19). HDL and apolipoprotein E (ApoE) suppress other laboratories, lipoproteins regulate HSPC biology sity lipoprotein (HDL; refs. 7, 13–16). As shown by

In this study, we investigated how hypercholesterolemia-induced alterations in HSPC homeostasis affect the inflammatory response and subsequent development of atherosclerosis in LDL receptor-knockout (Ldr−/−) mice.

MATERIALS AND METHODS

Mice

Male CD45.2-Ldr−/− and CD45.1-Ldr−/− mice were bred at the animal facilities of Maastricht University (Maastricht, The Netherlands) and the University of Amsterdam. The mice received chow or a high-fat diet (HFD) containing 16% fat and 0.15% cholesterol. The mice were given ad libitum access to food and were housed according to institutional guidelines. The animal experiment and care committees of Maastricht University and the University of Amsterdam approved the animal experiments.

Hematology and lipoproteins

Blood was obtained by venous or cardiac puncture and collected into ethylenediaminetetraacetic acid (EDTA)-containing tubes. Hematologic analysis was performed on a SciVet abc Plus+ (SciVet, Oostelbeers, The Netherlands). Individual lipoprotein levels were determined by fast-performance liquid chromatography (FPLC). In brief, the system contained a PU-980 ternary pump with an LG-980-02 linear degasser, an FP-920 fluorescence detector, and a UV 975 UV/VIS detector (Jasco, Tokyo, Japan). An extra P-50 pump (Pharmacia Biotech, Uppsala, Sweden) was used for inline addition of cholesterol PAP enzymatic reagent (Biomerieux, Marcy l’Etoile, France) at 0.1 ml/min. EDTA plasma was diluted 1:1 with Tris-buffered saline, and 30 μl sample/buffer mixture was loaded on a Superose 6 HR 10/30 column (GE Healthcare, Life Sciences Division, Diegem, Belgium) for lipoprotein separation at a flow rate of 0.31 ml/min.

Flow cytometry

BM from age- and sex-matched Ldr−/− mice was harvested in cold phosphate-buffered saline (PBS). BM cell (BMC) suspension was prepared and filtered through a 70-μm nylon mesh (Falcon; BD Biosciences, Breda, The Netherlands). Lineage depletion by magnetic bead isolation was performed according to the manufacturer’s instructions (Lineage Cell Depletion Kit; Miltenyi Biotec, Teterow, Germany). Blood was treated with red blood cell lysis buffer. Staining was performed with anti-mouse antibodies against the following antigens: CD3, 7-4, NK1.1, Ly6G, CD11b, CD5, and Gr-1 (all from BD Pharmingen, Breda, The Netherlands); CD117, Ter-119, CD45.1, CD45.2, B220 CD127, CD34, and CD32/16 (all from Ebiolscience, Vienna, Austria); CD135, CD150, and Sca-1 (all from Biologic, San Diego, CA, USA); and Ly6C (Miltenyi Biotech). Nonpecific binding was prevented by preincubuting the cells with an Fc receptor-blocking antibody. For cell-cycle analysis, lineage-negative (Lin−) BMCs were stained with the indicated primary antibodies, fixed in 70% ethanol for 24 h, and treated with propidium iodide/RNase buffer (PI/RNase; BD Biosciences; ref. 20). Staining was analyzed by flow cytometry (FACS Canto II; BD Biosciences).

Colony-forming unit in culture (CFU-C) assays

BM was isolated from normo- and hypercholesterolemic mice, and a single-cell suspension was prepared (20). BMCs (1×10^6) were cultured in 2 ml semisolid methylcellulose medium supplemented with growth factors (MethoCult; Stem Cell Technologies, Grenoble, France) at 37°C in 98% humidity and 5% CO2 for 7 d. CFU-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM), CFU-granulocyte-macrophage (CFU-GM), CFU-megakaryocyte (CFU-M), and CFU-granulocyte (CFU-G) colonies were scored after 7 d with an inverted microscope by T.S., in a blinded protocol.

Competitive BM transplantation (cBMT)

C57Bl6CD45.2-recipient mice were housed in filter-top cages and received drinking water containing antibiotics (polymyxin B sulfate, 6000 U/ml, and neomycin, 100 μg/ml) for 5 wk, starting 1 wk before the BMT. At 1 d before BMT, the mice were lethally irradiated (9.5 Gy, 0.5 Gy/min; MU15F/225 kV; Philips, Eindhoven, The Netherlands). The next day, the donor mice (CD45.2-Ldr−/− and normocholesterolemic and hypercholesterolemic CD45.1-Ldr−/− mice) were euthanized, and BMCs were obtained as just described. The donor mice were fed chow or an HFD for 4 wk. BM mononuclear cells were isolated via Lympholiter-M (Cedarlane-Sanbio, Uden, The Netherlands) and density centrifugation (20). Test cells (5×10^5) and competitor cells (1×10^6) were transplanted into the recipient mice. Reconstitution of peripheral blood leukocytes was analyzed by flow cytometry, as described above, at 4, 8, 12, 16, and 20 wk after BMT.
Bromodeoxyuridine (BrdU) incorporation

BrdU was injected intraperitoneally at 0.2 mg/g. BM was collected 16 h after injection, and Lin− cells were isolated as described earlier. BrdU incorporation was determined by intracellular staining with anti-BrdU antibodies, using the FITC BrdU Flow Kit (BD Biosciences).

Atherosclerosis

Male CD45.2-Ldlr−/− mice received HFD, starting at the age of 6 wk. At 2 wk after the start of the HFD, the mice underwent a cBMT, as just described. The donor mice were fed chow or an HFD for 4 wk. Test cells (5×10⁵) and competitor cells (1×10⁶) were transplanted into the recipient mice. The mice were euthanized at the age of 18 wk, and the arterial tree was perfused with PBS containing nitroprusside. The aortic arch and its main branch points were excised, fixed overnight in 1% paraformaldehyde in PBS, and embedded in paraffin. Twenty consecutive, longitudinal sections of the aortic arch were selected for histologic analysis. For plaque area and morphology, 4 sections (20 μm apart) were stained with hematoxylin and eosin (H&E), as described previously (21, 22). For phenotypic analysis, immunohistochemistry was performed for CD3 (Dako, Heverlee, Belgium), CD45 (BD Biosciences), Mac-3 (BD Biosciences), and α-smooth muscle actin (αSMA; Sigma-Aldrich, St. Louis, MO, USA). Sirius red staining was performed as described elsewhere (23). Morphometric analyses were performed with Las4.0 software (Leica, Wetzlar, Germany). Organs were analyzed by H&E staining, no abnormalities were observed. There were no differences between the experimental groups in body weight and cholesterol levels.

In vitro macrophage culture

BMCs were isolated from normo- and hypercholesterolemic mice and cultured in RPMI supplemented with 15% L929-conditioned medium to generate BM-derived macrophages.
(BMDMs; ref. 24). BMDMs were activated by lipopolysaccharide (LPS; 10 ng/ml; Sigma-Aldrich) for 6 h.

**Quantitative PCR (qPCR) and ELISA**

RNA was isolated from sorted Lin⁻/Sca1⁺/cKit⁻ (LSK) cells and BMDMs and reverse transcribed with an iScript cDNA synthesis kit (Bio-Rad, Veenendaal, The Netherlands). qPCR was performed with a SYBR Green PCR kit (Applied Biosystems, Leusden, The Netherlands) on a ViiA7 real-time PCR system (Applied Biosystems). Primer sequences are shown in Supplemental Table S1. Enzyme-linked immunosorbent assays (ELISAs) were used to determine monocyte chemotactic protein 1 (MCP1), tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and IL-10 levels (Invitrogen, Leusden, The Netherlands).

**Figure 2.** Hypercholesterolemic BM microenvironment activates HSPCs and promotes myelopoiesis. A) cBMTs were performed, in which normocholesterolemic CD45.1⁻/Ldr⁻/⁻ BMCs were transplanted into chow- or HFD-fed CD45.2⁻/Ldr⁻/⁻ recipients to analyze the effects of the hypercholesterolemic BM microenvironment on the development of HSPCs. B) Hypercholesterolemic BM microenvironment increased the number of LSK cells, CMPs, and GMPs. C) Hypercholesterolemic BM microenvironment activates HSPCs, as reflected by increased CD45.1⁺ leukocyte reconstitution of normocholesterolemic HSPCs in a hypercholesterolemic microenvironment. D, E) No differences in the reconstitution of T cells (D) and B cells (E) were observed. F–I) Transplantation of normocholesterolemic BM into a hypercholesterolemic microenvironment increased the reconstitution of myeloid cells, especially proinflammatory Ly6C<sup>high</sup> monocytes. F) CD45.1⁺CD11b⁺Ly6G⁻ cells. G) CD45.1⁺CD11b⁺Ly6G⁻ cells. H) CD45.1⁺ Ly6C<sup>high</sup> monocytes. I) CD45.1⁺ Ly6C<sup>low</sup> monocytes. *P < 0.05; **P < 0.01.
Chromatin immunoprecipitation (ChIP)

BMCs were isolated as described earlier. For ChIP, 1.5–2.0 × 10^7 BMCs were cross-linked with 1% formaldehyde. AcH3(K9/K14) ChIP was performed with 5 μg antibody (Cell Signaling Technology, Danvers, MA), as described previously (25). ChIP-qPCR was performed on an ABI ViiA 7 PCR system using SYBR Green Fast (Applied Biosystems). Relative enrichments are presented as the percentage of input. Primer sequences are shown in Supplemental Table S1.

Statistical analysis

Data are presented as means ± SEM and were analyzed by Student’s t test, or, when appropriate, by linear regression. Calculations were performed with GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Values of \( P < 0.05 \) were considered statistically significant.

RESULTS

Hypercholesterolemic mice have an expanded HSPC population

To investigate the effects of hypercholesterolemia on peripheral blood leukocyte numbers and HSPC responses, we fed Ldlr^{-/-} mice chow or an HFD, containing 16% fat and 0.15% cholesterol, for 10 wk and analyzed peripheral blood, spleen, and BM. The HFD induced a 3.5-fold increase in total plasma cholesterol levels, a 3.6-fold increase in LDL levels, and a 72-fold increase in VLDL levels (Supplemental Fig. S1 A, B). No changes in HDL levels were detected (Supplemental Fig. S1 A, B). Hypercholesterolemia reduced the HDL/LDL ratio from 0.73 to 0.30, reflecting a detrimental lipoprotein profile, comparable to human hypercholesterolemia (26).

No differences in platelet and erythrocyte numbers, spleen weights, and body weights were observed (data not shown). In addition, no differences in splenic leukocyte and HSPC populations were observed (data not shown). The absolute number of leukocytes showed a 2.2-fold increase in hypercholesterolemic mice (Fig. 1A).

Leukocyte subset analysis revealed that these mice exhibited a relative monocytosis, whereas relative lymphocyte counts were decreased (Supplemental Fig. S1C). Hypercholesterolemia increased the absolute number of monocytes by 6.3-fold, granulocytes by 2.7-fold, and lymphocytes by 1.7-fold (Supplemental Fig.

Figure 3. The hypercholesterolemic BM microenvironment expresses increased levels of proinflammatory cytokines. A–D) Hypercholesterolemia did not affect the expression of GM-CSF (A), M-CSF (B), G-CSF (C), or SCF (D) in the BM microenvironment. E–I) The expression of the cytokines TNF-α (E), IL-1β (F), and IL-6 (G), but not of IL-12 (H) and IL-10 (I), was increased in hypercholesterolemic BM. \( n = 4–6 \) /group. *\( P < 0.05 \); **\( P < 0.01 \).
Figure 4. Hypercholesterolemia induces a cell-intrinsic priming of HSPCs that persists during long-term normocholesterolemia and promotes myelopoiesis. A) cBMTs were performed, in which hypercholesterolemic CD45.1-Ldlr<sup>-/-</sup> BMCs were transplanted into chow-fed CD45.2-Ldlr<sup>-/-</sup> recipients, to analyze the development of hypercholesterolemia-primed HSPCs. B) Hypercholesterolemia-primed BM had an increased reconstitution of CMPs and GMPs, compared with nonprimed HSPCs. C) Hypercholesterolemia-primed BM had an increased total leukocyte reconstitution potential. D, E) T-cell (D) and B-cell (E) reconstitution was not affected. F–I) Hypercholesterolemia-primed HSPCs produced more granulocytes and monocytes, (continued on next page)
S1D). A strong correlation between total cholesterol levels and peripheral blood leukocyte count was observed, reflected by a Pearson’s correlation coefficient of 0.81 (Fig. 1B).

To investigate whether hypercholesterolemia-induced leukocytosis results from changes in HSPC homeostasis, we analyzed the BM. Flow cytometry revealed a 3.6-fold increase in the number of hematopoietic stem cells, defined as LSK cells, in hypercholesterolemic mice (Fig. 1C). Next, we analyzed the different subsets within the LSK population, including long-term hematopoietic stem cells (LT-HSCs; Lin− cKit+ Sca1+ CD150+ CD34− CD135− cells), short-term hematopoietic stem cells (ST-HSCs; Lin− cKit+ Sca1+ CD150+ CD34+ CD135− cells), early multipotent progenitors (E-MPPs; Lin− cKit+ Sca1+ CD150− CD34− CD135− cells), and late multipotent progenitors (L-MPPs; Lin− cKit+ Sca1+ CD150− CD34+ CD135− cells) (Supplemental Fig. S2A). No differences were observed, indicating that hypercholesterolemia did not affect the relative composition of the LSK population. However, because the LSK population was increased by 3.6-fold, the absolute number of LT-HSCs, ST-HSCs, E-MPPs, and L-MPPs was increased in the hypercholesterolemic Ldlr+/− mice. The number of common myeloid progenitors (CMPs; Lin− cKit+ Sca1+ CD16+ /32low), granulocyte–monocyte progenitors (GMPs; Lin− cKit+ Sca1− CD16− /32− cells), and mature BM granulocytes and monocytes were increased as well in hypercholesterolemic BM (Supplemental Fig. S2B–D). CFU assays showed that the myeloid colony-forming potential of hypercholesterolemic BM increased by 1.3-fold (Fig. 1D). Especially the CFU-G colonies and CFU-M colonies were increased (Supplemental Fig. S2E).

Together, these results show that hypercholesterolemia induces quantitative and functional alterations in the BM, characterized by an expanded HSPC population with an increased differentiation potential toward the myeloid lineages.

**Hypercholesterolemia disrupts HSPC quiescence**

To assess whether the expansion of the HSPC population is a result of increased proliferation, we analyzed BrdU uptake in LSK cells in normo- and hypercholesterolemic mice. BrdU incorporation was increased by 1.8-fold in hypercholesterolemic LSK cells compared with their normocholesterolemic counterparts (Fig. 1E). Flow cytometry using propidium iodide revealed that fewer hypercholesterolemic Lin− cells were in the G0/G1 phase, indicating that these cells were less quiescent than the normocholesterolemic Lin− cells (Fig. 1F). qPCR showed increased expression of *cyclin B1, D1, and E1* in hypercholesterolemic LSK cells, reflecting a proliferative cyclin profile (Fig. 1G).

Since Abca1, Abcg1, and increased expression of growth factors have been reported to be responsible for the hyperproliferative phenotype of HSPCs in *ApoE*−/− mice, we determined the expression of these genes on LSK cells of our hypercholesterolemic *Ldlr*+/− mice. However, we did not observe any differences in the expression of the *Abca1* and *Abcg1* transporters, or in the expression of the GM-colony-stimulating factor (GM-CSF) receptor, M-CSF receptor, or G-CSF receptor (Supplemental Fig. S3A–E). As the retinoblastoma (Rb) tumor suppressor family has a critical role in the regulation of both cell proliferation and differentiation of HSPCs (27), we analyzed the expression of Rb gene family members in normo- and hypercholesterolemic LSK cells. Hypercholesterolemic LSK cells showed reduced Rb expression, compared to normocholesterol LSK cells (Supplemental Fig. S3F). The expression of the Rb gene family members *p107* and *p130* was not affected (Supplemental Fig. S3G, H). ChIP revealed decreased acetylation of histone H3 on lysine 9/14 within the promoter of Rb, suggesting that epigenetic mechanisms regulate hypercholesterolemia-induced phenotype of HSPCs (Supplemental Fig. S3H).

Overall, these results show that hypercholesterolemic HSPCs are less quiescent than normocholesterol HSPCs and have a hyperproliferative phenotype, possibly mediated via epigenetic mechanisms.

**Naive HSPCs are activated by the hypercholesterolemic microenvironment**

HSPC homeostasis is tightly regulated by the BM microenvironment. To elucidate whether the BM microenvironment is involved in the hypercholesterolemia-induced activation of HSPCs, we performed cBMTs. To investigate the effect of a hypercholesterolemic microenvironment on HSPC differentiation, normocholesterolemic CD45.1-*Ldlr*−/− BMCs were transplanted into chow- or HFD-fed CD45.2-*Ldlr*−/− recipients (Fig. 2A). Transplantation of CD45.1-*Ldlr*−/− normocholesterolemic BM into a hypercholesterolemic BM microenvironment increased the reconstitution of CD45.1-*Ldlr*−/−/− LSK cells by 1.6-fold (Fig. 2B). In addition, the hypercholesterolemic niche induced a 2.3- and 2.8-fold increase in the reconstitution of CD45.1-*Ldlr*−/−/− CMP and GMP cells, respectively (Fig. 2B). Accordingly, the hypercholesterolemic niche increased the reconstitution of peripheral blood leukocytes at 2, 4, 6, and 10 wk after cBMT, compared with transplantation into a normocholesterolemic BM microenvironment (Fig.
Figure 5. Hypercholesterolemia-induced activation of HSPCs aggravates atherosclerosis. A) eBMTs were performed in which normo- or hypercholesterolemic CD45.1-\(Ldlr^{-/-}\) BMCs were transplanted into HFD-fed CD45.2-\(Ldlr^{-/-}\) recipients, to analyze the effects of hypercholesterolemia-induced HSPC activation on the development of atherosclerosis. B) Normocholesterolemic HSPCs, which were transplanted into an HFD-fed recipient, showed increased leukocyte reconstitution. C) Total atherosclerotic plaque area in the aortic arches of 18-wk-old \(Ldlr^{-/-}\) mice was increased in those that received normocholesterolemic BM. D) Representative H&E-stained sections of the aortic arch that show the branch point of the left common carotid artery. Mice that (continued on next page)
2O). No differences in the reconstitution of T and B cells were observed (Fig. 2D, E). However, the reconstitution of granulocytes and monocytes, especially inflammatory Ly6C<sup>high</sup> monocytes was increased, whereas patrolling Ly6C<sup>low</sup> monocytes were not affected (Fig. 2F–I). Thus, the hypercholesterolemic BM microenvironment activated HSPCs and skewed hematopoiesis specifically toward the proinflammatory myeloid lineages.

Next, we assessed the expression of myeloid growth factors in the normo- and hypercholesterolemic BM microenvironment. We observed a trend toward decreased expression of M-CSF and G-CSF under hypercholesterolemic conditions; no differences in the expression of GM-CSF and stem cell factor (SCF) were observed (Fig. 3A–D). It is known that hypercholesterolemia increases the expression of proinflammatory cytokines in the BM microenvironment (28). These cytokines are known to alter HSPC homeostasis, (e.g., increase proliferation and induce a myeloid lineage bias; refs. 29–31). Therefore, we analyzed the expression of these cytokines in the normo- and hypercholesterolemic BM microenvironments and found that hypercholesterolemia increased the expression of TNF-α, IL-1β, and IL-6 (Fig. 3E–G). No differences were observed in the expression of IL-12 and the anti-inflammatory cytokine IL-10 (Fig. 3H, I).

Together, these data demonstrate that the hypercholesterolemic BM microenvironment activates HSPCs and induces a myeloid lineage bias, possibly mediated by alterations in TNF-α, IL-1β, and IL-6 expression in the microenvironment.

**Hypercholesterolemia induces a long-term, cell intrinsic priming of HSPCs**

To investigate whether hypercholesterolemia results in cell intrinsic alterations in HSPC development, we performed a cBMT in which we transplanted normo- or hypercholesterolemic CD45.1<sup>Ldlr</sup><sup>−/−</sup> BM into chow-fed CD45.2<sup>Ldlr</sup><sup>−/−</sup> recipients (Fig. 4A).

At 10 wk after the cBMT, no differences were observed in the reconstitution of normo- or hypercholesterolemic CD45.1<sup>Ldlr</sup><sup>−/−</sup> LSK cells (Fig. 4B). However, hypercholesterolemic LSK cells gave rise to more myeloid progenitors, as indicated by a 1.9-fold increase in CMP and 2.2-fold increase in GMP (Fig. 4B). The reconstitution of CD45.1<sup>Ldlr</sup><sup>−/−</sup> leukocytes was increased by 1.3-fold in mice that received BM from hypercholesterolemic donors (Fig. 4C). We observed no differences in the reconstitution of T and B cells (Fig. 4D, E). However, hypercholesterolemic HSPCs exhibited an increased granulocyte and monocyte reconstitution 10 wk after transplantation into a normocholesterolemic microenvironment (Fig. 4F, G). Monocyte subset analysis revealed that especially the proinflammatory Ly6C<sup>high</sup> monocytes were increased, whereas the reconstitution of patrolling Ly6C<sup>low</sup> monocytes was not affected (Fig. 4H, I).

To determine the functional effects of hypercholesterolemia-induced HSPC priming, BMCs were isolated from chow- or HFD-fed Ldlr<sup>−/−</sup> mice and cultured under normocholesterolemic conditions in L929-conditioned medium to generate BMDMs. Macrophages differentiated from hypercholesterolemic HSPCs produced more TNF-α, IL-6, and MCP1 on activation (Fig. 4J). The production of the anti-inflammatory cytokine IL-10 was not affected.

Thus, hypercholesterolemia induces a cell-intrinsic priming of HSPCs, which persists under long-term normocholesterolemic conditions, is characterized by a hyperproliferative phenotype, and specifically promotes the development myeloid cells with an increased inflammatory propensity.

**Hypercholesterolemia-induced activation of HSPCs aggravates atherosclerosis**

To determine the effects of hypercholesterolemia-induced activation and priming of HSPCs on the development of atherosclerosis, we performed a cBMT in which HFD-fed CD45.2<sup>Ldlr</sup><sup>−/−</sup> recipients received normo- or hypercholesterolemic CD45.1<sup>Ldlr</sup><sup>−/−</sup> BMCs (Fig. 5A). Normocholesterolemic HSPCs showed an increased leukocyte reconstitution (Fig. 5B), which reflects the activation of normocholesterolemic HSPCs by the hypercholesterolemic microenvironment (Fig. 3A).

The aortic arch of the recipients was harvested 10 wk after the cBMT, and the extent of atherosclerosis was determined. A total of 56 lesions of mice that received hypercholesterolemic HSPCs and 63 lesions of mice that received normocholesterolemic HSPCs were analyzed by histology. Hypercholesterolemia-induced activation by the BM niche of naive HSPCs resulted in a 2.1-fold increase in atherosclerotic plaque size (Fig. 5C). Morphologic analysis revealed that these plaques were characterized by a more advanced phenotype (pathologic intimal thickening), whereas plaques in mice that received hypercholesterolemic HSPCs had a more initial phenotype (intimal xanthoma; Fig. 5D and Supplemental Fig. S4A). Hypercholesterolemia-induced activation of HSPCs increased the number of CD45.1<sup>+</sup> plaque leukocytes by 1.6-fold, indicating that the progeny of the hypercholesterolemia-primed HSPCs promoted the inflammatory response under-
ing atherogenesis (Fig. 5E). In accordance, these plaques had a more inflammatory phenotype, characterized by more macrophages (Mac3⁺), more granulocytes (Ly6G⁺), and more T cells (CD3⁺) (Fig. 5F–H) and contained 1.9-fold more collagen, reflecting the progressed plaque phenotype (Supplemental Fig. S4B). No differences in smooth muscle cell (αSMΑ⁺) content were observed (Supplemental Fig. S4C). Together, these data show that hypercholesterolemia-induced priming by the niche of naïve HSPCs aggravates the development of atherosclerosis, possibly by increasing the accumulation of proinflammatory myeloid progeny in the plaques.

DISCUSSION

Over the past decades, numerous studies have reported the association between peripheral blood leukocyte counts, especially myeloid cells, and the occurrence of CVD (10, 13, 31–34). For example, patients with an increased granulocyte/lymphocyte ratio (>3) have more advanced coronary artery disease [odds ratio (OR)=2.45; 95% confidence interval (CI), 1.76–3.42; P<0.001] and a higher risk of major cardiovascular events in the next 3 yr [hazard ratio (HR)=1.55; 95% CI, 1.09–2.2; P=0.01] compared with patients with a lower ratio (<2) (11). The mechanisms that link leukocyte counts to the development of atherosclerosis are poorly understood (11). Hypercholesterolemia, a major risk factor for atherosclerosis, may promote leukocytosis by altering HSPC homeostasis (16–19). However, the role of hypercholesterolemia-associated alterations in HSPC homeostasis in the development of atherosclerosis is unknown.

In this study, hypercholesterolemia induced a pathologic HSPC phenotype, which is characterized by reduced HSPC quiescence and a biased development toward the myeloid lineages. Increased LDL and VLDL levels and decreased HDL levels characterize hypercholesterolemia. Recent studies have demonstrated a role for lipoproteins in the regulation of HSPC biology. HDL and ApoE suppress HSPC proliferation in ApoE−/− mice by promoting ABCa1- and ABCg1-mediated cholesterol efflux, thereby decreasing the expression of growth factor receptors on the cell surface, including the IL-3 and GM-CSF receptors (17, 18). In our study, in which we used Ldlr−/− mice, we observed that the hypercholesterolemia-induced hyperproliferative HSPC phenotype was independent of ApoE. Moreover, we found that hypercholesterolemia did not affect the gene expression of growth factor receptors such as GM-CSF, G-CSF, and M-CSF and ABC transporters, such as ABCA1 and ABCG1 on HSPCs, or the expression of hematopoietic growth factor in the BM microenvironment, indicating that other mechanisms are involved. As the Rb tumor-suppressor family is known for its role in the integration of multiple cellular signals that control cell proliferation and differentiation, especially under stress conditions (27, 35, 36), we analyzed the expression of Rb family members in normo- and hypercholesterolemic HSPCs. We observed decreased gene expression of Rb in hypercholesterolemic HSPCs, whereas p107 and p130 were not affected. Our results indicate that hypercholesterolemia-induced epigenetic mechanisms may be involved in the down-regulation of Rb, which may explain why hypercholesterolemic-primed HSPCs maintain their pathologic phenotype under long-term normocholesterolemic conditions.

Besides the effects of direct priming of HSPCs by hypercholesterolemia, we also showed a prominent role for the hypercholesterolemic niche in altering the biology of HSPCs. Using cBMTs, we showed that the hypercholesterolemic BM environment promoted HSPC proliferation and myeloid differentiation. We observed elevated expression of IL-1β and TNF-α, cytokines known to promote HSPC proliferation and myeloid differentiation (3, 4, 28–30, 37, 38), suggesting that these cytokines are responsible for the hypercholesterolemia-induced activation and priming of HSPCs. Interestingly, TNF-α is also known to increase cell proliferation by promoting RAF-1-mediated inactivation of the Rb protein in vascular smooth muscle cells (39). A similar mechanism may also be true of myeloid cells, and may be an alternative explanation of the reduced Rb expression that we observed in hypercholesterolemic BM.

Our data show that there is a difference in dynamic responses of HSPC priming in terms of proliferation and differentiation over time. The effects of niche-mediated alterations on mature leukocyte reconstitution occur sooner after the BM transplantation, and are even more prominent than the effects of hypercholesterolemia-primed HSPCs. This suggests more a continuous role of the niche in the regulation of HSPC proliferation and shows that both direct and niche-induced hypercholesterolemic priming of HSPCs are important in HSPC proliferation and myeloid differentiation.

In early atherogenesis, myeloid cells are actively recruited to sites of vascular inflammation and critically contribute to the initiation of plaque development (8, 9). We observed that hypercholesterolemia-induced activation of HSPCs not only increased the development of proinflammatory myeloid cells, but also increased leukocyte accumulation in the atherosclerotic plaques. After homing to the inflamed endothelium, these cells produce proinflammatory chemokines and cytokines, which further propagate atherogenesis. We observed increased T-cell accumulation in plaques of mice that received normocholesterolemic BM compared with mice that received hypercholesterolemic BM. As shown by Kidani et al. and others (40, 41), hypercholesterolemia may directly affect T-cell progenitors and effector T cells, however, we observed that the lipid background of the transplanted BM did not affect circulating T-cell reconstitution or thymocyte reconstitution (data not shown). This suggests that the increased plaque T-cell content resulted from increased recruitment of these cells by proinflammatory plaque.
macrophages and not from alterations in T-cell development. As macrophages are the most abundant leukocyte subset in atherosclerotic plaques, we analyzed whether hypercholesterolemia-induced priming of HSPCs affects the inflammatory propensity of these cells. Compared to macrophages differentiated from normocholesterolemia-primed HSPCs, those derived from hypercholesterolemia-primed HSPCs produced increased levels of TNF-α, IL-6, and MCP1, thereby increasing plaque inflammation and disease progression.

In summary, we have shown that hypercholesterolemia induces a pathologic HSPC phenotype state that persists under long-term normocholesterolemic conditions and favors the development of proinflammatory myeloid cells that aggravate the development of atherosclerosis. Inhibition of this novel proinflammatory mechanism on the HSPC level harbors the potential to reduce atherosclerosis.

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