Hematopoietic Stem/Progenitor Cells Directly Contribute to Arteriosclerotic Progression via Integrin β_2

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ABSTRACT

Recent studies described the association between hematopoietic stem/progenitor cell (HSPC) expansion in the bone marrow (BM), leukocytosis in the peripheral blood, and accelerated atherosclerosis. We hypothesized that circulating HSPC may home to inflamed vessels, where they might contribute to inflammation and neointima formation. We demonstrated that Lin$^-$ Sca-1$^+$ cKit$^+$ (LSK cells) in BM and peripheral blood of LDLr$^{-/-}$ mice on high fat diet expressed significantly more integrin β_2, which was responsible for LSK cell adhesion and migration toward ICAM-1 in vitro, and homing to injured arteries in vivo, all of which were blocked with an anti-CD18 blocking antibody. When homed LSK cells were isolated from ligated artery and injected to irradiated recipients, they resulted in BM reconstitution. Injection of CD18$^{+/+}$ LSK cells to immunodeficient Balb/C Rag2$^-$ ɣC$^{-/-}$ recipients resulted in more severe inflammation and reinforced neointima formation in the ligated carotid artery, compared to mice injected with PBS and CD18$^{-/-}$ LSK cells. Hypercholesterolemia stimulated ERK phosphorylation (pERK) in LSK cells of LDLr$^{-/-}$ mice in vivo. Blockade of pERK reduced ARF1 expression, leading to decreased integrin β_2 function on HSPC. In addition, integrin β_2 function could be regulated via ERK-independent LRP1 pathway. Integrin β_2 expression on HSPC is regulated by hypercholesterolemia, specifically LDL, in pERK-dependent and -independent manners, leading to increased homing and localization of HSPC to injured arteries, which is highly correlated with arteriosclerosis. STEM CELLS 2015;33:1230–1240

INTRODUCTION

Inflammatory cells in atherosclerotic plaques are exclusively derived from hematopoietic stem/progenitor cells (HSPC). Recently, others and we reported that hypercholesterolemia promotes HSPC proliferation and differentiation toward atherogenic monocytes and granulocytes; of which elevated peripheral white blood cell levels have been described in atherosclerotic patients [1–3]. Although the link among HSPC proliferation in bone marrow (BM), leukocytosis in peripheral blood (PB), and accelerated atherosclerosis progression was noted, there is to date no evidence demonstrating the direct involvement of HSPC in plaque development.

It is well-known that HSPC reside in the hypoxic BM niche and give rise to all blood cells in postnatal life [4]. Under steady state conditions, a fraction of HSPC can be found in PB [5]. Circulating HSPC can migrate into peripheral tissues such as spleen, liver, lymph node, and aortic adventitia [6–9]. Although circulating HSPC migrate into lymph nodes and proliferate and differentiate in situ to become resident myeloid cells for host defense [6], the fate of HSPC that traffic into damaged tissues remains largely unknown. Nevertheless, there is evidence that when CD34+ hematopoietic progenitor cells migrate into the injured spinal cord, exacerbated neuroinflammation and progression of multiple sclerosis are observed [10]. Likewise presence of HSPC in asthmatic airways is associated with asthma progression [11]. HSPC trafficking in injured liver, by contrast, appears to be associated with improved liver function, even if the mechanism is unknown [8, 9]. We here tested the hypothesis that HSPC, under the influence of hypercholesterolemia, can home into the intima of inflamed arteries and contribute to arteriosclerotic plaques formation.

HSPC self-renewal versus differentiation and retention of HSPC within the BM niche are regulated by HSPC intrinsic factors as well as by extrinsic factors via engagement of

cytokine and growth factor receptors and adhesion receptors by their specific ligands. The integrins including $\alpha4\beta1$, $\alpha5\beta1$, $\beta2$, $\alpha6$ and some chemokines such as C-X-C chemokine receptor type 4 (CXCR4) and CD192 (i.e. CCR2) play important roles in retention and homing of HSPC from blood to the BM niche [12–15], mobilization of HSPC from the BM into the PB [16–19], and trafficking into peripheral tissues [8–10, 20]. In addition, CXCR4 has been identified as a key factor in hypercholesterolemia-induced HSPC mobilization from BM into PB [17, 21, 22]. However, it remains unknown what other key adhesion molecules are also affected and thus modify HSPC function by hypercholesterolemia. Expression and function of these adhesion receptors are regulated by a variety of signaling cascades, including the mitogen activated protein kinases (MAPK) signaling pathway [23–25]. Recently, Yves-Charvet reported extracellular signal regulated kinases 1/2(ERK1/2) activation in HSPC from hypercholesterolemic $Abca1^{-/-}$ $Abcg1^{-/-}$ mice [3]. In line with this study, our data demonstrated that low-density lipoprotein (LDL)-mediated differentiation of HSPC to granulocytes occurs in response to LDL-stimulated ERK1/2 activation [2]. This led us to determine if LDL affects integrin function and hence migration of HSPC into arteriosclerotic plaques via activation of the ERK pathway.

LDL receptor-related protein (LRP) is a member of the LDL-receptor family. It is expressed in a variety of cell types such as hepatocytes and leukocytes. More than 30 ligands have been discovered which explains the multiple functions of LRP [26]. Like other members in this family, LRP1 mediates cholesterol uptake via endocytosis. Aside from its function in cholesterol homeostasis, LRP1 has been found to interact with integrin $\beta2$ in leukocytes and therefore modulate integrin clustering on the membrane [27]. LRP1 deficiency abrogated integrin $\beta2$-dependent adhesion of leukocytes to endothelial cells [28]. Interestingly, an intimate association between LRP1 expression and ERK phosphorylation has been noticed in different cell types, all of which modulate cell adhesion and migration [29–31]. However, it is currently unknown if LRP1 regulates HSPC adhesion, migration or homing.

Here we report that hypercholesterolemia increased the percentage of integrin $\beta2^+$ Lin$^-$ Sca-1$^+$ cKit$^+$ (LSK) cells in LDLr$^{-/-}$ mice. Integrin $\beta2$ regulated LSK cell adhesion and migration toward to ICAM and homing to injured artery. Grafted integrin $\beta2^{+/+}$ LSK cells resulted in enhanced inflammation and neointima formation in the ligated artery, compared to injection of PBS and integrin $\beta2^{-/-}$ LSK cells. Finally, we demonstrate that LDL effects on integrin $\beta2$ expression and function are mediated by the ERK/ADP-ribosylation factor 1 (ARF1)-dependent and ERK-independent LRP1 pathway.

MATERIALS AND METHODS

Integrin $\beta2$ expressing HSPC were studied in LDLr$^{-/-}$ mice fed on chow or high fat diet (HFD) (34% fat, 1% cholesterol, Catalog no. D12492 mod, BioServices, The Netherlands, http://www.researchdiets.com/collection1?q=D12492). Complete ligation of right carotid artery was performed on B.6SJL-PTPRCA (CD45.1) mice, wild type (WT) C57BL/6J (CD45.2, H-2kb) mice, CD18$^{-/-}$ mice and their littermates or Balb/c Rag2$-$ ɣC$^{-/-}$ mice (H-2kd) mice for HSPC homing and injection experiment. Detailed methods are shown in Supporting Information data.

RESULTS

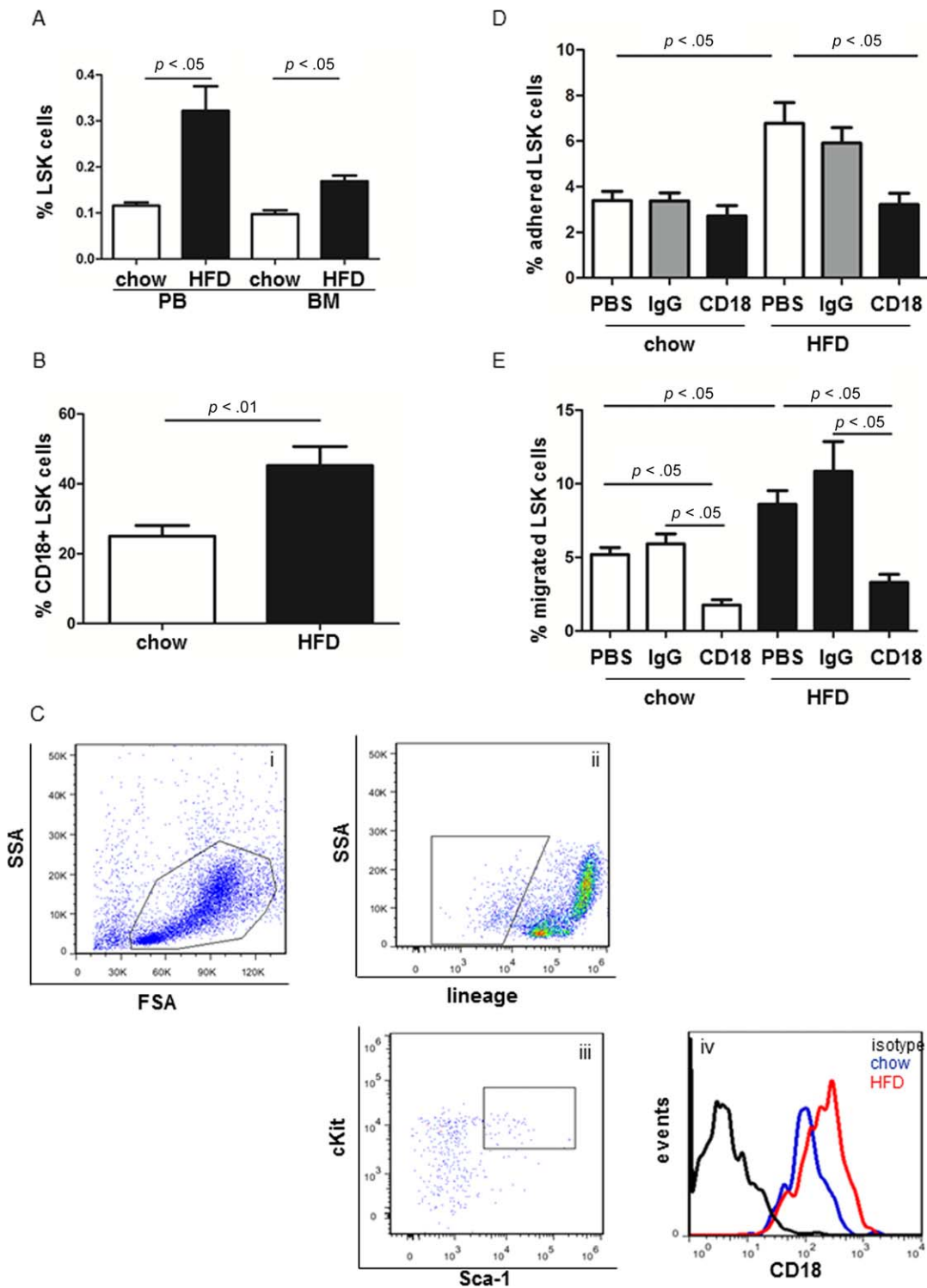### Hypercholesterolemia Increased Integrin β_2 Expression on LSK Cells

Adhesion molecules play critical roles in LSK function. Therefore, we first screened integrin expression on HSPC in LDLr$^{-/-}$ mice on chow diet and HFD. After 8 weeks of HFD, total cholesterol, LDL-c and high-density lipoprotein cholesterol (HDL-c) were dramatically increased in LDLr$^{-/-}$ mice compared to mice on chow diet (Supporting Information Fig. 1). Consistent with our previous findings [2], the frequency of LSK cells was significantly increased in PB and BM of LDLr$^{-/-}$ mice on HFD compared to those on chow diet (PB: $0.32\% \pm 0.053\%$ vs. $0.12\% \pm 0.007\%$, $n = 8$–10, $p < .01$; BM: $0.17\% \pm 0.012\%$ vs. $0.10\% \pm 0.008\%$, $n = 8$–10, $p < .01$) (Fig. 1A). When BM cells (BMCs) were stained with anti-CD18 and LSK Abs, the percentage of CD18$^+$ LSK cells in the LSK population from mice on HFD was higher than that of the chow diet group (%CD18$^+$ LSK cells: Chow: $25.04\% \pm 3.02\%$; HFD: $45.25\% \pm 5.41\%$, $n = 7$–8, $p < .01$) (Fig. 1B, 1C). Integrin $\beta1$ and $\alpha5$ expression was not different between LDLr$^{-/-}$ mice on chow and HFD (data not shown).

To determine if β_2 on LSK cells was functional, LSK cells from LDLr$^{-/-}$ mice on chow and HFD were isolated by fluorescence-activated cell sorting (FACS), incubated with or without a blocking anti-CD18 Ab or IgG control Ab for 20 minutes prior to the adhesion assay. LSK cells were plated on intercellular adhesion molecular-1 (ICAM-1)-coated 96-well plate for 2 hours. After nonadherent LSK cells were removed, adherent LSK cells were mixed with beads and enumerated by FACS. LSK cells from LDLr$^{-/-}$ mice on HFD displayed a 2.6-fold greater adhesion capacity to ICAM-1 compared with those from mice on chow diet, which was inhibited by a blocking anti-CD18 Ab ($n = 5$–8, Fig. 1D).

To further evaluate whether increased integrin $\beta2$ expression was associated with enhanced LSK migration over ICAM-1, Lin$-$ cells were isolated from LDLr$^{-/-}$ mice on chow and HFD, treated with phosphate buffered saline (PBS), anti-CD18 or IgG control Abs for 20 minutes, and then plated in transwells coated with ICAM-1. After 6 hours, cells in the lower chamber were collected, counted, and stained with anti-LSK Abs. The input Lin$-$ population was also stained with LSK Abs to enumerate the absolute number of LSK cells that had migrated to the lower chamber. LSK cells from HFD mice displayed a 1.6-fold higher migration capacity through ICAM-1-coated transwells compared to those from mice on chow diet. Anti-CD18 Abs inhibited LSK migration from mice on chow diet and HFD ($n = 5$–11, Fig. 1E).

### HSPC Could Home to Inflamed Arteries via Integrin $\beta2$

We next assessed the role of integrin β_2 on HSPC homing to injured arteries in vivo. Ligation of carotid artery has been shown to induce ICAM-1 expression on injured endothelial cells [32, 33]. Therefore, this model was used in the entire study. Lin$-$ cells isolated from LDLr$^{-/-}$ mice on HFD were labeled with PKH26 and then injected intravenously into mice wherein the carotid artery was ligated 3 days earlier. Prior to injection, cells were incubated with PBS, IgG Ab, or anti-CD18 Ab for 20 minutes. The following day, the injured and

Figure 1. In vivo hypercholesterolemia increases integrin β_2 expression on LSK cells. LDLr$^{-/-}$ mice were fed on chow or HFD for 8 weeks. **(A):** Frequency of LSK cells in PB and BM cells (BMC). **(B):** The percentage of integrin β_2^+ LSK cells in the LSK population. **(C):** Representative fluorescence-activated cell sorting (FACS) analysis of integrin β_2^+ LSK cells: (a) living BMC were shown in the box; (b) Lin− cells when gated on living BMC; (c) LSK cells when gated on Lin− cells; (d) integrin β_2 expressing LSK cells when gated on LSK population. **(D):** To evaluate the adhesion capacity of LSK cells from mice on HFD vs. chow diet, LSK cells were isolated by FACS and allowed to adhere to ICAM-1-coated plates. After 2 hours, adherent LSK cells were detached with 0.25% trypsin and counted by FACS. $n = 5–13$. **(E):** Lin− cells isolated from chow and HFD mice were plated in transwells coated with ICAM-1 for 6 hours. The percentage of LSK cells that migrated through the transwells was assessed. $n = 5–11$. Abbreviations: BM, bone marrow; FSA, forward scatter area; HFD, high fat diet; LSK, Lin$^-$ Sca-1$^+$ cKit$^+$; PB, peripheral blood; PBS, phosphate buffered saline; SSA, side scatter area.
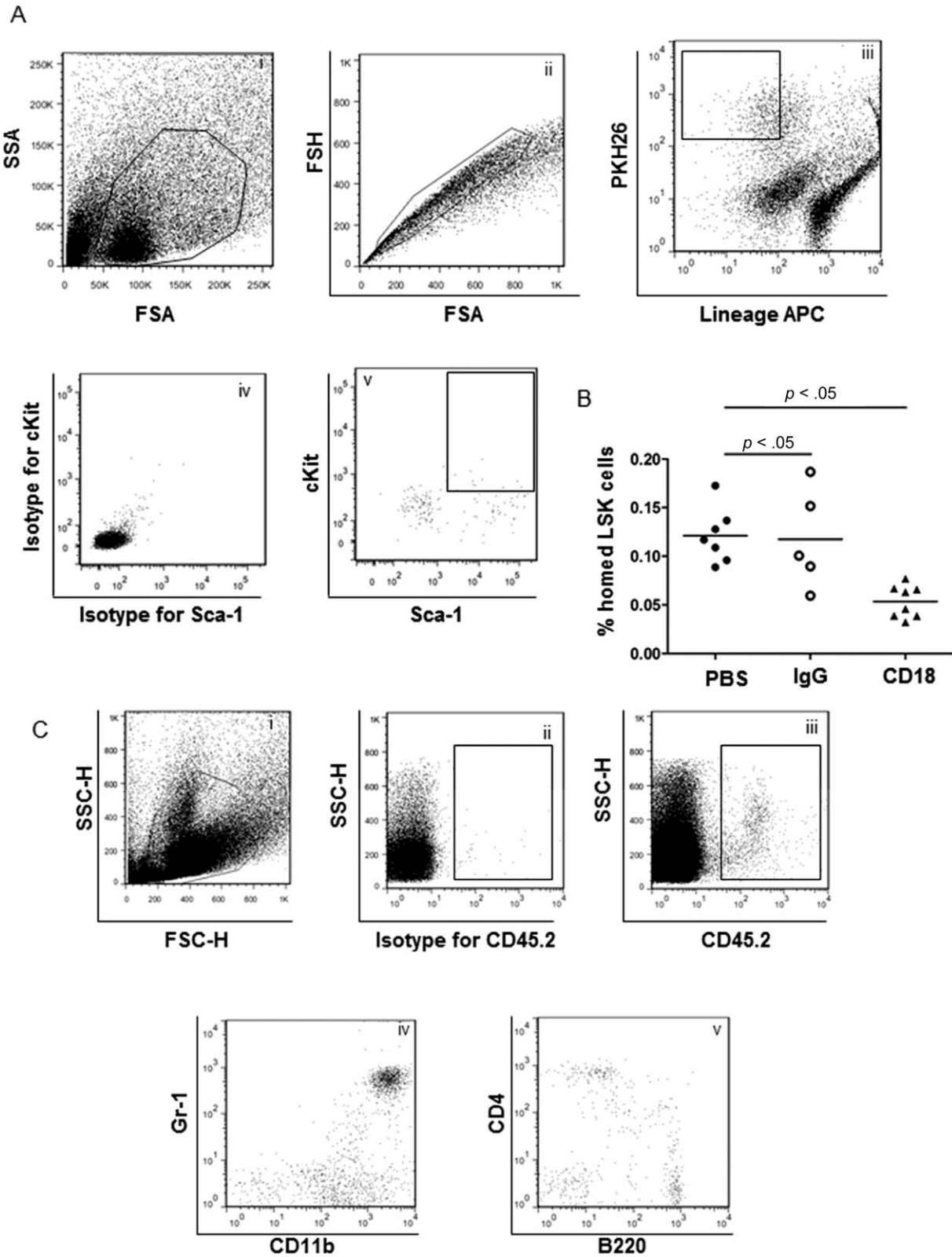
Figure 2. Hematopoietic stem/progenitor cell (HSPC) could home to lesion site via integrin β_2. **(A):** Lin$-$ cells from LDLr$^{-/-}$ mice on high fat diet were labeled with PKH26 and then injected by tail vein in mice that had undergone carotid artery ligation. The fraction of homed LSK cells was identified by fluorescence-activated cell sorting (FACS). (i) Living artery cells shown in the box; (ii) single artery cell suspension; (iii) PKH26+ Lin$-$ cells were shown when gated on single cells; when compared to the isotype (iv), homed LSK cells were identified (v). **(B):** Quantification of homed LSK cells in recipients that were injected with PBS, Lin$-$ cells treated with IgG Ab or CD18 Ab. **(C):** Homed PKH26+/LSK cells were sorted out by FACS and injected in irradiated CD45.1 mice via tail vein. After 16 weeks, donor-derived myeloid and lymphoid cells were identified by FACS. (i) Living bone marrow cell shown in the box; compared with cells stained with isotype IgG PerCP-Cy5.5 (ii), CD45.2+ cells were identified (iii); (iv) CD45.2+ derived granulocytes and monocytes; (v) CD45.2-derived B cells and T cells. Abbreviations: APC, allophycocyanin; FSA, forward scatter area; FSH, forward scatter height; LSK, Lin$^-$ Sca-1$^+$ cKit$^+$; PBS, phosphate buffered saline; SSA, side scatter area; SSC, side scatter.
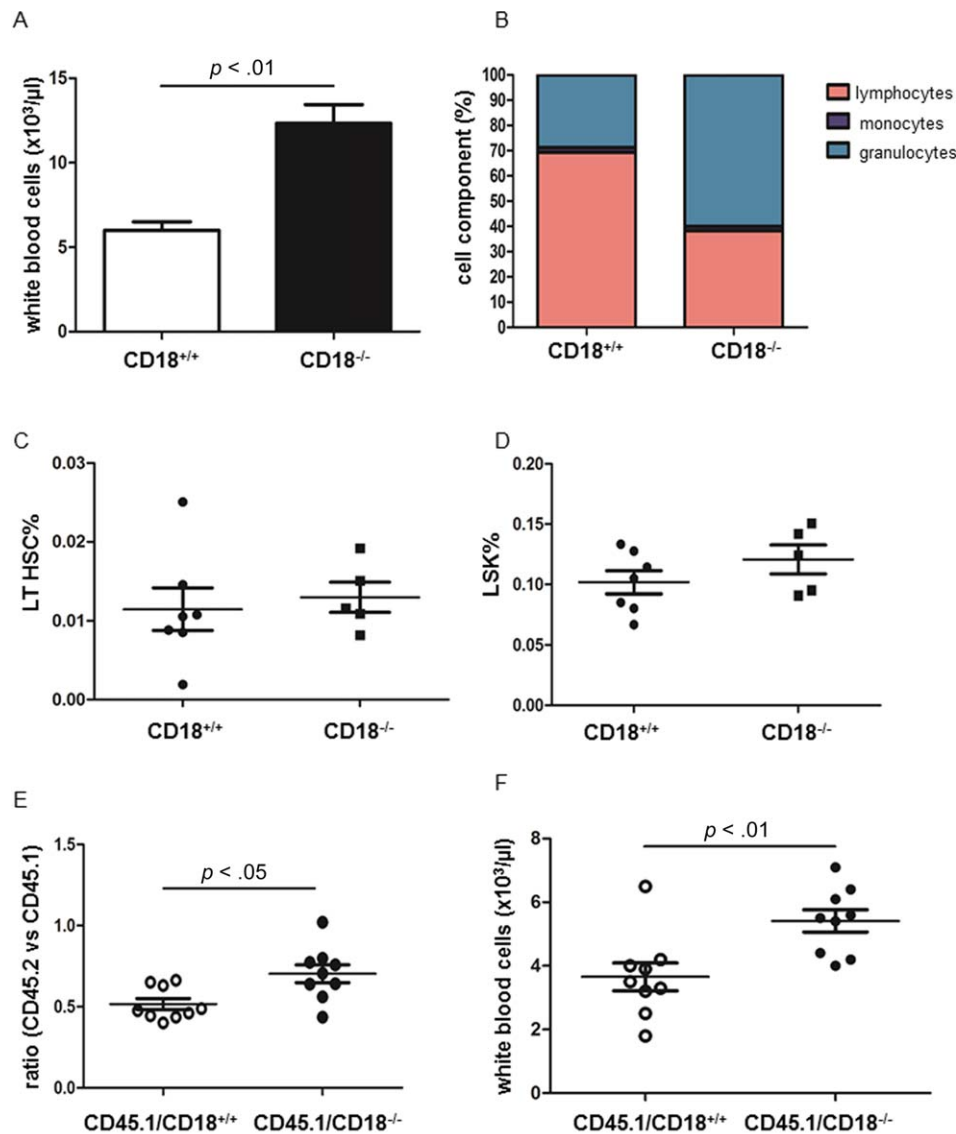
Figure 3. Phenotypic characterization of CD18$^{-/-}$ hematopoietic stem/progenitor cell. **(A):** WBC count of CD18$^{+/+}$ and CD18$^{-/-}$ mice. **(B):** The percentage of lymphocytes, monocytes, and granulocytes in white blood cell (WBC) of CD18$^{+/+}$ and CD18$^{-/-}$ mice. **(C, D):** The frequency of LT HSC and LSK cells LSK frequency in bone marrow (BM). **(E):** CD18$^{+/+}$ or CD18$^{-/-}$ BM cell (BMC) were mixed with equal amount of CD45.1 BMC before transplanted to irradiated CD45.1 recipients. Eight weeks after transplantation, peripheral blood of recipients was stained with CD45.1 and CD45.2 for chimerism (E). The WBC count of CD45.1 recipients was shown 8 weeks after BM transplantation. Abbreviations: LSK, Lin$^-$ Sca-1$^+$ cKit$^+$; LT HSC, long-term hematopoietic stem cell.

noninjured carotid arteries were dissected after extensive perfusion to remove blood cells in the arteries, digested, and stained with anti-LSK Abs. FACS data demonstrated that homing of LSK cells to injured artery was blocked by CD18 antibody (% homed LSK cells: PBS group: 0.120% ± 0.0107%; IgG group: 0.118% ± 0.0228%; CD18 group: 0.053 ± 0.0059; $n = 5$–8) (Fig. 2A, 2B).

To explore the kinetics of homed LSK cells to injured arteries, carotid artery ligation was performed on CD45.1 mice and then they were received Lin− cells isolated from LDLr$^{-/-}$ mice on HFD. One and ten days after cell injection, CD45.1 mice were sacrificed, and both injured and uninjured carotid arteries were dissected. After digestion, artery cells were numerated and stained with anti-mouse CD45.2 and LSK markers for FACS. The absolute number of homed LSK cells in uninjured and injured arteries was calculated. The number of

donor-derived LSK cells was higher in injured arteries compared to uninjured arteries at both day 1 and day 10 after injection (day 1: 34 ± 10.3 cells per graft vs. 308 ± 63.6 cells per graft, $n = 7$, $p < .01$; day 10: 41 ± 10.1 cells per graft vs. 219 ± 34.5 cells per graft, $n = 6$, $p < 0.01$) (Supporting Information Fig. 2).

To prove that LSK cells homed to the injured arteries were HSPC, PKH26+ LSK cells were isolated by FACS and injected together with CD45.1 BMCs to irradiated CD45.1 recipients. After 16 weeks of BM transplantation, recipient blood cells were stained with anti-CD45.1 FITC, anti-CD45.2 PerCP-Cy5.5 together with myeloid and lymphoid markers for chimerism analysis. CD45.2-derived granulocytes, monocytes, B cells, and T cells were identified by FACS ($n = 5$, Fig. 2C). These data indicate that homed PKH26$^+$ LSK cells are indeed HSPC.

CD18 Deficiency Was Associated with Leukocytosis But No Difference in HSPC Frequency in BM

To further investigate the role of CD18$^{-/-}$ HSPC in grafting and arterosclerosis development, CD18$^{-/-}$ mice were generated as described before [34]. CD18$^{-/-}$ mice displayed dramatic increased white blood cells in PB with skewed granulocyte production (Fig. 3A, 3B). Despite leukocytosis, percentage of long-term HSC and LSK in BMC were not differed between CD18$^{+/+}$ and CD18$^{-/-}$ mice (Fig. 3C, 3D). When equal amount of CD18$^{+/+}$ BMC or CD18$^{-/-}$ BMC were competitively transplanted to irradiated CD45.1 recipients, transplantation of CD18$^{-/-}$ BMC resulted in more white blood cells (WBC) count in the recipients (Fig. 3E, 3F). Despite greater amount of WBC in CD18$^{-/-}$ mice, when carotid artery ligation was performed in CD18$^{-/-}$ mice and their littermates, neointima formation was not differed between two groups after 28 days of surgery (neointima area: 41,770 $\pm$ 12,207.0 μm^2 vs. 41,814 $\pm$ 9,322.8 μm^2, $n = 4$–5).

Injection of CD18$^{+/+}$ LSK Cells Resulted in More Neointima Formation than CD18$^{-/-}$ LSK Cells

To assess the fate of HSPC homing to injured artery, we grafted HSPC to immunodeficient Balb/C Rag2$^-$ ɤC$^{-/-}$ recipients (H-2kd) that underwent a complete ligation of the right carotid artery. Three days after ligation, splenectomy was performed to improve HSPC homing (Fig. 4A). Seven days after artery ligation, 40,000 LSK cells isolated from CD18$^{+/+}$ or CD18$^{-/-}$ mice were injected to Balb/C Rag2$-$ ɤC$^{-/-}$ recipients. Four weeks after ligation surgery, recipients were sacrificed and the histology of the plaques was examined. H&E analysis showed that administration of CD18$^{+/+}$ LSK cells resulted in greater neointima formation compared to mice injected with PBS or CD18$^{-/-}$ LSK cells (n=4–7, Fig. 4B, 4C). Cryosections were stained with antibodies against H-2kb and CD45 and inflammation index was assessed. Quantification of CD45+ cells illustrated that mice that were injected with CD18$^{+/+}$ LSK cells developed aggravated neointima inflammation compared to mice received PBS and CD18$^{-/-}$ LSK cells (Fig. 4D). In addition, the percentage of H-2kb+ CD45+ cells among CD45+ cells was twofold higher in mice injected with CD18$^{+/+}$ LSK cells compared to mice received CD18$^{-/-}$ LSK cells (Fig. 4E). Identification of donor-derived CD45+ cells, that is, H-2kb$^+$ CD45$^+$ cells was illustrated in Figure 4F.

ERK1/2 Phosphorylation Is Involved in LDL-Mediated Integrin β2 Induction on HSPC

Thereafter, to gain insight in how hypercholesterolemia induces β_2 expression, we first stained BMC harvested from LDLr$^{-/-}$ mice on chow and HFD with anti-pERK (p42/p44) and LSK Abs as described before [2]. Consistent with our previous in vitro findings [2], we found a 1.4-fold increase in the percentage of pERK$^+$ LSK cells in LDLr$^{-/-}$ mice on HFD compared with chow diet (% pERK$^+$ LSK in total LSK cells: chow diet: 11.7% $\pm$ 1.18%; HFD: 16.4% $\pm$ 1.74%, $n = 8$–9, $p < .05$) (Fig. 5A, 5B).

We established in vitro assays to explore the effects of lipoproteins on LSK cells. Lin$-$ cells were isolated from LDLr$^{-/-}$ mice and exposed to equal amount of LDL or HDL (0–100 μg/ml) for 24 hours. Cells were harvested and stained with anti-mouse CD18 and LSK markers for FACS as described above.

The percentage of CD18+ LSK cells in LSK population was 1.4-fold higher at 25 μg/ml LDL, 1.5-fold higher at 50 μg/ml LDL, and 1.6-fold higher at 100 μg/ml LDL than non-LDL treated cells. Distinct from LDL, HDL treatment did not increase CD18 expressing LSK cells in vitro (Supporting Information Fig. 3).

We next assessed if pERK is involved in the induction of β_2 integrin expression by LDL. Lin$-$ cells were cultured with 100 μg/ml LDL in the presence or absence of pERK inhibitor, U0126, for 24 hours. Cells were stained with Abs against LSK and integrin β_2. As was seen in vivo, LDL increased the fraction of LSK cells that expressed CD18 ($n = 5$–6, Fig. 5C). Culture of LSK cells with LDL in combination with U0126 attenuated these changes (Fig. 5C). By contrast, incubation with LDL did not alter the percentage of integrin $\alpha5^+$ LSK cells nor the percentage of integrin β_1^+ LSK cells (%integrin $\alpha5^+$ LSK cells: 89% $\pm$ 3.0% vs. 89% $\pm$ 1.8%; %integrin β_1^+ LSK cells: 96.7% $\pm$ 1.16% vs. 95.8 $\pm$ 1.74, $n = 4$).

Presentation of adhesion molecules on the cell membrane is a complex process which starts with gene transcription, translation, and transportation. The small G protein ARF1 has been shown to mediate receptor trafficking to the membrane, including the chemokine receptor CXCR4, integrin β1, and CD11a [35–38]. We thus evaluated whether LDL induced any change in ARF1 expression in Lin$-$ cells. Western blot data illustrated that exposure of LDL to Lin$-$ cells increased ARF1 expression in a pERK-dependent manner (Fig. 5D, 5E). To dissect whether ARF1 facilitated CD18 expression or function, Lin$-$ cells were exposed to PBS or 5 μM Brefeldin A (BFA), an ARF1 inhibitor [36], for 8 hours and then stained with anti-CD18 and LSK Abs. BFA treatment neither altered the percentage of LSK cells in the population (control: 3.8% $\pm$ 0.44%; BFA: 3.5% $\pm$ 0.59%, $n =$ 7) nor led to significant change of CD18 expression on LSK cells. However, blocking ARF1 expression by BFA significantly reduced LSK cell adhesion to ICAM-1 in vitro and LSK cell homing to ligated arteries in vivo ($n = 6$–7, Fig. 5F, 5G).

LRP1 is also in Part Responsible for LDL-Mediated Integrin β2 Induction on HSPC

LRP1 belongs to the LDL receptor superfamily. LRP1, together with LDLr, regulate cholesterol homeostasis via endocytosis. Different from LDLr, LRP1 has multiple functions such as regulation of integrin expression and function [27, 28, 39]. We first determined if LRP1 is expressed on Lin$-$ cells by Western blot analysis. LRP1 could not be detected in Lin$-$ cells under control conditions but was induced by exposure of Lin$-$ cells to LDL. By contrast, exposure to LDL did not significantly affect LDL receptor expression in Lin$-$ cells (Fig. 6A–6C). Of note, inhibition of ERK by U0126 did not prevent induction of LRP1 expression in Lin$-$ cells. To further assess the possible involvement of LRP1 in integrin-mediated HSPC homing to injured arteries, we isolated LSK cells from LRP1 mutant mice (LRP1$^{n2/n2}$) [31] and repeated the function assays described above. LSK cells isolated from LRP1$^{n2/n2}$ mice showed decreased adhesion to ICAM-1 in vitro and reduced homing to injured arteries in vivo, compared to WT LSK cells ($n = 5$–7, Fig. 6D, 6E). These data indicate that LRP1 is at least in part responsible for the regulation of integrin β2 function, while induction of LRP1 by LDL is ERK-independent.
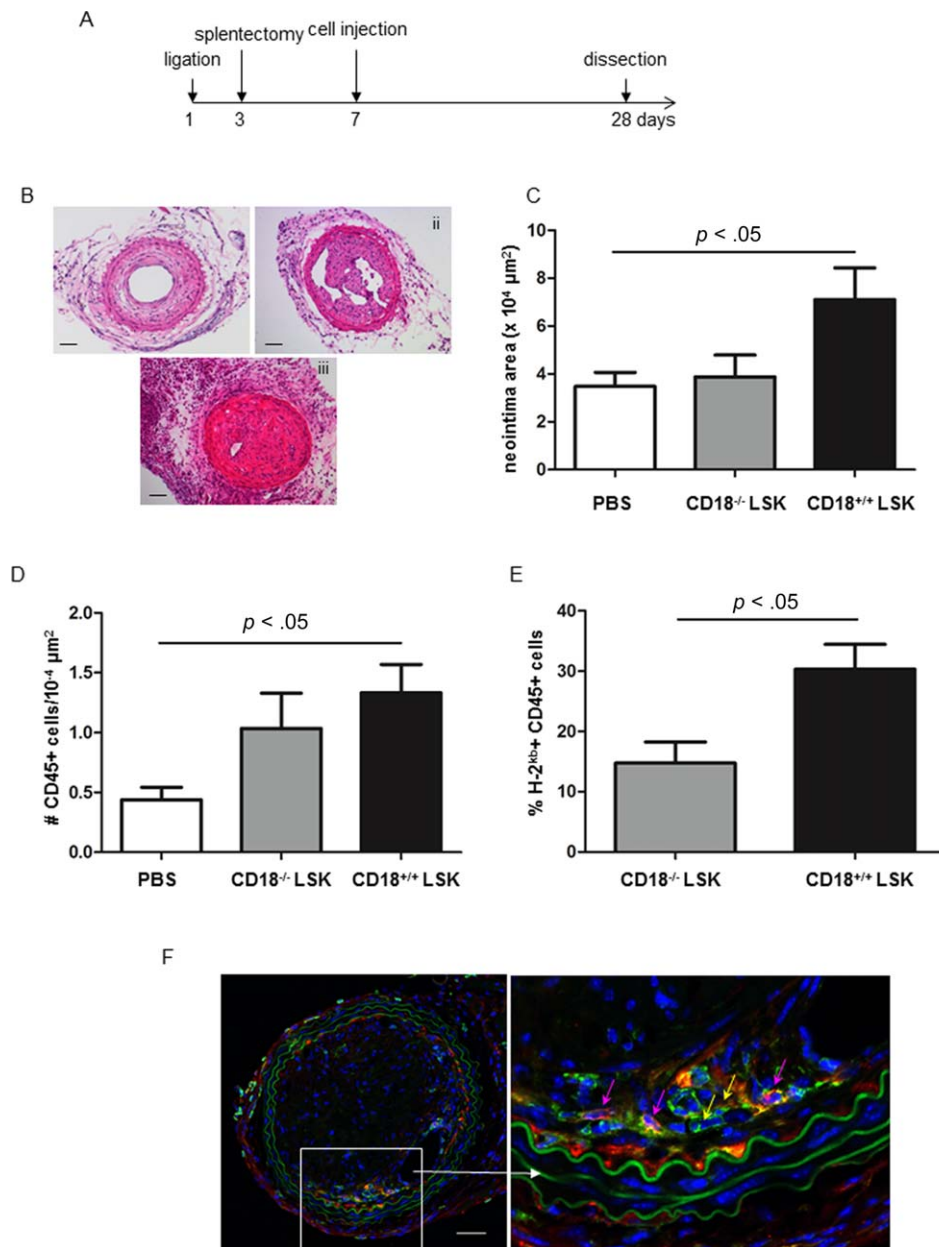
Figure 4. Hematopoietic stem/progenitor cell injection reinforced inflammation and accelerated neointima formation in mice that underwent carotid artery ligation. **(A):** The scheme of the cell injection experiment. **(B):** Representative H&E sections of carotid arteries of Balb/C Rag2$-$ γC$^{-/-}$ mice injected with PBS (i), CD18$^{-/-}$ LSK cells (ii), and CD18$^{+/+}$ LSK cells (iii). Scale bar = 50 μm. **(C):** Neointima area in the ligated artery of Balb/c Rag2$-$ γC$^{-/-}$ mice received saline, CD18$^{+/+}$ LSK, and CD18$^{-/-}$ LSK cells. n = 4–7 per group. **(D):** Cryosections were stained with rat anti-mouse CD45 and mouse anti-mouse H-2kb Abs, and then goat anti-rat Alexa 488 and goat anti-mouse Alexa 555 Abs. CD45+ cell density was obtained by the number of CD45+ cells divided by neointima area. **(E):** The percentage of donor-derived CD45+ cells in the lesion site. **(F):** Identification of donor-derived CD45+ cells. Yellow arrows indicate CD45+ cells derived from recipients, whereas pink arrows indicate CD45+ cells originated from donor HSPC. Scale bar = 100 μm. Abbreviations: LSK, Lin$^-$ Sca-1$^+$ cKit$^+$; PBS, phosphate buffered saline.

DISCUSSION

Leukocytosis and monocytosis contribute substantially to arteriosclerotic progression [40–42]. Others and we have previously described that hypercholesterolemia induces HSPC proliferation and differentiation, resulting in expansion of the inflammatory cell pool in PB. However, it remains unclear if and how HSPC can migrate into lesion where they may locally expand and differentiate into leukocytes that further reinforce plaque formation. Till present, CXCR4 is the main factor identified to be responsible for HSPC mobilization in hypercholesterolemia. It would be very interesting to explore if other key factors that are modulated and in turn regulate HSPC function in the context of hypercholesterolemia. Therefore, we assessed if (a) HSPC directly contribute to arteriosclerosis progression; (b) hypercholesterolemia and LDL modulate
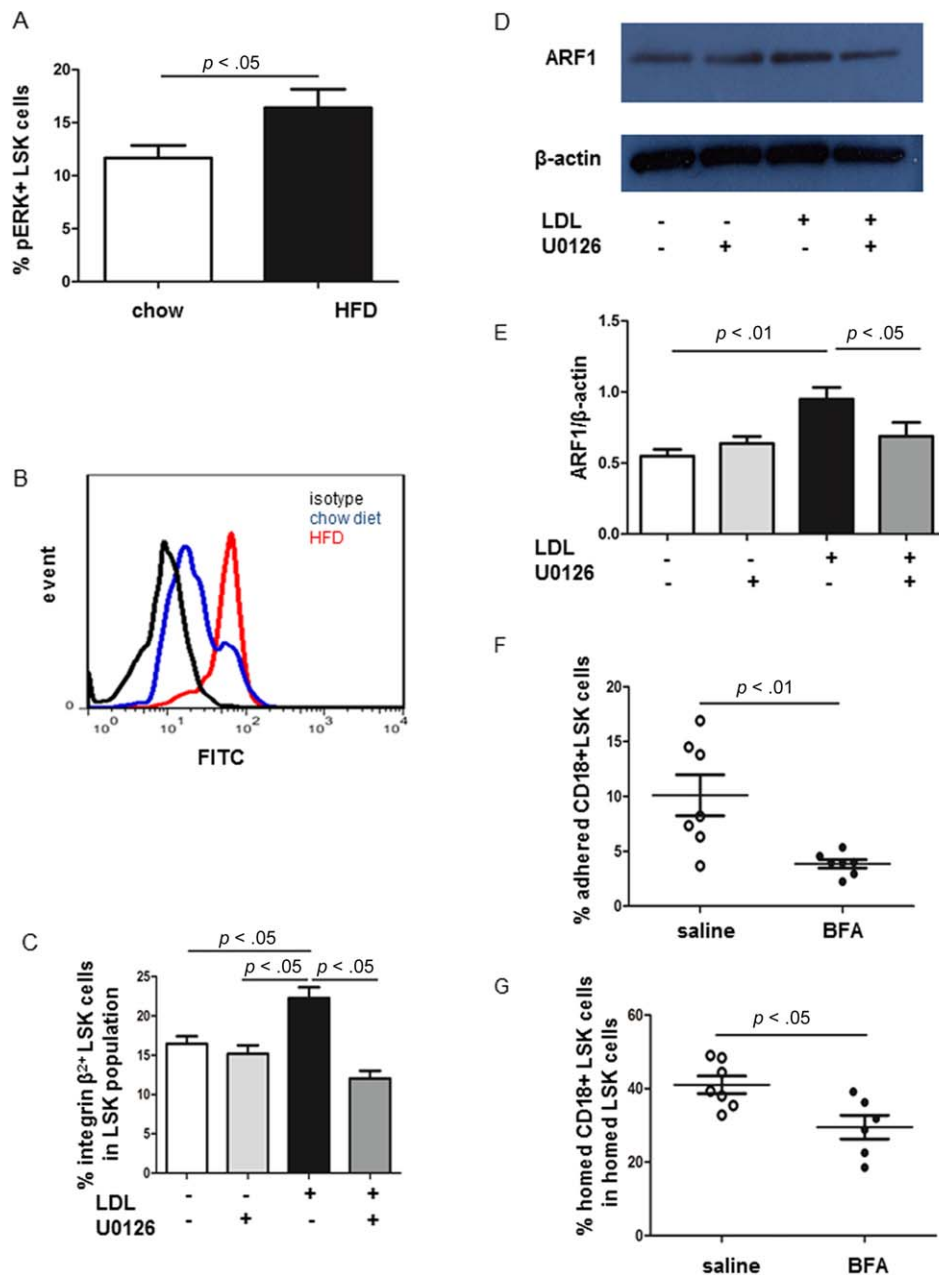
Figure 5. Role of pERK on integrin β_2 expression. **(A):** Quantification of pERK+ LSK cells in LDLr$^{-/-}$ mice on chow or HFD. **(B):** Fluorescence-activated cell sorting analysis of pERK+ LSK cells. **(C):** Lin− cells were exposed to 0 or 100 μg/ml LDL with or without 10 μM U0126 for 24 hours. Cells were stained with Abs against LSK cells and anti-CD18 for FACS. The percentage of integrin β_2^+ LSK cells in the LSK population; $n = 5$–6. **(D):** Representative Western blot of ARF1 and β-actin expression in Lin− cells that were treated with 0 or 100 μg/ml LDL in the presence or absence of 10 μM U0126 for 24 hours. **(E):** Quantification of ARF1 protein expression normalized with β-actin. $n = 6$. **(F):** Lin− cells were exposed to 0 or 5 μM BFA overnight. Equal amounts of cells were plated into ICAM-1-coated plates and allowed to adhere for 2 hours. Data are expressed as the percentage of adhered integrin β_2^+ LSK cells divided by the total number of seeded integrin β_2^+ LSK cells. $n = 7$. **(G):** Lin− cells were treated with or without BFA as described above and labeled with PKH26 before injection into ligated CD45.2 recipients. The percentage of integrin β_2^+ LSK cells within the LSK cell population was quantified. $n = 6$–7. Abbreviations: ARF1, ADP-ribosylation factor 1; BFA, Brefeldin A; FITC, fluorescein; HFD, high fat diet; LDL, low density lipoprotein; LSK, Lin$^-$ Sca-1$^+$ cKit$^+$; pERK, phospho ERK.

migration, attachment, and homing of HSPC to injured artery, and if so by which mechanism.

Inflammation of blood vessels, which underlies the formation of atherosclerotic plaques, leads to upregulation of ICAM-1, the ligand for integrin β_2. It is well-known that integrin β_2 facilitates neutrophil and monocyte attachment and then transmigration through inflamed endothelium, which is

why integrin $\beta2$ deficiency attenuates neointima formation in mice [43–45]. We demonstrate here that HSPC express significantly higher levels of CD18, when harvested from hypercholesterolemic mice, or following exposure of HSPC to LDL in vitro. We further demonstrate that homing of LSK to injured arteries occurs, which can be inhibited with blocking anti-CD18 antibodies. Grafting CD18$^{+/+}$ LSK cells to recipients
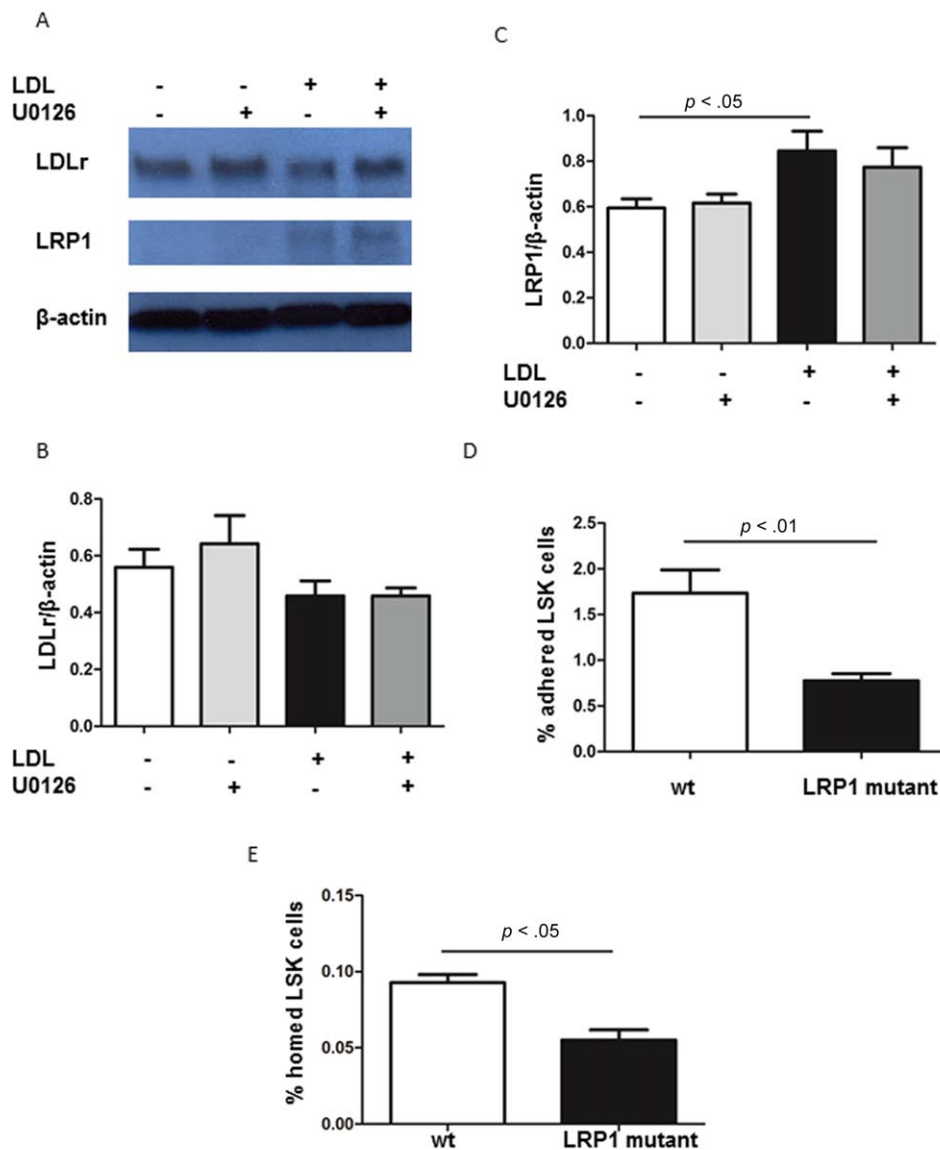
Figure 6. pERK independent regulation of integrin β_2 expression on LSK cells. **(A):** Lin− cells from wild type mice were exposed to 0 or 100 μg/ml LDL with or without 10 μM U0126. Western blot was performed to study LDLr, LRP1, and β-actin expression. Representative Western blot of LDLr, LRP1, and β-actin were shown. **(B, C):** Quantification of the expression levels of LDLr and LRP1, normalized with β-actin in (B) and (C), respectively. $n = 6$. **(D):** LSK cells from LRP1 mutant versus wild type (WT) mice were selected by FACS and seeded onto ICAM-1 coated 96-well plates. Two hours later, adherent LSK cells were harvested and enumerated by FACS. Data are expressed as the percentage of adherent LSK cells in the input number of LSK cells. $n = 5$. **(E):** Lin− cells were isolated from LRP1 WT and mutant mice. After labeling with PKH26, cells were injected in mice with carotid artery ligation. Homed LSK cells to the ligated artery were quantified by FACS. $n = 6$–7. Abbreviations: LDL, low density lipoprotein; LRP1, LDL receptor-related protein 1; LSK, Lin⁻ Sca-1⁺ cKit⁺.

accelerated inflammation and arteriosclerosis in the injured artery, which is partially due to donor-derived inflammatory cells in the lesion. These data implicate the critical regulation of integrin β_2 in HSPC adhesion, migration, and homing and further identify pro-atherogenic property of HSPC in arteriosclerosis development. Due to technical limitations, it is at present not feasible to trace HSPC proliferation and differentiation following homing to the lesion. Nevertheless, we postulate that both LSK proliferation and differentiation might occur in situ.

We previously demonstrated that pERK is responsible for LDL-mediated increased differentiation of HSPC in vitro. In this

study, we reported that hypercholesterolemia is associated with pERK activation in LSK cells in vivo. We found that pERK is responsible for upregulation of the G protein ARF1, known to mediate receptor trafficking to the membrane such as CXCR4, integrin β_1, and CD11a [35–38]. We here demonstrate that ARF1 is also responsible for induction of functional β_2 integrin on HSPC. We also identified a second signaling pathway, LRP1, to be involved in mediating integrin β_2 function on HSPC. Aside from LDL uptake, LRP1 also can affect integrin expression and/ or function. In macrophages, LRP1 interacts with multiple sites in $\alpha_M\beta_2$, which results in integrin clustering leading to cell adhesion and migration [27, 28]. LRP1 also plays a role in inducing
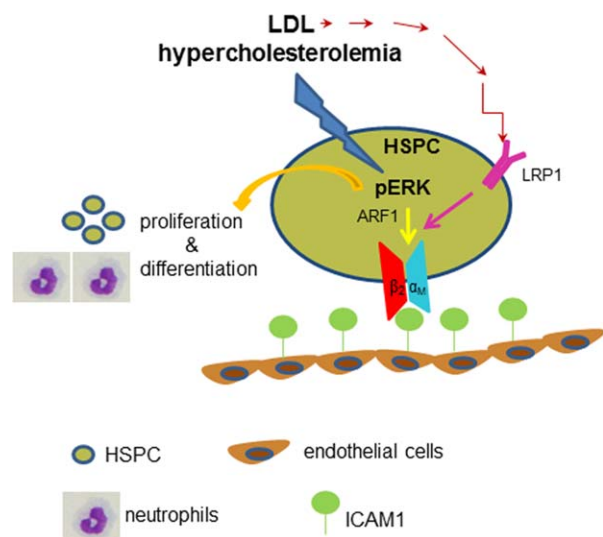
Figure 7. Proposed model of how hypercholesterolemia and LDL modulate HSPC homing to lesion site. Hypercholesterolemia and LDL stimulate ERK phosphorylation in HSPC. ERK activation promotes HSPC proliferation and differentiation into atherogenic myeloid cells. Apart from that, ERK activation enhances ARF1-mediated integrin β_2 function of HSPC. In parallel, LDL increases LRP1 expression on HSPC, which in turn potentiates integrin β2 activity. As results from these changes, HSPC can attach to ICAM-1-expressing endothelial cells in lesion site where they may further contribute to inflammation and arteriosclerosis progression. Abbreviations: ARF1, ADP-ribosylation factor 1; HSPC, hematopoietic stem/progenitor cell; ICAM1, intercellular cell adhesion molecular-1; LDL, low density lipoprotein; LRP1, LDL receptor-related protein 1; pERK, phospho ERK.

maturation of the β1 integrin, causing a substantial increase of this receptor on myeloid cells [39]. In this study, we found that LDL induces expression of LRP1, and that deficiency of LRP1 significantly inhibits HSPC adhesion to ICAM-1 in vitro. LRP1 induction by LDL was, however, independent on pERK. Deficiency of LRP1 did not affect expression levels of β_2 but reduced the adhesion capacity of LRP1-defieicnt LSK cells to ICAM-1. This would be consistent with the fact that LRP1 can not only cause integrin maturation and therefore expression [23] but also integrin function, as was shown in macrophages [27]. How LDL induces LRP1 expression remains unknown. Likewise, whether other forms of modified LDLs, aside from LDL isolated from plasma used in this study, influence LRP1 expression would be of interest. Finally, as LRP1 has multiple functions, we cannot exclude that other functions of LRP1 may play additional role in HSPC biology.

Our proposed model of the molecular mechanisms underlying LDL-mediated effects on HSPC biology is depicted in Figure 7. In vivo hypercholesterolemia and in vitro exposure to LDL causes phosphorylation of ERK1/2 in LSK cells. Phospho-ERK is, as others and we previously showed, responsible for LSK proliferation and differentiation [2, 3] and as shown here, adhesion, migration, and homing. Phospho-ERK induces HSPC migration via ARF1, which increases expression of functional

β_2 integrin on LSK cells. LDL also increases integrin function, not expression, on HSPC by interacting with LRP1 known to increase integrin function [27]. The proposed model extends our knowledge on the pathogenesis of hypercholesterolemia-associated arteriosclerosis in the context of HSPC, which may provide novel therapeutic targets for the treatment of arteriosclerosis.

SUMMARY

Hypercholesterolemia-associated leukocytosis accelerates arteriosclerosis progression. The link among HSPC proliferation and differentiation in the BM, leukocytosis, and reinforced arteriosclerosis has been noted in hypercholesterolemic mice. However, it remains undefined whether and how circulating HSPC could directly participate in arteriosclerosis. In addition, how hypercholesterolemia, specifically LDL, affects HSPC trafficking to injured arteries is largely unknown. Using LDLr$^{-/-}$ mice model, we identified that integrin β_2 was upregulated on HSPC of LDLr$^{-/-}$ on HFD. Increased integrin β_2 expression enhanced HSPC function including their adhesion and migration toward ICAM-1 and homing to injured arteries. Strikingly, grafting HSPC to injured arteries accelerated inflammation and arteriosclerosis progression. Taken together, HSPC bear proatherogenic property and could directly participate in arteriosclerosis. Hypercholesterolemia stimulates arteriosclerosis progression could be partially via its regulation of HSPC as well as mature myeloid cells.

AUTHOR CONTRIBUTIONS

Y.F.: initiated, conducted the study, analyzed the data, and drafted the manuscript; X.W., M.G., S.S., K.E., and Y.F.: performed the experiments; A.R.: generated LRP1 mutant mice and an antibody against LRP1; P.P.V.V.: generated FPLC data; T.P.: generated CD18$^{-/-}$ mice and the littermates; G.L. and C.V.: provided intellectual input and participated in manuscript preparation.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

REFERENCES

1 Murphy AJ, Akhtari M, Tolani S et al. ApoE regulates hematopoietic stem cell proliferation, monocytosis, and monocyte accu-

mulation in atherosclerotic lesions in mice. J Clin Invest 2011;121:4138–4149.

2 Feng Y, Schouteden S, Geenens R et al. Hematopoietic stem/progenitor cell proliferation and differentiation is differentially regu-

lated by high-density and low-density lipoproteins in mice. PLoS One 2012;7: e47286.

3 Yvan-Charvet L, Pagler T, Gautier EL et al. ATP-binding cassette transporters and

HDL suppress hematopoietic stem cell proliferation. Science 2010;328:1689–1693.

4   Dao MA, Creer MH, Nolta JA et al. Biology of umbilical cord blood progenitors in bone marrow niches. Blood 2007;110:74–81.

5   Prosper F, Stroncek D, McCarthy JB et al. Mobilization and homing of peripheral blood progenitors is related to reversible downregulation of alpha4 beta1 integrin expression and function. J Clin Invest 1998; 101:2456–2467.

6   Massberg S, Schaerli P, Knezevic-Maramica I et al. Immunosurveillance by hematopoietic progenitor cells trafficking through blood, lymph, and peripheral tissues. Cell 2007;131:994–1008.

7   Psaltis PJ, Harbuzariu A, Delacroix S et al. Identification of a monocyte-predisposed hierarchy of hematopoietic progenitor cells in the adventitia of postnatal murine aorta. Circulation 2012;125:592–603.

8   Si Y, Tsou CL, Croft K et al. CCR2 mediates hematopoietic stem and progenitor cell trafficking to sites of inflammation in mice. J Clin Invest 2010;120:1192–1203.

9   Dalakas E, Newsome PN, Harrison DJ et al. Hematopoietic stem cell trafficking in liver injury. FASEB J 2005;19:1225–1231.

10   Palazuelos J, Davout N, Julien B et al. The CB(2) cannabinoid receptor controls myeloid progenitor trafficking: Involvement in the pathogenesis of an animal model of multiple sclerosis. J Biol Chem 2008;283: 13320–13329.

11   Fischer KD, Agrawal DK. Hematopoietic stem and progenitor cells in inflammation and allergy. Front Immunol 2013;4:428.

12   Qian H, Tryggvason K, Jacobsen SE et al. Contribution of alpha6 integrins to hematopoietic stem and progenitor cell homing to bone marrow and collaboration with alpha4 integrins. Blood 2006;107:3503–3510.

13   Teixido J, Hemler ME, Greenberger JS et al. Role of beta 1 and beta 2 integrins in the adhesion of human CD34hi stem cells to bone marrow stroma. J Clin Invest 1992;90:358–367.

14   Bonig H, Watts KL, Chang KH et al. Concurrent blockade of alpha4-integrin and CXCR4 in hematopoietic stem/progenitor cell mobilization. Stem Cells 2009;27:836–837.

15   Taniguchi Ishikawa E, Chang KH, Nayak R et al. Klf5 controls bone marrow homing of stem cells and progenitors through Rab5-mediated beta1/beta2-integrin trafficking. Nat Commun 2013;4:1660.

16   Papayannopoulou T, Priestley GV, Nakamoto B et al. Synergistic mobilization of hemopoietic progenitor cells using concurrent beta1 and beta2 integrin blockade or beta2-deficient mice. Blood 2001;97:1282–1288.

17   Gomes AL, Carvalho T, Serpa J et al. Hypercholesterolemia promotes bone marrow cell mobilization by perturbing the SDF-1:CXCR4 axis. Blood 2010;115:3886–3894.

18   Pruijt JF, van Kooyk Y, Figdor CG et al. Anti-LFA-1 blocking antibodies prevent mobilization of hematopoietic progenitor cells induced by interleukin-8. Blood 1998;91: 4099–4105.

19   Asaumi N, Omoto E, Mahmut N et al. Very late antigen-5 and leukocyte function-associated antigen-1 are critical for early stage hematopoietic progenitor cell homing. Ann Hematol 2001;80:387–392.

20   Shao H, Tan Y, Eton D et al. Statin and stromal cell-derived factor-1 additively promote angiogenesis by enhancement of progenitor cells incorporation into new vessels. Stem Cells 2008;26:1376–1384.

21   Crysandt M, Hilgers RD, von Hobe S et al. Hypercholesterolemia and its association with enhanced stem cell mobilization and harvest after high-dose cyclophosphamide+G-CSF. Bone Marrow Transplant 2011;46:1426–1429.

22   Westerterp M, Gourion-Arsiquaud S, Murphy AJ et al. Regulation of hematopoietic stem and progenitor cell mobilization by cholesterol efflux pathways. Cell Stem Cell 2012; 11:195–206.

23   Woods D, Cherwinski H, Venetsanakos E et al. Induction of beta3-integrin gene expression by sustained activation of the Ras-regulated Raf-MEK-extracellular signal-regulated kinase signaling pathway. Mol Cell Biol 2001;21:3192–3205.

24   Lai CF, Chaudhary L, Fausto A et al. Erk is essential for growth, differentiation, integrin expression, and cell function in human osteoblastic cells. J Biol Chem 2001;276: 14443–14450.

25   Langlois A, Chouinard F, Flamand N et al. Crucial implication of protein kinase C (PKC)-delta, PKC-zeta, ERK-1/2, and p38 MAPK in migration of human asthmatic eosinophils. J Leukoc Biol 2009;85:656–663.

26   Herz J, Strickland DK. LRP: A multifunctional scavenger and signaling receptor. J Clin Invest 2001;108:779–784.

27   Spijkers PP, da Costa Martins P, Westein E et al. LDL-receptor-related protein regulates beta2-integrin-mediated leukocyte adhesion. Blood 2005;105:170–177.

28   Ranganathan S, Cao C, Catania J et al. Molecular basis for the interaction of low density lipoprotein receptor-related protein 1 (LRP1) with integrin alphaMbeta2: Identification of binding sites within alphaMbeta2 for LRP1. J Biol Chem 2011;286:30535–30541.

29   Langlois B, Perrot G, Schneider C et al. LRP-1 promotes cancer cell invasion by supporting ERK and inhibiting JNK signaling pathways. PLoS One 2010;5:e11584.

30   Geetha N, Mihaly J, Stockenhuber A et al. Signal integration and coincidence detection in the mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) cascade: Concomitant activation of receptor tyrosine kinases and of LRP-1 leads to sustained ERK phosphorylation via down-regulation of dual specificity phosphatases (DUSP1 and −6). J Biol Chem 2011;286: 25663–25674.

31   Gordts PL, Reekmans S, Lauwers A et al. Inactivation of the LRP1 intracellular NPxYxxL motif in LDLR-deficient mice enhances postprandial dyslipidemia and atherosclerosis. Arterioscler Thromb Vasc Biol 2009;29:1258–1264.

32   Noma K, Rikitake Y, Oyama N et al. ROCK1 mediates leukocyte recruitment and neointima formation following vascular injury. J Clin Invest 2008;118:1632–1644.

33   McPherson JA, Barringhaus KG, Bishop GG et al. Adenosine A(2A) receptor stimulation reduces inflammation and neointimal growth in a murine carotid ligation model. Arterioscler Thromb Vasc Biol 2001;21:791–796.

34   Oreshkova T, Wang H, Seier AM et al. Beta(2) integrin deficiency yields unconventional double-negative T cells distinct from mature classical natural killer T cells in mice. Immunology 2009;128:271–286.

35   D'Souza-Schorey C, Chavrier P. ARF proteins: Roles in membrane traffic and beyond. Nat Rev Mol Cell Biol 2006;7:347–358.

36   Dong C, Zhang X, Zhou F et al. ADP-ribosylation factors modulate the cell surface transport of G protein-coupled receptors. J Pharmacol Exp Ther 2010;333:174–183.

37   Powelka AM, Sun J, Li J et al. Stimulation-dependent recycling of integrin beta1 regulated by ARF6 and Rab11. Traffic 2004;5: 20–36.

38   Quast T, Tappertzhofen B, Schild C et al. Cytohesin-1 controls the activation of RhoA and modulates integrin-dependent adhesion and migration of dendritic cells. Blood 2009; 113:5801–5810.

39   Salicioni AM, Gaultier A, Brownlee C et al. Low density lipoprotein receptor-related protein-1 promotes beta1 integrin maturation and transport to the cell surface. J Biol Chem 2004;279:10005–10012.

40   Swirski FK, Libby P, Aikawa E et al. Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. J Clin Invest 2007;117:195–205.

41   Tacke F, Alvarez D, Kaplan TJ et al. Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. J Clin Invest 2007;117:185–194.

42   Dutta P, Courties G, Wei Y et al. Myocardial infarction accelerates atherosclerosis. Nature 2012;487:325–329.

43   Kevil CG, Patel RP, Bullard DC. Essential role of ICAM-1 in mediating monocyte adhesion to aortic endothelial cells. Am J Physiol Cell Physiol 2001;281:C1442–1447.

44   Jozsef L, Khreiss T, Fournier A et al. Extracellular signal-regulated kinase plays an essential role in endothelin-1-induced homotypic adhesion of human neutrophil granulocytes. Br J Pharmacol 2002;135:1167–1174.

45   Simon DI, Dhen Z, Seifert P et al. Decreased neointimal formation in Mac-1(−/−) mice reveals a role for inflammation in vascular repair after angioplasty. J Clin Invest 2000;105:293–300.

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