Loss of the Liver X Receptors Disrupts the Balance of Hematopoietic Populations, With Detrimental Effects on Endothelial Progenitor Cells

Adil Rasheed, PhD; Ricky Tsai, BSc; Carolyn L. Cummins, PhD

**Background**—The liver X receptors (LXRs; α/β) are nuclear receptors known to regulate cholesterol homeostasis and the production of select hematopoietic populations. The objective of this study was to determine the importance of LXRs and a high-fat high-cholesterol diet on global hematopoiesis, with special emphasis on endothelial progenitor cells (EPCs), a vasoreparative cell type that is derived from bone marrow hematopoietic stem cells.

**Methods and Results**—Wild-type and LXR double-knockout (Lxrβ−/−) mice were fed a Western diet (WD) to increase plasma cholesterol levels. In WD-fed Lxrαβ−/− mice, flow cytometry and complete blood cell counts revealed that hematopoietic stem cells, a myeloid progenitor, and mature circulating myeloid cells were increased; EPC numbers were significantly decreased. Hematopoietic stem cells from WD-fed Lxrαβ−/− mice showed increased cholesterol content, along with increased myeloid colony formation compared with chow-fed mice. In contrast, EPCs from WD-fed Lxrαβ−/− mice also demonstrated increased cellular cholesterol content that was associated with greater expression of the endothelial lineage markers Cd144 and Vegfr2, suggesting accelerated differentiation of the EPCs. Treatment of human umbilical vein endothelial cells with conditioned medium collected from these EPCs increased THP-1 monocyte adhesion. Increased monocyte adhesion to conditioned medium–treated endothelial cells was recapitulated with conditioned medium from Lxrαβ−/− EPCs treated with cholesterol ex vivo, suggesting cholesterol is the main component of the WD inducing EPC dysfunction.

**Conclusions**—LXRs are crucial for maintaining the balance of hematopoietic cells in a hypercholesterolemic environment and for mitigating the negative effects of cholesterol on EPC differentiation/secretome changes that promote monocyte-endothelial adhesion. (J Am Heart Assoc. 2018;7:e007787. DOI: 10.1161/JAHA.117.007787.)

**Key Words:** cholesterol • endothelial progenitor cells • hematopoietic stem cells • liver X receptors • nuclear receptor

The liver X receptors (LXRs) belong to the nuclear receptor superfamily of ligand-activated transcription factors. LXRs are present in 2 isoforms: LXRβ (NR1H2) is ubiquitously expressed throughout the body, whereas LXRα (NR1H3) is most highly expressed in the liver, kidney, adrenals, and intestine.1,2 Widely referred to as the “cholesterol-sensing nuclear receptors,” LXRs are endogenously activated by oxysterols, which are oxidized derivatives of cholesterol. LXRα and LXRβ share 77% sequence homology in their ligand-binding domain and largely appear to have overlapping transcriptional functions.3,4 Interest in LXRs as potential drug targets stems from their potent capability to induce reverse cholesterol transport by increasing expression of the cholesterol efflux transporters Abca1 and Abcg1.5,6 Furthermore, LXRs decrease the expression of proinflammatory cytokines, such as Mcp-1, Il1β, and Tnfα, through transcriptional repression.7

A role for LXRs in endothelial cell homeostasis has been previously described in which LXRs were beneficial in preventing endothelial cell activation and senescence, as well as in promoting overall endothelial health.8–10 However, preservation of the endothelium is not solely mediated by direct effects on endothelial cells. Bone marrow (BM)–derived endothelial progenitor cells (EPCs) are also responsible for promoting endothelial cell maintenance.11 EPCs were initially discovered in 1997 by Asahara and colleagues.12 Identification of these cells in vivo is performed by flow cytometry, using a combination of markers for stem cells and endothelial cells.11,12

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Clinical Perspective

What Is New?

- Our data demonstrate that liver X receptors (LXRs) protect hematopoietic stem cells against hypercholesterolemia-induced shifts in hematopoietic cell fate.
- In the absence of LXRs, hypercholesterolemia increases myeloid cell numbers while decreasing endothelial progenitor cell (EPC) numbers.
- EPCs from hypercholesterolemic mice lacking LXRs have a more pathogenic secretome that promotes binding of monocytes to endothelial cells.
- LXRs protect EPCs from cell-intrinsic defects in differentiation and function, resulting from elevated intracellular cholesterol.

What Are the Clinical Implications?

- Previous studies have documented a decrease in EPC function and EPC numbers in cardiovascular disease.
- Our data show that LXRs play a protective role against cholesterol-induced defects in EPC differentiation and vasoreparative factor secretion.
- We have uncovered a new mechanism by which LXRs could be atheroprotective in hypercholesterolemia (ie, via protection of EPC numbers/function).

After 7 to 10 days in culture, whereas the late EPCs arise after 14 to 21 days in culture. Early EPCs possess a lower expression of endothelial lineage markers and secrete more vasoreparative factors when compared with late EPCs. In vivo, circulating EPC numbers and the function of EPCs are decreased in patients with cardiovascular disease or diabetes mellitus. Reports have demonstrated inverse correlations between EPC numbers/function and low-density lipoprotein cholesterol levels, suggesting a potential role for cholesterol in the dysfunction of EPCs. To date, only a few studies have investigated the specific role of BM-derived EPCs in the in vivo resolution of atherosclerosis using mouse models. Infusion of cultured EPCs was reported to improve proinflammatory and oxidative stress markers in splenectomized Apoe−/− mice and decrease atherosclerotic lesion area. Studies examining atherosclerotic regression (using Reversa mice) found that treatment with AMD3100 (a C-X-C chemokine receptor type 4 [CXCR4] antagonist that mobilizes stem cells) or infusion of GFP-labeled EPCs resulted in enhanced regression. These studies strongly support a positive role for EPCs in supporting atherosclerotic regression, but they do not inform us of the role of EPCs during the early stages of atherogenesis.

EPCs are derived from hematopoietic stem cells (HSCs). Although HSCs can also differentiate into the common lymphoid or common myeloid progenitors, EPCs are more related to the myeloid lineage and, thus, we focused our investigation on this subpopulation. Previously, we and others have described a role for LXRs in promoting migration of progenitor cells in a diabetic animal model. In addition, the importance of LXRs has been investigated in mature hematopoietic populations; however, a global examination of the role of LXRs in regulating hematopoietic cell types, including EPCs, has not been undertaken, particularly in the context of hypercholesterolemia. In this article, we demonstrate that the LXR-knockout mice fed a Western diet (WD) had an increased propensity to form myeloid populations compared with EPCs, changes that were associated with increased HSC cholesterol content. EPCs derived from LXR-knockout mice exposed to a cholesterol-rich environment showed accelerated endothelial differentiation and an increase in secretory factors that promoted monocyte-endothelial cell adhesion, a key initiating step during atherogenesis. These results demonstrate the crucial role for LXRs in regulating hematopoietic cell numbers and EPC function, especially in the context of elevated cellular cholesterol.

Methods

The data, analytic methods, and study materials will be made available to other researchers on request for purposes of reproducing the results or replicating the procedure.

Mice

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Toronto (Toronto, ON, Canada). Wild-type (WT) and LXR double-knockout (Lxrgβ−/−) mice were backcrossed >10 generations on a C57Bl/6 background and were maintained on a chow diet (no. 2016; 4.0% fat; Harlan Teklad) in a temperature- and light-controlled environment. For WD feeding experiments, 8- to 9-week-old mice were placed on a WD (TD 88137; 21.2% fat, 0.2% cholesterol; Harlan Teklad) for 12 weeks. To assess in vivo proliferation of hematopoietic populations, each mouse was injected intraperitoneally with 1 mg of bromodeoxyuridine (BD Biosciences) 24 hours before euthanasia. The mice were housed 1 to 2 mice per cage. Food intake was calculated as an average per mouse housed in each cage and reported as energy content consumed per mouse each day. For all other experiments, 12-week-old chow-fed WT and Lxrgβ−/− mice were used. Mice were euthanized by exsanguination or cervical dislocation under isoflurane anesthesia. All mice were euthanized between 9 and 11 AM to ensure consistency between experiments, because hematopoietic egress from the BM highly follows a circadian rhythm. This specific time point (between

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Zeitgeber time-3 and Zeitgeber time-5) was selected to detect circulating levels of rare hematopoietic populations, including EPCs. The number of mice used per experiment is specified in the legend of each data figure.

Whole blood obtained at euthanasia was centrifuged at 500g for 20 minutes at 4°C for the separation of plasma. Plasma cholesterol levels were determined by enzymatic assay using the Cholesterol E kit (Thermo Scientific).

**Flow Cytometry**

Red blood cells in the BM and peripheral blood were lysed using room temperature 1× Pharm Lyse (BD Biosciences). To lyse red blood cells, the BM was resuspended in 1 mL Flow Cytometry Buffer (PBS ±3% fetal bovine serum [FBS] ±1× antibiotic/antimycotic), and 3 mL Pharm Lyse was added to each sample. Lysing occurred for 3 minutes. For lysing peripheral blood red blood cells, 100 µL peripheral blood was added to 100 µL deionized water, and 2 mL of Pharm Lyse was added to each sample. Lysing occurred for 10 minutes. Red blood cell lysing in both the BM and peripheral blood was quenched with an excess volume of Flow Cytometry Buffer, and the cells were passed through a 40-µm cell strainer as a single-cell suspension (Fisher Scientific). Lysed cells were resuspended in 100 µL Brilliant Violet Buffer (BD Biosciences) and transferred into 5-mL polystyrene round-bottom tubes (Fisher Scientific). Fc block (1 µg/10⁶ cells) or CD16/32 (FcgR) Brilliant Violet 605 (0.25 µg/10⁶ cells; BD Biosciences) was used to prevent nonspecific antibody binding. The cells were stained with the following antibodies at 4°C for 20 minutes: lineage antibody cocktail PerCP-Cy5.5 (CD3e, CD11b, CD45R/B220, Ly-76, Ly-6G, Ly-6C; 20 µL/10⁶ cells), Ly6A/E (Sca-1) PE-Cy7 (0.06 µg/10⁶ cells), CD117 PE (0.06 µg/10⁶ cells), CD127 Brilliant Violet 510 (0.1875 µg/10⁶ cells), CD34 AlexaFluor647 (0.75 µg/10⁶ cells), and Flk-1 APC-Cy7 (0.109 µg/10⁶ cells). Dead cell identification was performed by staining with LIVE/DEAD Violet Fixable Dead Cell Stain (1 µL/10⁶ cells; Life Technologies) at 4°C for 30 minutes. To determine apoptosis, an active caspase-3 fluorescein isothiocyanate kit (no. 550480, BD Biosciences) was used, as per manufacturer's instructions. To determine proliferation, a bromodeoxyuridine fluorescein isothiocyanate detection kit (no. 559619, BD Biosciences) was used, as per manufacturer's recommendations. If apoptosis or proliferation was not assessed in the flow cytometry panels, the cells were fixed with IC Fixation Buffer (eBioscience) at 4°C for 15 minutes. Samples were resuspended in 150 µL of Flow Cytometry Buffer for sample acquisition on an LSR Fortessa flow cytometer (BD Biosciences). Analysis of the flow cytometry data was performed using FlowJo (Tree Star). HSCs were identified as lineage-negative cells (Lin⁻) CD117⁺ Sca-1⁺, common myeloid progenitors as Lin⁻ CD127⁻ CD117⁺ Sca-1⁻ CD34⁺ FcgR⁺, megakaryocyte-erythroid progenitors as Lin⁻ CD127⁻ CD117⁺ Sca-1⁻ CD34⁻ FcgR⁺, granulocyte-monocyte (GM) progenitors as Lin⁻ CD127⁻ CD117⁺ Sca-1⁻ CD34⁺ FcgR⁺, and EPCs as Flk-1⁺ Sca-1⁺.

**Complete Blood Cell Counts**

Whole blood was collected from live mice via the saphenous vein in EDTA-coated microvettes. Samples were kept at room temperature until analysis on a Hemavet 950 (Drew Scientific).

** Colony Formation Assays**

BM HSCs (Lin⁻) were immunomagnetically isolated using the Mouse Hematopoietic Progenitor Cell Isolation Kit (StemCell Technologies) following manufacturer’s recommendations. For the EPC colony formation assay, Lin⁻ cells were resuspended in Iscove’s modified Dulbecco’s medium (Life Technologies) supplemented with 10% FBS. Then, they were plated in mouse serum/factor free methylcellulose-based medium (StemCell Technologies) supplemented with 50 ng/mL vascular endothelial growth factor, 50 ng/mL basic fibroblast growth factor, 50 ng/mL endothelial growth factor, 50 ng/mL insulin-like growth factor-1, 100 ng/mL stem cell factor, 20 ng/mL interleukin-3 (R&D Systems), and 2 U/mL heparin (Sigma-Aldrich). For the GM colony formation assay, Lin⁻ cells were resuspended in Iscove’s modified Dulbecco’s medium supplemented with 2% FBS and plated in mouse methylcellulose-based medium containing FBS, stem cell factor, interleukin-3, interleukin-6, and erythropoietin (StemCell Technologies), supplemented with 10% interleukin-3 culture supplement (VWR) and 2 ng/mL GM colony-stimulating factor (R&D Systems). The cell suspension for either assay was plated at a concentration of 10³ Lin⁻ cells/mL in 6-well SmartDish plates using 16-gauge blunt-ended needles (StemCell Technologies) at a volume of 1 mL per well and incubated at 37°C with 5% CO₂ for 7 days (EPC colony formation assay) or 10 days (GM colony formation assay). For activation of LXRs, 1 µmol/L GW3965 (Tocris) was added to each medium mix before plating.

**BM Harvesting and EPC Culture**

EPCs were cultured from the BM of the tibias and femurs, as previously described. The BM cells were seeded on human-fibronectin-coated (Sigma-Aldrich) tissue culture dishes and differentiated for 7 days to EPCs in endothelial basal growth supplemented with growth factors/cytokines (EGM-2 Bullet Kit; Lonza) at 37°C with 5% CO₂. Medium was changed every other day.

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RNA Isolation, cDNA Synthesis, and Real-Time Quantitative Polymerase Chain Reaction Analysis

Total RNA was extracted from cells using RNA Stat-60 (Tel-Test), per manufacturer’s recommendations. Extracted RNA was treated with DNaseI (Life Technologies), followed by reverse transcription to cDNA using the High Capacity Reverse Transcription System (Applied Biosystems). Quantitative polymerase chain reaction was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems) in a 384-well plate. Reactions in each well contained cDNA, 150 nmol/L of each primer, and 5 μL 2× SYBR Green PCR Master Mix (Life Technologies) in a 10-μL volume, and they were plated in triplicate. Relative levels of each gene were calculated using the comparative Cycle time method normalized to cyclophilin expression. Primer sequences are listed in Table 1.

Treatment of Culture-Derived EPCs

For short-term activation of LXRs, EPCs were treated with 1 μmol/L GW3965 on day 6, 24 hours before harvesting RNA. For induction of inflammation, EPCs were pretreated with LXR agonist, as described, followed by an additional 6 hours of cotreatment with 1 μmol/L GW3965 and 10 ng/mL lipopolysaccharide (Sigma-Aldrich). To determine the role of exogenous cholesterol, EPCs were treated with 25 μg/mL cholesterol (BioShop) dissolved in ethanol and treated at a 1:1000 dilution throughout the 7-day EPC culture period.

Oxidative Stress Assay

Malondialdehyde content of the EPCs was determined using the thiobarbituric acid reactive substances assay kit (Cayman Chemical), according to the manufacturer’s recommendations.

Lipid Extraction and Quantification of Cholesterol Content

Before lipid extraction, all samples were spiked with 5 μg of the internal standard cholesterol-d7 (Avanti Polar Lipids). Lipids were extracted twice in a 1:10 ratio with methyl tert-butyl ether (VWR). Extracted lipids were dried down and resuspended in high-performance liquid chromatography grade methanol (Caledon Laboratory Chemicals). Samples were analyzed by liquid chromatography/tandem mass spectrometry with a 6410 Triple Quadrupole liquid chromatography/tandem mass spectrometry instrument using an electrospray ionization source and Zorbax XDB-C18 column (4.6×50 mm, 3.5 μm) (Agilent Technologies). Sample acquisition was performed in multiple reaction monitoring mode using transitions for cholesterol (m/z 404→369) and the internal standard cholesterol-d7 (m/z 411→376).

Conditioned Medium Collection

After 7 days of differentiation, EPCs were washed twice with cold PBS and incubated in factor and ligand free medium (EBM-2; Lonza) for 30 minutes at 37°C and 5% CO2. The medium was discarded, and the cells were incubated in fresh EBM-2 medium. The medium was collected after 24 hours, centrifuged at 700g for 5 minutes to remove any nonadherent cells, and syringe filtered. The conditioned medium (CM) was frozen at −80°C until use.

Cell Lines

Human umbilical vein endothelial cells (HUVECs) and medium (EGM-2 Bullet Kit) were purchased from Lonza. THP-1 monocytes (received as a gift from Dr Myron I. Cybulsky, University of Toronto) were maintained in RPMI 1640 medium with L-glutamine (Sigma-Aldrich) supplemented with 10% FBS and 0.05 mmol/L β-mercaptoethanol (Sigma-Aldrich). All cell lines were used between passages 4 and 5.

Monocyte-Endothelial Adhesion Assay

HUVECs were grown in 12-well plates on glass coverslips and were treated at 50% to 60% confluence with 20% CM for 20 hours in fully supplemented endothelial medium (EGM-2 Bullet Kit), followed by 4-hour cotreatment with 20% CM and 5 ng/mL tumor necrosis factor-α (Life Technologies). After treatment, the CM was removed, 10^5 5-chloromethylfluorescein diacetate (Life Technologies) labelled THP-1 monocytes were added to each well, and adhesion was allowed to occur for 90 minutes. After coculture, the cells were washed and adherent THP-1 cells were fixed using 4% paraformaldehyde. Images were taken on a laser confocal LSM700 microscope operated by Zen 2011 software (Zeiss).

Statistical Analysis

Parametric unpaired 2-tailed t test was used for comparisons between 2 groups. One-way ANOVA, followed by the Holm-Sidak’s post hoc test, was used for comparisons between >2 groups. All values are expressed as mean±SEM. P<0.05 was considered statistically significant. Statistical tests were performed using GraphPad Prism (GraphPad Software Inc). When a large number of statistical tests are performed, we acknowledge that some results may be statistically significant by chance alone.
| Gene   | Forward (5′→3′)                  | Reverse (5′→3′)              |
|--------|----------------------------------|------------------------------|
| Abca1  | TCCTCATCCTGCTGATTCAA             | GGACTTGGTAGACGGAACCT         |
| Abcg1  | GTTCAGAGGCCAAGCTGATTG            | CTGGTCTGCTGCTGCTGCTC         |
| Ar     | TGCAACTCCAGAAGCTGCTGCTC          | TGACGGAACGTGGAACCT           |
| Car    | GCTCAAGAGGCTCTTCAAG              | AAGGACAGATGACGGAACCT         |
| Cdf14  | TGCCAAGAGCAGCTGAGAC             | ACTGGGCTCGAGGAGGTGA          |
| Cdf45  | AAGCAGCTCCTCAGGACCA              | CATGGAAGCATGTTGGA            |
| CoupT2 | GCTGAGACGGAGAAGCTGAC            | GCTGGTAGGACGGAACCT           |
| CoupTl | TGCATTAGCTCAGTCTGCTC            | AGGGGCACAGAGTCTGCTC          |
| Cyclo  | CAACGATAAGAAGAGGACCTAA          | GGGGCTCAGAGAGCAGAA           |
| Dax    | AAGGACGAGCAGCTGCTTAA            | TGCCGTAGGAGCTGCTC            |
| Ear2   | GAGGGCTGACAGGAGCTGCTC           | TGCCGTAGGAGCTGCTC            |
| Errα   | AGCAAGCCCGCATGGA                | GAGAAGCTGATGCTCTT            |
| Errβ   | CAGATGCGGAGCTGCTGCTC            | TGCCGACAGAGTCTGCTC           |
| Errγ   | ACTTGTCGACGGAGAGTG              | GGCAAGAGATGACGGAAGCT         |
| Ers    | GCAGATAGGGAGCTGCTGCA            | GCTGGTAGGACGGAACCT           |
| Eryα   | GCACACCTCCGTAGTCTGCTGCTC        | TGCCACAGAGTCTGCTGCTC         |
| Eryβ   | CAGAAGCCAGGACCTGCTGCTGCTG       | TGCCACAGAGTCTGCTGCTGCTG      |
| Eryγ   | CATACTGCTGCTGGAGCTACAA          | TGCCACAGAGTCTGCTGCTGCTG      |
| Gcnf   | CCGAACAAGAGCATTTGGGA            | GAGAAGGAGCTGATGCTCTT          |
| Gr     | GCAAGTGGAAACCTGCTGATG           | TGCCGAGAGCTGATGCTC           |
| Hnf4α  | CCAGATGTCAGTAGTCTGCTGCTGCTG     | TGCCGAGAGCTGATGCTC           |
| Hnf4β  | CATGTGCTGCTGGAGCTACAA           | TGCCGAGAGCTGATGCTC           |
| Hnf4γ  | AAGGACAGAGGAGCTGCTGCTGCTG       | TGCCGAGAGCTGATGCTC           |
| Inox   | AGAGAGCTGCTGCTGCTGCTGCTGCTG     | TGCCGAGAGCTGATGCTC           |
| Lrt1   | CGATGTCCTACTGCTGATTCA           | TGCCGAGAGCTGATGCTC           |
| Lrxα   | AGGATGTCCTCTGCTGCTGCTGCTG      | TGCCGAGAGCTGATGCTC           |
| Lrxβ   | AAGATGTCCTCTGCTGCTGCTGCTG      | TGCCGAGAGCTGATGCTC           |
| Mr     | AGGACGACCCGCTGCTGCTGCTGCTG     | TGCCGAGAGCTGATGCTC           |
| Nrf2   | ACAGATGTCCTACTGCTGCTGCTGCTG    | TGCCGAGAGCTGATGCTC           |
| Nor1   | AGGTCTGAGAGGAGTTAAGGA           | AGCATGTCCTCTGCTCCTTCA        |
| Nox2   | GCACACCCGCACTCACC              | CCCGACCAAGAGCTGACG           |
| Nurr1  | GCACCTTGCGAGGAGTG              | GGAATCAGGGAGCTGACG           |
| Pnr    | AGATGTCCTGCTGCTGCTGCTGCTGCTG  | GGAATCAGGGAGCTGACG           |
| Ppara  | CAGGGTCACACTCGGGAGTTCC         | CCGAATAGTGCAGGGAAGAA         |
| Pparaα/β| GCCTGGGGCTGCTGCTGCTGCTG       | AGATGTCCTGCTGCTGCTGCTG      |
| Pparaγ | GATGTCCTGCTGCTGCTGCTGCTGCTG  | GGAATCAGGGAGCTGACG           |
| Pr     | GCTGGTAGATGCTGCTGCTGCTGCTG    | GGAATCAGGGAGCTGACG           |
| Pparα  | CAGGGACAGGAGGAGTTAAGGA         | CCGAATAGTGCAGGGAAGAA         |
| Pparαβ/δ| GCCTGGGGCTGCTGCTGCTGCTG       | AGATGTCCTGCTGCTGCTGCTG      |
| Pparγ  | GATGTCCTGCTGCTGCTGCTGCTGCTG  | GGAATCAGGGAGCTGACG           |
| Prx    | CAGGGACAGGAGGAGTTAAGGA         | CCGAATAGTGCAGGGAAGAA         |
| Ranα   | CCAGATGTCCTGCTGCTGCTGCTGCTG  | GGAATCAGGGAGCTGACG           |
| Ranβ   | ACAGATGTCCTGCTGCTGCTGCTGCTG  | GGAATCAGGGAGCTGACG           |
| RevErbα| CCAGATGTCCTGCTGCTGCTGCTGCTG  | GGAATCAGGGAGCTGACG           |
Results

Loss of LXRs Shifts the Balance of Hematopoietic Cell Types

Previous studies have established a role for LXRs in decreasing the numbers of mature hematopoietic cell types in vivo, through inhibiting lymphocyte proliferation and increasing clearance of apoptotic neutrophils.\(^\text{35,36}\) However, a global examination of the role of LXRs in regulating the numbers of progenitor and mature hematopoietic populations has not been directly examined. To address this, we enumerated these cell types in 12-week-old chow-fed WT and Lxr\(^{\text{ab/C0/C0}}\) mice. Using flow cytometry, hematopoietic stem and progenitor populations were enumerated at the site of formation, in the BM. HSC numbers were increased 1.5-fold, accompanied by increases in myeloid progenitor populations (common myeloid progenitors, megakaryocyte-erythrocyte progenitors, and GM progenitors), in the Lxr\(^{\text{ab/C0/C0}}\) mice compared with their WT counterparts (Figure 1A). These changes are consistent with previous reports in which absence of the LXR target genes, Abca1 and Abcg1 or Apoe, allows for proliferation of the upstream HSCs and expansion of the myeloid progenitor cell populations.\(^\text{37,38}\) There was no significant change in BM EPC numbers (Figure 1A), whereas in circulation, EPC numbers were reduced by 27% in the Lxr\(^{\text{ab/C0/C0}}\) compared with WT mice (Figure 1B). Consistent with increases in their progenitor populations in the BM, circulating levels of neutrophils and monocytes were increased by 1.4- and 1.5-fold in the Lxr\(^{\text{ab/C0/C0}}\) mice, respectively (Figure 1C). These data indicate that the loss of LXRs increases HSC and myeloid progenitor numbers while decreasing EPC numbers.

To probe the cell autonomous effects of LXRs on hematopoietic differentiation, HSCs (Lin\(^{-}\)) isolated from the BM were used for colony formation assays. LXR activation (1 \(\mu\text{mol/L GW3965}\)) in WT, but not Lxr\(^{\text{ab/C0/C0}}\) HSCs, increased EPC colony numbers by 36% compared with vehicle. Conversely, the number of GM colonies was decreased by 25% in the WT GW3965-treated HSCs (Figure 1D). Taken together, these data suggest that LXRs can shift HSC differentiation towards EPCs and away from myeloid cell types.

EPCs Express Both LXR Isoforms and LXR Target Genes

The gold standard method to study EPCs in vitro is to differentiate them ex vivo from BM or peripheral blood mononuclear cells. To this effect, ex vivo activation of multiple nuclear receptors has been shown to enhance the functions of EPCs, including migration, proliferation, and angiogenesis.\(^\text{39-41}\) Specifically, activation of LXRs decreases...
high glucose-induced reactive oxygen species in EPCs by enhancing AMP kinase signaling and increases EPC migration and proliferation by activating the protein kinase B/endothelial nitric oxide synthase signaling pathway.42,43 In our studies, gene expression analysis revealed that Lxrα was the most highly expressed of the nuclear receptor family members, indicating it may play an important role in EPCs. Lxrβ was the third highest expressing receptor behind Lxrα and Reverbβ (Figure 2A). To assess the function of LXRs in EPCs, we examined the expression of known LXR target genes. Treatment of WT EPCs with GW3965 upregulated expression of the cholesterol efflux transporters Abca1 and Abcg1 (Figure 2B). In addition, treatment of WT EPCs with GW3965 repressed lipopolysaccharide-induced expression of the proinflammatory cytokines Inos and Il1β (Figure 2C). These studies confirm the ability of the LXRs to regulate gene targets important for cholesterol efflux and repression of inflammation in this novel cell type.

**LXRs Are Important for Maintaining the Balance of Hematopoietic Cell Populations in a Hypercholesterolemic Environment**

Given the known role of LXRs in maintaining cholesterol homeostasis, we set out to investigate the role of LXRs in regulating hematopoietic cell populations in a hypercholesterolemic environment. WT and Lxrβ−/− mice were placed on a WD for 12 weeks. Although food intake was similar between genotypes on their respective diets, only WT mice gained weight on the WD compared with their chow-fed...
counterparts (Table 2). These data are consistent with previous observations that on a WD, Lxrαβ—/— mice had greater peripheral energy expenditure than WT mice. Nevertheless, total plasma cholesterol levels in both genotypes were similarly increased on WD feeding (Table 2).

In the BM of WD-fed Lxrαβ—/— (Lxrαβ—/— WD) mice, HSCs and common myeloid progenitors were significantly increased 2.2- and 1.3-fold, respectively, relative to their chow-fed counterparts, with corresponding increases in circulating numbers of mature myeloid populations, in particular the monocytes and neutrophils. Conversely, EPCs in the BM and peripheral blood were decreased by 24% and 52%, respectively, in the Lxrαβ—/— mice fed a WD versus chow (Figure 3A and 3B). To determine whether these decreases in EPC numbers were the result of decreased proliferation, we performed bromodeoxyuridine injections and quantified its incorporation by flow cytometry as a marker of proliferation. We also measured active caspase-3 expression by flow cytometry to investigate whether EPC numbers were decreased because of an increase in apoptosis. Surprisingly, differences observed in these markers were not consistent with the enumeration results for EPCs (Figure 4A and 4B). To assess whether intrinsic HSC differentiation was perturbed after WD feeding, colony formation assays were performed.

**Figure 2.** Liver X receptors (LXRs) are highly expressed in culture-derived endothelial progenitor cells (EPCs) and induce cholesterol efflux transporter expression, while repressing proinflammatory gene expression. A, Gene expression of all 49 mouse nuclear receptors in culture-derived wild-type (WT) EPCs. n=6 per group. B, Expression of the LXR target genes, Abca1 and Abcg1, in culture-derived EPCs treated with LXR agonist (1 μmol/L GW3965) for 24 hours. n=8 to 9 per group. C, Expression of proinflammatory genes in culture-derived EPCs after treatment with GW3965 and lipopolysaccharide (10 ng/mL). n=5 to 6 per group. Lxrαβ—/— indicates LXR double knockout; Veh, vehicle. **P<0.01, ***P<0.001, and ****P<0.0001.
HSCs from the \( \text{Lxr}^{-/-} / \text{C0}^{-/-} / \text{C0}^{-/-} \) WD mice formed 1.4-fold more GM colonies compared with their chow-fed counterparts. In contrast, the number of EPC colonies was unchanged between treatment groups (Figure 3C). These data suggest that LXRs are protective against WD-induced (1) increases in myeloid cell numbers by limiting the differentiation of HSCs and (2) decreases in EPC numbers through an as yet undetermined mechanism.

**Table 2.** Physiologic Parameters of WT and LXR-Knockout Mice After WD Feeding

| Parameters                | WT Chow       | WT WD         | \( \text{Lxr}\beta^{-/-} \text{ Chow} \) | \( \text{Lxr}\beta^{-/-} \text{ WD} \) |
|---------------------------|---------------|---------------|----------------------------------------|--------------------------------------|
| Final body weight, g      | 27.4±0.3      | 31.2±0.5*     | 28.3±0.2                               | 27.7±0.2†                            |
| Food intake, kcal/mouse per d | 10.6±0.2     | 12.3±0.3*     | 11.0±0.2                               | 11.5±0.2                             |
| Plasma cholesterol, mg/dL | 146±5         | 232±7*        | 101±3†                                 | 212±8‡                               |

Body weight (n=45–56 mice per group), food intake (n=25–30 cages per group), and plasma cholesterol (n=50–56 mice per group) were analyzed after WT and \( \text{Lxr}\beta^{-/-} \) mice were fed a chow or WD for 12 weeks. LXR indicates liver X receptor; \( \text{Lxr}\beta^{-/-} \), LXR double knockout; WD, Western diet; WT, wild type.

*P<0.0001 compared with chow, †P<0.0001 compared with WT, ‡P<0.05 compared with WT.

**Figure 3.** Western diet (WD) feeding to liver X receptor double-knockout (\( \text{Lxr}\beta^{-/-} \)) mice alters the levels of hematopoietic populations in the bone marrow and peripheral blood. Wild-type (WT) and \( \text{Lxr}\beta^{-/-} \) mice were fed a WD for 12 weeks. A, Hematopoietic progenitor populations in the bone marrow (BM) and peripheral blood (PB) of 20-week-old mice were enumerated by flow cytometry. n=11 to 12 per group. B, Mature myeloid cell types in the PB were quantified by complete blood cell counts. n=14 to 17 per group. C, Hematopoietic stem cells (HSCs) isolated from the BM were used for the endothelial progenitor cell (EPC) and granulocyte-monocyte (GM) colony formation assays. n=6 per group. BA indicates basophil; CMP, common myeloid progenitor; EO, eosinophil; GMP, GM progenitor; LY, lymphocyte; MEP, megakaryocyte-erythrocyte progenitor; MO, monocyte; NE, neutrophil; PLT, platelet; RBC, red blood cell; WBC, white blood cell. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001.
WD Feeding Increases EPC Cholesterol Content in Lxrβ−/− Mice, Altering EPC Identity

Two populations of culture-derived EPCs have been described, distinguished by their expression of endothelial lineage markers and function.13 The culture-derived EPC population with lower expression of the endothelial markers, Cd144 and Vegfr2, has been of particular therapeutic focus because these cells facilitate endothelial repair by the secretion of vasoreparative factors. The expression levels of Cd144 and Vegfr2 were significantly increased (2.1- and 1.9-fold, respectively) in culture-derived EPCs from Lxrβ−/− WD mice compared with their chow-fed counterparts. These changes were specific to the endothelial lineage because there were no changes in the expression of the pan-leukocyte marker Cd45 (Figure 5A). WD feeding is known to precipitate a proinflammatory and oxidative stress response in multiple cell types. We examined the expression of the proinflammatory genes, Tnfα and Il1β, and found no significant increase with WD in the Lxrβ−/− EPCs (Figure 5B). In contrast, the oxidative stress marker Nox2 was increased in the Lxrβ−/− WD EPCs compared with other treatment groups (Figure 5C). However, accumulation of the oxidative species malondialdehyde was similarly increased in EPCs of both genotypes after WD feeding (Figure 5D). We found the cellular cholesterol content was increased in multiple cell types from the Lxrβ−/− WD mice, including the following: (1) HSCs, the cells from which all other hematopoietic cells are derived; (2) the BM from which the
EPCs were differentiated; and (3) the EPCs after 7 days in culture (Figure 5D). Together, these data suggest that increased EPC cholesterol content, rather than inflammation or oxidative stress, is more closely correlated with increased expression of EPC endothelial markers.

**EPCs From WD-Fed LXR-Knockout Mice Secrete Factors That Increase Monocyte-Endothelial Adhesion**

Culture-derived EPCs that express lower levels of endothelial markers are associated with improved endothelial repair by the secretion of vasoreparative factors. To investigate whether the accelerated endothelial differentiation we observed in the Lxrαβ−/− WD EPCs was associated with a detrimental change in the secretome of these cells, we used the monocyte-endothelial cell adhesion assay. Briefly, tumor necrosis factor-α–activated HUVECs were treated with EPC CM, before the addition of labelled THP-1 monocytes. HUVECs treated with the CM from the Lxrαβ−/− WD EPCs potentiated monocyte adhesion by 1.4-fold compared with EPC CM derived from chow-fed mice (Figure 6). No differences in total protein levels were noted in the EPC CM between any of the feeding groups (data not shown), suggesting that qualitative differences in the factors secreted contributed to this endothelial phenotype.

**Treatment With Exogenous Cholesterol Recapitulates the EPC Defects of WD Feeding**

To determine whether treatment with exogenous cholesterol could phenocopy the effects of WD on Lxrαβ−/− EPC function, we performed ex vivo assays with EPCs of chow-fed WT and Lxrαβ−/− mice that were differentiated in the presence of exogenous cholesterol (25 μg/mL). The cholesterol content of both WT and Lxrαβ−/− EPCs was significantly increased after treatment (Figure 7A). Interestingly, despite similar increases in cholesterol content, only cholesterol treatment to Lxrαβ−/− EPCs accelerated differentiation towards the endothelial lineage (ie, increased expression of Cd144 and Vegfr2), as previously observed with WD feeding (Figure 7B). This observation is consistent with our in vivo model, in which WD feeding increased plasma hypercholesterolemia in WT and Lxrαβ−/− mice but only produced defects in the endothelial differentiation markers of Lxrαβ−/− EPCs. To explore whether the EPC secretome could be selectively altered by the addition of cholesterol, we assessed monocyte adherence to HUVECs treated with the CM from these ex vivo cholesterol-treated EPCs. CM from the cholesterol-treated Lxrαβ−/− EPCs increased monocyte adhesion to the treated endothelial cells (Figure 7C). Together, these results indicate that LXRs play a protective role against cholesterol-induced defects in EPC differentiation and vasoreparative factor secretion.

**Discussion**

LXRs remain therapeutic targets of interest in the context of atherosclerosis, because of their potent capability to promote cholesterol efflux from macrophages. Although studies using knockout mice for the LXR target genes Abca1/g1−/− or Apoe−/− have shown important roles for cholesterol in the development of monocytecytosis, a role for LXRs per se in regulating global hematopoiesis, particularly within a hypercholesterolemic environment, has not been explored. Likewise, to our...
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Furthermore, the culture-derived EPCs from hypercholesterolemic mice lost LXRβ, while decreasing the numbers of the vasoreparative EPCs.

While decreasing the numbers of the vasoreparative EPCs.

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knowledge, quantitation of EPCs relative to other hematopoietic cell types has also not yet been investigated in any of these contexts. In this study, we demonstrate that in the absence of LXRs, a hypercholesterolemic environment alters hematopoietic balance, increasing the numbers of proinflammatory cell types (namely, the monocytes and neutrophils).

In this study, we demonstrate that in the absence of LXRs, a hypercholesterolemic environment alters hematopoietic balance, increasing the numbers of proinflammatory cell types (namely, the monocytes and neutrophils), while decreasing the numbers of the vasoreparative EPCs.

Monocytes and neutrophils, as well as other hematopoietic cell types, have been shown to be affected by cholesterol levels. However, the role of LXRs in the regulation of these cell types has not been fully elucidated.

Figure 7. Treatment of liver X receptor double-knockout (Lxrβ/−/−) culture-derived endothelial progenitor cells (EPCs) with cholesterol (Chol) phenocopies the dysfunction observed with Western diet feeding. Culture-derived EPCs from 12-week-old Chow-fed wild-type (WT) and Lxrβ/−/− mice were differentiated in the presence of exogenous Chol (25 μg/mL Chol). A, Chol content of the treated EPCs was determined by mass spectrometry. n=6 per group. B, Gene expression of endothelial (Cd144 and Vegfr2) and leukocyte (Cd45) markers in the EPCs. n=8 to 12 per group. C, Quantification of monocyte adherence to human umbilical vein endothelial cells incubated with the conditioned medium (CM) from the Chol-treated EPCs. n=6 per group. Veh indicates vehicle. *P<0.05, **P<0.01, and ****P<0.0001.

Herein, we have shown that WD feeding also increases the cholesterol content and proliferation of HSCs in Lxrβ/−/− mice (Figure 4C), not only pushing the fate of these HSCs towards the myeloid population but also away from the formation of EPCs (Figure 3A and 3B), suggesting that LXRs play a basal role in hematopoietic differentiation within the individual lineages.

Although colony formation assays showed increased myeloid cell formation in WD Lxrβ/−/− mice, no differences in EPC colony formation were observed between any of the feeding groups (Figure 3C). We also found no increase in EPC apoptosis or decrease in EPC proliferation that could account for their lower numbers in WD Lxrβ/−/− mice. In the 12-week-old cohort of mice, enumeration by flow cytometry or complete blood cell counts revealed significant increases in myeloid populations, with significant decreases in EPCs in the Lxrβ/−/− mice (Figure 1A through 1C), demonstrating the importance of LXRs in preserving hematopoietic balance. The colony formation assays showed no basal differences between the vehicle-treated WT and Lxrβ/−/− HSCs; however, ex vivo pharmacologic activation of LXRs in HSCs increased EPC colony formation (Figure 1C). Thus, although we were able to show that LXRs are important for modulating HSC differentiation to EPCs, the formation of EPCs from HSCs in vivo may be strongly affected by microenvironmental cues within the BM niche that could not be recapitulated in the basal ex vivo colony formation assay. As an example, microenvironmental cues were shown to be critical for the expression of context-dependent lineage-determining transcription factors in tissue-resident macrophages.

Stem cells are particularly sensitive to the effects of aging, where paradoxically, aging of HSCs increases their numbers in the BM. In accordance with this, we observed a 2-fold increase in BM HSCs in the 20-week-old versus 12-week-old WT mice (Figures 1A and 3A). However, the increase in HSC numbers we observed in the 12-week-old Lxrβ/−/− mice, compared with their WT counterparts, was lost in the Chow-fed 20-week-old cohort. Taken with our previous observation that BM HSCs are increased in WT mice with aging, these data suggest that the Lxrβ/−/− mice may show a premature aging phenotype at 12 weeks. Furthermore, with aging, previous studies have also demonstrated preferential differentiation of HSCs toward the myeloid lineage over those in the lymphoid lineage. This is also consistent with the shift in myeloid cell numbers we observed in the Lxrβ/−/− versus WT mice basally at 12 weeks. Accelerated aging of Lxrβ/−/− HSCs, leading to increases in their numbers in the BM and preferential differentiation to the myeloid lineage, may have occurred because of changes in the intracellular distribution of cholesterol within the HSCs that, in turn, influenced their ability to differentiate to the various lineages.
The cellular cholesterol levels of culture-derived EPCs from Lxrβ−/− WD mice were significantly elevated compared with WT WD mice, and this elevation was associated with increased expression of markers of terminally differentiated endothelial cells. Our data would suggest that this accelerated differentiation to endothelial cells is detrimental to the reparative function of EPCs because EPCs from Lxrβ−/− WD mice possessed a different secretome, which resulted in increased in vitro monocyte-endothelial cell adhesion, an initiating step in atherogenesis. Thus, despite removal from a hypercholesterolemic environment, defects from the Lxrβ−/− WD mice persist in EPCs after culture, suggesting cell-intrinsic defects are present. These changes in EPC differentiation and secretome were recapitulated when Lxrβ−/− chow EPCs (but not WT chow EPCs) were treated with cholesterol in culture, suggesting that this was the component of the WD mediating this change. Interestingly, previous clinical studies of EPCs from patients with cardiovascular disease or diabetes mellitus provide evidence that EPCs may passively contribute to disease progression by reducing the migration, proliferation, or tube formation capacity of the cells. However, herein we show that the loss of LXRs combined with an increase in EPC cholesterol content produces an EPC population that may actively contribute to disease progression by directly promoting monocyte adhesion to activated endothelial cells. Additional studies will be required to identify the specific factor or set of factors in the secretome that are responsible for enhancing the binding of monocytes to endothelial cells.

Numerous studies have reported inverse correlations of EPC numbers and migratory function with low-density lipoprotein cholesterol. The data we present in this study are the first to demonstrate a cell autonomous role for LXRs in the cholesterol-mediated dysfunction of EPCs. To study the effects of cholesterol on EPCs, we derived EPCs from WT and Lxrβ−/− mice after WD feeding, or we derived EPCs from chow-fed mice and treated them exogenously with cholesterol. In vivo, the WD did not increase the cholesterol content of the WT EPCs. We originally attributed this finding to enhanced cholesterol efflux possible by increasing Abca1/g1 by LXRs in the WT EPCs, which, in turn, would prevent the accumulation of cholesterol and subsequent dysfunction of the EPCs. However, this idea was challenged by the ex vivo treatment of EPCs with cholesterol, in which the intracellular cholesterol levels of both WT and Lxrβ−/− EPCs were increased, yet only the Lxrβ−/− EPCs showed defects in their differentiation and secretome. This suggests that, apart from their capacity to regulate bulk cholesterol levels via cholesterol efflux, LXRs regulate the actions of intracellular cholesterol, which may, in turn, affect the differentiation of EPCs. One hypothesis, consistent with previous studies performed in mouse models absent LXR target genes, is that LXRs influence the subcellular distribution of cholesterol within EPCs. Indeed, Apoe−/− and Abca1/g1−/− mice were shown to have an increase in cholesterol accumulation within lipid rafts of HSCs, leading to increased signaling through the common β subunit to enhance the production of myeloid cells. In contrast, other studies demonstrated that LXR agonist treatment disrupted the localization of key signaling molecules vascular endothelial growth factor receptor 2 and protein kinase B within lipid rafts, suggesting that activation of LXRs decreases the intracellular distribution of cholesterol to the rafts. Thus, LXRs may be modulating lipid raft cholesterol content, which indirectly affects the identity and function of EPCs.

In conclusion, we have demonstrated that LXRs are essential for maintaining the balance of hematopoietic cells under WD feeding. Previous studies have demonstrated the importance of LXRs in BM macrophages on atherogenesis. Herein, we focused on the impact of LXRs on another BM-derived cell type, the EPCs. WD feeding of Lxrβ−/− mice produced EPCs ex vivo that had increased cellular cholesterol and produced CM that increased monocyte-endothelial adherence. These data suggest that dysfunction of these cells may contribute to early atherogenesis. Further mechanistic studies are required to determine how LXRs specifically regulate cholesterol homeostasis in this cell type and determine whether EPCs contribute to endothelial repair during the early stages of atherogenesis in vivo. Nevertheless, these studies highlight the important role of LXRs in the differentiation and function of EPCs and the potential for therapeutic manipulation of these cells via ex vivo LXR targeting.

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Disclosures

None.

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